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IMPACTS OF CROWDING STRESS ON AQUATIC ANIMALS AND ITS MITIGATION THROUGH FEED ADDITIVES SUPPLEMENTATION – A REVIEW

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Abstract

Continuous and sustainable aquaculture development is essential to provide affordable protein sources for the global population. Nevertheless, the intensification of fish farming at high stocking density led to chronic crowding stress that could impair the growth performance, immune system, and antioxidant capacity of an aquaculture species. Consequently, the prevalence of disease outbreaks increases, resulting in low aquaculture production. Various countries remain highly dependent on antibiotics and chemotherapies to overcome this problem. Recently, researchers have proposed and developed alternative solutions using environmentally-friendly feed additives, such as probiotics, phytobiotics, and synthetic immune stimulants, to mitigate crowding stress. The feed additives reportedly alleviate crowding stress, promote growth performance, enhance gut microbiota, immune system, and antioxidant capacity, and stimulate disease resistance, contributing to the overall improvement of the health status of aquaculture species and boosting aquaculture production. The application of feed additives in aquafeed has been investigated in various aquaculture species to explore their beneficial effects at optimum doses. The study outcomes illustrated that feed additives in aquafeed are promising in mitigating crowding stress among aquaculture species. Herein, this review paper discussed and summarised the impacts of crowding stress on the growth, immune system, and antioxidant capacity of aquatic animals. In addition, this review highlighted the potential of feed additives such as probiotics, phytobiotics, and synthetic immune stimulants in mitigating crowding stress in aquatic animals.

Key words: crowding stress, probiotic, phytobiotic, synthetic immune stimulant, growth performance, immune system, antioxidant

Water quality, nutritious feed, and genetics are crucial in ensuring the success of aquaculture. On top of that, stocking density could impact the welfare and productivity of an aquaculture species. A non-optimal stocking density will result in poor water quality due to ammonia and nitrite accumulation and dissolved oxygen depletion in an aquaculture system (Amano et al., 2022). Moreover, the aquaculture species will exhibit abnormal behaviour and appetite loss, thus reducing farm productivity (Amano et al., 2022). As aquaculture is gearing towards intensification, the risk of crowding stress has become a setback for aquaculture industry development (Lin et al., 2018; Liu et al., 2014). Previous studies reported that crowding stress significantly compromised the immune system of aquaculture species, exposing them to a higher risk of disease infection (Adineh et al., 2021 a; Lin et al., 2018; Paray et al., 2020).

Cortisol is a hormone for stress regulation in aquatic animals (Gabr et al., 2023). Fish under stress exhibit high blood cortisol and glucose levels, which impairs their growth, physiology, and immune system (Espelid et al., 1996; Esteban et al., 2004; Sadoul and Vijayan, 2016; Schreck, 2010; Schreck and Tort, 2016), thus, impacting the fish welfare. Fish welfare is the primary for a successful aquaculture activity; hence, fish farmers are required to practice good stress management for their aquaculture species. Nonetheless, farmers face challenges in preventing crowding stress among the aquatic species as the aquaculture industry intensifies. Therefore, several strategies were utilised to maximise production while maintaining good welfare for the aquaculture species, although farmers are still struggling to find the right balance between the two components. Besides improving the fish farming facilities, incorporating feed additives is a practical and cost-effective strategy to maintain fish welfare in intensive aquaculture farming (Herrera et al., 2019).

Feed additives application is currently trending among aquaculturists. Studies have demonstrated the efficacy of feed additives in aquatic animals associated with handling (Costas et al., 2012), salinity (Do Huu et al., 2016), anoxia (Pahor-Filho et al., 2017), ammonia (Rama and Manjabhat, 2014), hypoxia (Henrique et al., 1998), temperature (Chen et al., 2004), pH (Wang et al., 2014), starvation (Yengkokpam et al., 2016) and crowding stress (Castro-Osses et al., 2017; Chen et al., 2016; Ganga et al., 2011). The benefits of feed additives in aquaculture include improvements in fish growth performance (Güroy et al., 2012; Hamid et al., 2022), nutritional profile (Sukri et al., 2022), reproduction (Dabrowski and Ciereszko, 2001), immune system (Sukri et al., 2023), disease resistance (Abdul Kari et al., 2022; Goh et al., 2023), and mitigating stressor impacts (Kari et al., 2022; Wei et al., 2022). A recent study revealed the potential of phytobiotics and probiotics as feed additives to mitigate crowding stress in aquaculture.

Phytobiotics contain bioactive compounds, such as tannins, flavonoids, carotenoids, phenolic compounds, and terpenes, that can reduce stress and modulate the immune system (Kari et al., 2024; Seong Wei et al., 2024; Tungmunnithum et al., 2018; Wei et al., 2022). Furthermore, phytobiotics could promote the growth of gut microbiota, enhance feed utilisation efficiency, and stimulate disease resistance in aquatic animals (Abdul Kari et al., 2022; Goh et al., 2023; Kari et al., 2022; Wei et al., 2022). Meanwhile, probiotics reportedly enhance the gut microbiota balance (Kirihara et al., 2018) and intestinal microflora (Gatesoupe, 1999), eliminate gut pathogens, and stimulate the innate immune system (Gómez and Balcázar, 2008; Wee et al., 2024). Examples of probiotics in aquaculture are *Bacillus*, *Lactobacillus*, *Lactococcus*, *Clostridium*, *Leuconostoc*, *Enterococcus*, *Aeromonas*, *Shewanella*, and *Carnobacterium* (Takanashi et al., 2014). Synthetic immune stimulants (SIS) were also used as a feed additive in mitigating stress among aquatic animals, including gallic acid in *Cyprinus carpio* (Ghafarifarsani et al., 2023) and vitamin E and C in gilthead seabream, *Sparus aurata* (Montero et al., 1999).

Given the accumulating evidence of crowding stress impact on aquatic species, the present review discussed and summarised the detrimental effects of this condition on animal growth and their immune system. Additionally, this review highlighted the potential of feed additives in mitigating crowding stress in aquaculture species.

Impacts of high stocking density on aquatic animals

Stocking density is critical in determining a successful aquaculture practice (Bi et al., 2023). A high stocking density may be profitable and cost-effective in maximising space utilisation in an aquatic environment, but this practice forces the farmed species to compete for basic necessities such as food, space, and dissolved oxygen (Bi et al., 2023). Earlier studies claimed that a high stocking density could reduce fish growth performance and immunity, thus diminishing the total aquaculture production (Dediu et al., 2021). Therefore, stocking density has been recognised as a major source of chronic stress, which entails proper management for good aquaculture practices.

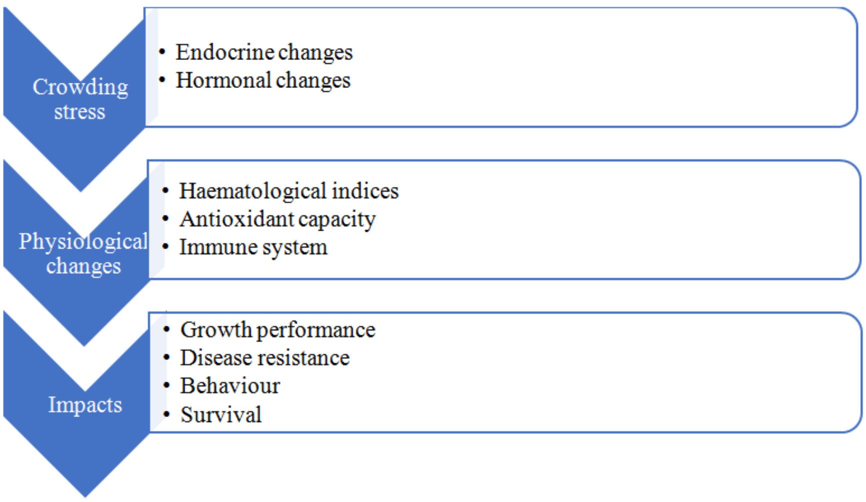

Figure 1. Impacts of crowding stress on aquatic animals

Stocking density may lead to biological or crowding stress, directly impacting fish gene expression, welfare, and production (Dediu et al., 2021; Jia et al., 2016). Furthermore, crowding stress disturbs the normal physiological response required for internal homeostasis maintenance (Bayunova et al., 2002; Long et al., 2019). Crowding stress also causes endocrine changes related to stress hormones, namely corticosteroids and catecholamines (Herrera et al., 2019). Endocrine alterations can influence an organism's physiological aspects, such as haematological indices, antioxidant capacity, and immune system (Herrera et al., 2019), manifested as adverse effects on growth performance, disease resistance, behaviour, and survival (Barton, 2002) (see Figure 1).

Impacts of crowding stress on the growth performance of aquatic animals

Crowding stress harms the growth performance of aquatic animals (see Table 1). Current research on the impact of crowding stress is limited to several aquatic species, such as sturgeon, *Acipenser queldenstaedtii*, Nile tilapia, *Oreochromis niloticus*, Senegalese sole, *Solea senegalensis*, Chinese sturgeon, *Acipenser sinensis*, hybrid sturgeon and minor carp, *Labeo bata*. The study outcomes indicated that high stocking density causes crowding stress, increased stress hormones, and glucose imbalance in aquatic animals, negatively impacting their growth performance. Nonetheless, there are several cases where crowding stress had no adverse effects on the farmed aquatic animals. These discrepancies warrant further investigation to substantiate the impact of crowding stress on the growth performance of other aquatic animal species, particularly crustaceans, due to the lack of literature.

At low stocking density, sturgeons (*A. queldenstaedtii*) demonstrated superior growth performance compared to their counterpart at high stocking density (Çelikkale et al., 2005). Despite the insignificant results, the low-stocking density group recorded a better final weight and survival rate than the high-density group. Likewise, studies on juvenile of Senegalese sole, *Solea senegalensis* (Andrade et al., 2015; Costas et al., 2013, 2008; Salas-Leiton et al., 2008), larvae and fingerling of lake sturgeon, *A. fulvescens* (Fajfer et al., 1999), Atlantic sturgeon, *A. oxyrinchus* (Mohler et al., 2000) reported similar findings. The insignificant results in growth performance between the low and high stocking density groups may be attributed to the adaptation of the experimental fish to the high stocking density during the acclimatisation period. Another possible explanation is the fish farmed at high stocking density were fed with higher feed ration to fulfil their dietary requirements, thus sustaining themselves even under non-optimal conditions as observed in the Senegalese sole (Salas-Leiton et al., 2008). Similarly, (Costas et al., 2013) reported that Senegalese sole farms in high and low stocking exhibited comparable growth performance with the condition that the high stocking density fish are supplemented with a higher protein diet.

In some cases, high stocking density can promote the growth performance of aquatic animals, as reported in juvenile Siberian sturgeon, *A. baerii*. Despite that, high stocking density remains a risk for causing significantly ($P<0.05$) lower growth performance as observed in hybrid sturgeon farming (Bi et al., 2023; Dediu et al., 2021), Chinese sturgeon (Long et al., 2019), Atlantic cod (Lambert and Dutil, 2001), Nile tilapia (Gibtan et al., 2008), European seabass (Saillant et al., 2003) and rainbow trout (Ellis et al., 2002). In severe cases, crowding stress caused mortality in hybrid sturgeon (Bi et al., 2023), Nile tilapia, *Oreochromis niloticus* (Garcia et al., 2013), and minor carp, *Labeo bata* (Karnatak et al., 2021). Thus, researchers proposed the use of the biofloc system to ease crowding stress, which was proven effective in juvenile common carp farmed at high stocking density (Adineh et al., 2022; Yousefi et al., 2023 a).

Glucose is an essential nutrient for animals that provides energy in the form of adenosine triphosphate (ATP). Many studies utilised the blood glucose level to indicate chronic stress, particularly crowding stress (Iwama et al., 2015). High blood glucose levels have been linked to high stocking density (Long et al., 2019), but this is not the case for some studies as the species has acclimatised itself in a crowded environment (Dediu et al., 2021). Therefore, blood glucose levels may not be the most precise measurement of stress (Dediu et al., 2021).

Cortisol is an important hormone that regulates glucose production in aquatic animals via gluconeogenesis and glycogenolysis pathways (Iwama et al., 2015). This stress hormone level can be used to reflect the stress experienced by an organism (Adineh et al., 2021 b; Fatima et al., 2017) and has been applied in earlier studies (Mommsen et al., 1999). An organism under stress will trigger the secretion of catecholamines hormone into the body (see Figure 2). Once present in blood, catecholamines stimulate the hypothalamic-pituitary-interrenal (HPI) axis to release cortisol into the circulatory system. Cortisol is responsible for mobilising energy reserves in the body by regulating and activating liver glycogenolysis and inhibiting glycolysis (Mazeaud et al., 1977). A high cortisol level will activate glycogenolysis and inhibit glycolysis, resulting in higher blood glucose production, which is common in aquatic animals under high stocking density (Dediu et al., 2021; Jia et al., 2016) and crowding stress (Ghafarifarsani et al., 2023; Hoseini et al., 2019 b; Hosseini and Hoseini, 2012). These adaptive responses provide extra energy for cells in their bodies to handle crowding (Barton, 2002), hence reducing the growth performance of aquatic animals in high stocking density environments.

Table 1. Impacts of crowding stress on the growth performance of aquatic animals

Species (stage)	Stocking density	Environment/ Duration	Impact on growth performance	References
Sturgeon, *Acipenser queldenstaedtii* (juvenile)	8 fish/m³ (low density) and 12 fish/m³ (high density)	Net cage (2 m × 2 m × 2 m) 203 days	No significant difference between groups	(Çelikkale et al., 2005)
Nile tilapia, *Oreochromis niloticus* (juvenile)	50, 100, 150 and 200 fish/m³	Net cage 150 days	Low-density group demonstrated significantly better outcomes	(Gibtan et al., 2008)
Nile tilapia, *Oreochromis niloticus* (juvenile)	Stage I: 800, 2000, 2500 and 3000 fish/cage (6 m³)	Cage Stage I: 41 days	Low density showed significantly better results, and fish in uniform size	(Garcia et al., 2013)
	Stage II: 80, 100 and 120 kg/m³	Stage II: endpoint when the fish reached 800 g weight	Disease prevalence was higher in the high stocking density group	
Senegalase sole, *Solea senegalensis* (juvenile)	Initial densities: 7, 17 and 24 kg/m² Final densities: 13, 31 and 40 kg/m²	Raceway system 60 days	No significant difference between groups	(Andrade et al., 2015)
Chinese sturgeon, *Acipenser sinensis* (juvenile)	Low density: 4.80 kg/m² Medium density: 8.99 kg/m² High density: 12.68 kg/m²	Recirculating aquaculture system (RAS) 3 months	Low-density group exhibited significantly superior outcomes	(Long et al., 2019)
Hybrid sturgeon (Bester ♀ × Beluga ♂ juvenile)	Trial 1: 2 and 4 kg/m² Trial 2: 5 and 12 kg/m²	Recirculating aquaculture system (RAS) 6 weeks	Low-density group performed significantly better outcomes	(Dediu et al., 2021)
Minor carp, *Labeo bata* (juvenile)	50, 75 and 100 fish/m³	Cage (5 m × 5 m × 3 m) 180 days	Low-density group showed significantly better result	(Karnatak et al., 2021)
Hybrid sturgeon (*Acipenser baerii* ♀ × *Acipenser schrenckii* ♂ juvenile)	Low density: 100–150 fish/m³ Medium density: 200–250 fish/m³ High density: 300–350 fish/m³	In flow water pond 60 days	Low density group exhibited significantly better result	(Bi et al., 2023)

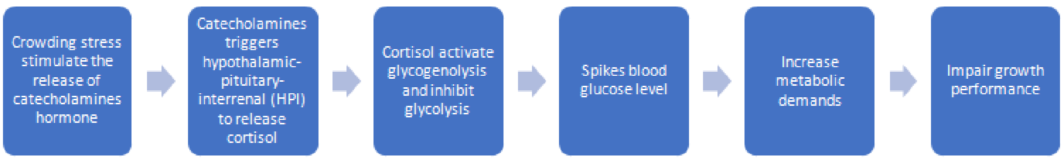

Figure 2. Mechanism of growth impairment of aquatic animals under crowding stress

Haematological indices are also good stress indicators (Jeney and Jeney, 2002) for aquatic animals under high stocking density, including total haemoglobin, total red blood cells, high mean corpuscular haemoglobin concentration, and low mean corpuscular volume (Dediu et al., 2021). The changes in haematological indices are adaptive responses for aquatic animals with high oxygen requirements and metabolic demands to maintain haemostasis (Dediu et al., 2021), particularly under crowding stress (Kleinert et al., 2019). Therefore, low-density aquatic animal farming remains ideal in aquaculture production, as Garcia et al. (2013) described in Nile tilapia, yielding superior production and uniform fish size. Nevertheless, several aquatic animals are adaptive to high-density farming, such as African catfish (Abdul Kari et al., 2021) and Senegalese sole (Andrade et al., 2015), with the condition that the farmed aquatic animals must be supplemented with high protein diet to mitigate crowding stress.

Impacts of crowding stress on the immune system and antioxidant capacity of aquatic animals

Aquatic animals farmed at high stocking density face the risk of immunosuppression and reduced antioxidant levels due to crowding stress (see Table 2), characterised by inhibiting metabolic and antioxidant enzyme activities. Despite evidence on the adverse effects of crowding stress on animal health and antioxidant status, several studies have reported no significant impacts on the farmed species. Therefore, further study is required to elucidate the effects of crowding stress on aquatic animals, particularly crustaceans.

Table 2. Impacts of crowding stress on the immune system and antioxidant capacity of aquatic animals

Species (stage)	Stocking density	Environment/ Duration	Impacts on the immune system and antioxidant capacity	References
Rainbow trout, *Oncorhynchus mykiss* (juvenile)	Low stocking density: 10 kg/m^3	9 months	Blood cortisol level increases in the high stocking density group	(North et al., 2006)
	High stocking density: 40 and 80 kg/m^3		Lower lysozyme activity in the high stocking density group	
Rainbow trout (juvenile)	Control group: 15 kg/m^3	Fiber glass tank 1 m (diameter) × 1.2 m (depth)	High stocking density inhibits metabolic and antioxidant enzyme activities	(Aksakal et al., 2011)
	High stocking density: 20, 25 and 30 kg/m^3	2 months	High stocking density upregulates heat shock protein (Hsp70) expression	
Senegalase sole, *Solea senegalensis* (juvenile)	Initial densities: 7, 17 and 24 kg/m^2	Raceway system	No significant difference between groups	(Andrade et al., 2015)
	Final densities: 13, 31 and 40 kg/m^2	60 days		
Turbot, *Scophthalmus maximus* (juvenile)	Low density: 9.3 kg/m^2	Recirculating aquaculture system (RAS)	High stocking density inhibits mucus enzyme activities	(Jia et al., 2016)
	Medium density: 13.6 kg/m^2	120 days	High stocking density induces oxidative stress on the skin	
	High density: 19.1 kg/m^2		High stocking density downregulated the expression of skin immune-related gene expression	
			High stocking density decreases lysozyme activity	
Grass carp, *Ctenopharyngodon idella* (juvenile, 98.48 g)	Low density: 0.9 kg/m^2 Medium density: – 2.97 kg/m^2 High density: 5.9 kg/m^2	Tank 10 weeks	High stocking density induces immunosuppression	(Lin et al., 2018)
Chinese sturgeon, *Acipenser sinensis* (juvenile)	Low density: 4.80 kg/m^2 Medium density: 8.99 kg/m^2 High density: 12.68 kg/m^2	RAS 3 months	High stocking density decreases lysozyme activity	(Long et al., 2019)
Hybrid sturgeon (*Acipenser baerii* ♀ × *Acipenser schrenckii* ♂ juvenile)	Low density: 100–150 fish/m^3 Medium density: 200–250 fish/m^3 High density: 300–350 fish/m^3	In-flow water pond 60 days	No significant difference in immune status High stocking density inhibits antioxidant capacity	(Bi et al., 2023)
Grass carp, *Ctenopharyngodon Idella* (712.70 g)	Low density: 10 kg/m^3 Medium density: 15 kg/m^3 High density: 20 kg/m^3	120 days	High stocking density induces immunosuppression	(Li et al., 2023)

Aquatic animals farmed in a high-density environment generally exhibit poorer immune function and a higher prevalence of disease infection (Aranguren et al., 2002; Mauri et al., 2011). For instance, Nile tilapia farmed in a high-density cage culture can exhibit higher disease infection and mortality (Garcia et al., 2013). High stocking density can also lead to immunosuppression, as observed in the common carp, *Cyprinus carpio* (Adineh et al., 2021 b;

Hoseini et al., 2019 a), turbot, *Scophthalmus maximus* (Jia et al., 2016), grass carp, *Ctenopharyngodon idella* (Li et al., 2023; Lin et al., 2018), and gilthead seabream, *Sparus aurata* (Montero et al., 1999). In addition, crowding stress diminishes red and white blood cells, leading to immune system impairment in grass carp, *Ctenopharyngodon idella* (Lin et al., 2018). Nonetheless, immunosuppression did not occur in several studies on crowding stress, indicating the plasticity of the immune system that could be influenced by certain factors and metabolic changes (Al-Bakheit et al., 2016; Bi et al., 2023).

Crowding stress reportedly elevated serum biochemical parameters, such as glucose, cortisol, alkaline phosphatase (ALP), lactate dehydrogenase (LDH), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) (Ghafarifarsani et al., 2023). Likewise, crowding stress elevated cortisol and glucose levels in common carp, *Cyprinus carpio* (Ghafarifarsani et al., 2023; Hoseini et al., 2019 b; Hosseini and Hoseini, 2012) as an adaptive response and provided additional energy (Barton, 2002). A high cortisol level in fish leads to an impaired immune system (Tort, 2011). Crowding stress did not adversely impact the immune and antioxidant status of several aquatic animals, such as Senegalese sole, *Solea senegalensis* (Andrade et al., 2015). There were no significant differences ($P>0.05$) between the high and low stocking density groups, and the haematocrit results demonstrated blood parameters such as cortisol and glucose levels remained constant in both groups (Andrade et al., 2015). Similar cases were also recorded in other aquaculture species, including European seabass, *Dicentrarchus labrax* (Pascoli et al., 2011), gilthead seabream, *Sparus aurata* (Tort et al., 1998), and rainbow trout, *Oncorhynchus mykiss* (Morgan et al., 2008). These discrepancies suggest that aquatic animals are adaptive to a high stocking-density environment (Andrade et al., 2015).

Low lysozyme activity has been reported in aquatic animals under stressful environments with high stocking density or infected by disease, including Siberian sturgeon, *Acipenser baerii* (Chesneau, 2018) and Chinese sturgeon, *Acipenser sinensis* (Long et al., 2019) at high stocking density. Thus, lysozyme activity is a suitable indicator of the health status of aquatic animals. Moreover, a high stocking density could inhibit and block metabolic and antioxidant enzyme activities, inducing oxidative stress (Gao et al., 2017; Junqueira et al., 2004) and physiological imbalances in aquatic animals (Aksakal et al., 2011). Despite that, several studies discovered no significant ($P>0.05$) difference in the antioxidant capacity of tongue sole, *Cynoglossus semilaevis* (Liu et al., 2016), largemouth bass, *Micropterus salmonides,* and Senegalese sole, *Solea senegalensis* (Andrade et al., 2015) at different stocking densities, suggesting the adaptability of farmed fish under high stocking density.

High stocking density also reportedly triggers oxidative stress and induces lipid peroxidation activity. For instance, antioxidant capacity induced lipid peroxidation and reduced superoxide dismutase (SOD) and glutathione (GSH-PX) activities in the serum of hybrid sturgeon farmed under high stocking density (Bi et al., 2023; Liu et al., 2014; Long et al., 2019; Yousefi et al., 2020). Lipid peroxidation produces malondialdehyde (MDA), a metabolite that can be measured in blood serum to indicate oxidative stress level. In addition, downregulation of skin immune-related genes, such as interleukin-1β (IL-1β), insulin-like growth factor – (IGF-), and lysozyme (LZM), were observed in juvenile turbot, *Scophthalmus maximus* under high stocking density (Jia et al., 2016). The study also found that high stocking density inhibits mucus enzyme activities, induces oxidative stress in the skin, and decreases lysozyme activity in juvenile turbot (Jia et al., 2016).

Heat shock protein (Hsp) expression parallels stress exposure; hence, it is a good stress indicator for aquatic animals (Sanders, 1993). The Hsps are classified based on molecular weight: small Hsps (< 40kDa), Hsp60, Hsp70, Hsp90, and Hsp100. These proteins are produced as a protective mechanism against protein denaturation in the events of hyperthermia, cytotoxicity from pesticides, heavy metals, ethanol, and hypoxia (Dorts et al., 2009; Lee and Corry, 1998; Lewis et al., 1999; Lindquist and Craig, 1988), and crowding stress (Aksakal et al., 2011).

Roles of phytobiotics in mitigating crowding stress among aquatic animals

Various plant extracts can be used as phytobiotics to mitigate crowding stress in aquaculture (see Table 3). Phytobiotics can be utilised as a feed additive between 40 and 112 days to enhance the immune system and antioxidant capacity of the aquatic species. Furthermore, phytobiotics could improve blood parameters, immune system, antioxidant capacity, and disease resistance and mitigate crowding stress in aquaculture. Nevertheless, studies in this area are still in their infancy and require further investigation to explore the potential of phytobiotics in aquaculture.

Earlier studies have reported that oak, *Quercus castaneifolia* leaf extract has high antioxidant and antibacterial properties (Almeida et al., 2008; Bahador and Baserisalehi, 2011) that could reduce free radical formation and inhibit the growth of pathogenic bacteria. Besides improving growth performance, the plant extract enhanced the antioxidant enzyme activities such as SOD, CAT, and GPx to mitigate oxidative stress in the common carp (Paray et al., 2020). Oak leaf extract may also stimulate immune components, such as lysozyme elevation, to increase disease resistance in the fish (Hoseinifar et al., 2019; Paray et al., 2020). Conversely, oak leaf extract could not regulate the cortisol level of fish under stress for faster recovery compared to the untreated control group (Paray et al., 2020). Meanwhile, dietary *Rheum officinale* extract significantly regulated cortisol and glucose levels to mitigate crowding stress in common carp (Xie et al., 2008). Green tea extract also mitigated anaesthesia-induced stress in black rockfish, *Sebates schlegeli* (Hwang et al., 2013), while *Urtica dioica* extract significantly reduced crowding stress in Victoria labeo, *Labeo victorianus* by lowering cortisol and glucose levels (Ngugi et al., 2015).

Table 3. Roles of phytobiotics in mitigating crowding stress among aquatic animals

Phytobiotics/ (dosage)	Application/Duration	Aquatic animals – Stage	Impacts	References
Anthraquinone extract from rhubarb *Rheum officinale* bail (1% and 2%)	Feed additive 70 days	Common carp, *Cyprinus carpio* var. Jian Juvenile (5.39 g)	Improve blood parameters Stimulate disease resistance against *Aeromonas hydrophila* Mitigate crowding stress	(Xie et al., 2008)
Stinging nettle, *Urtica dioica* (1%, 2% and 5% of diet)	Feed additive 16 weeks	Victoria labeo, *Labeo victorianus* Juvenile (25 g) Female adult (3.4 kg) Male adult (470 g)	Improve blood parameters Stimulate disease resistance against *A. hydrophila*	(Ngugi et al., 2015)
Saint John's wort, *Hypericum perforatum*, lemon balm, *Melissa officinalis*, rosemary, *Rosmarinus officinalis* (ratio 3:2:1, 6 g/kg of feed)	Feed additive 40 days	Atlantic salmon, *Salmo salar* Juvenile (4.83 g)	Enhance antioxidant capacity Mitigate crowding stress	(Reyes-Cerpa et al., 2018)
Rosemary, *Rosmarinus officinalis* leaf (1–3% of feed)	Feed additive 65 days	Common carp, *Cyprinus carpio* Juvenile (12.93 g)	Improve growth, enhance immune system, increase antioxidant capacity, mitigate crowding stress	(Yousefi et al., 2019)
Oak, *Quercus castaneifolia* leaf extract (0.5, 1 and 2 g/kg of feed)	Feed additive 60 days	Common carp, *Cyprinus carpio* Juvenile (20 g)	Enhance antioxidant and immune system No significant improvement in growth performance Mitigate crowding stress	(Paray et al., 2020)
Dehydrated *Citrus lemon* peel (DLP) (0.5–1.5% of diet)	Feed additive 45 days	Rainbow trout, *Oncorhynchus mykiss* Juvenile (26.8 g)	No significant improvement in growth performance Enhance antioxidant and immune system Improve blood parameters Mitigate crowding stress	(Chekani et al., 2021)
Pomegranate peel (0.5% of diet)	Feed additive 56 days	Common carp, *Cyprinus carpio* Juvenile (18.1 g)	No adverse impact on the growth performance Enhance antioxidant and immune system Improve blood parameters Mitigate crowding stress	(Yousefi et al., 2023 b)

Rheum officinale bail is a Chinese herbal medicine for constipation, jaundice, ascites, and asthma (Akbar, 2020). Previous studies have claimed this herbal remedy enhances the immune system and relieves stress (Jian and Wu, 2004; Yin et al., 2006). Anthraquinone extract from rhubarb bail potentially improves blood parameters, disease resistance against *Aeromonas hydrophila*, and crowding stress in aquaculture (Xie et al., 2008). Nevertheless, overusing Chinese herbs in farmed aquatic species causes gut microbiota imbalance (Liu et al., 2004) and affects the palatability of medicated feed, reducing their growth performance (Xie et al., 2008).

Lemon (*Citrus limon*) peel reportedly possesses medicinal value such as antimicrobial, anticancer, and antioxidant properties (Espina et al., 2011). This high-fibre agricultural waste has been widely explored as a feed additive in aquaculture. Previous studies revealed that dried lemon peel (DLP) positively impacted the growth performance, haematological parameters, and immune system in various aquatic animals, such as common carp, *Cyprinus carpio* (Sadeghi et al., 2021), Nile tilapia, *Oreochromis niloticus*, African catfish, *Clarias gariepinus* (Abdel Rahman et al., 2019), gilthead seabream, *Sparus aurata* (Beltrán et al., 2017) and rohu, *Labeo rohita* (Harikrishnan et al., 2020).

Table 4. Roles of probiotics in mitigating crowding stress among aquatic animals

Probiotics/prebiotics – Dosage	Application/Duration	Aquatic animals/stages (weight)	Impacts	References
Shewanella putrefaciens Pdp11 – 10^9 CFU/g	Feed additive 30 days	*Solea senegalensis* Juvenile (14.6 g)	Mitigate crowding stress Upregulate immune gene transcription Modulate gut microbiota Stimulate disease resistance against *Vibrio harveyi* and *V. parahaemolyticus*	(Tapia-Paniagua et al., 2014)

DLP potentially mitigated crowding stress in rainbow trout, *Oncorhynchus mykiss,* by enhancing their immune system and antioxidant capacity, besides lowering the blood cortisol level (Chekani et al., 2021). The cortisol level is significantly lower in the group of fish that received DLP than the fish from the control group, which was also observed in juvenile *Labeo victorianus* (Ngugi et al., 2017). On the other hand, there was no significant (P>0.05) difference in cortisol levels between the DLP-treated and untreated Nile tilapia and African catfish (Abdel Rahman et al., 2019). Dietary DLP also significantly (P<0.05) increased the total immunoglobulin, alternative complement (ACH50), and lysozyme activity in rainbow trout compared to the control group. Currently, the mechanism of DLP in boosting the immune system remains clear, but studies have explained that these enhancements are caused by the bioactive compounds found in DLP (Baba et al., 2016; Beltrán et al., 2017; Xi et al., 2017). The DLP also enhances the antioxidant capacity of aquaculture species by significantly increasing the SOD and CAT activities (P<0.05) compared to the control group. The SOD and CAT are the frontliners of the antioxidant defence against pathogenic stress (Zhang et al., 2013).

Rosemary, *Rosmarinus officinalis*, leaf is another phytobiotic that can be used as a feed additive to reduce crowding stress in common carp due to the high stocking density farming system. This medicinal herb contains bioactive compounds with various health benefits, including flavonoids and tannins, which act as free radical scavengers and inhibit lipid peroxidation (Almeida et al., 2008; Benedí et al., 2004; Zheleva-Dimitrova et al., 2010). For instance, dietary rosemary leaf demonstrated the ability to control cortisol and glucose levels in fish under crowding stress (Yousefi et al., 2019). Furthermore, combining Saint John's wort, *Hypericum perforatum*, lemon balm, *Melissa officinalis*, and rosemary promoted the antioxidant capacity and mitigated crowding stress in juvenile Atlantic salmon, *Salmo salar*. Phenolic compounds, particularly in rosemary and lemon balm, contributed to antioxidant capacity enhancement in fish (Barros et al., 2013; Peng et al., 2007).

Pomegranate peel is another promising feed additive to ease crowding stress. This plant possesses various bioactive compounds, such as polyphenols, which are known for their pharmacological effects, including anti-diabetic, antioxidant and immunostimulatory (Akhtar et al., 2015; Malviya et al., 2014; Singh et al., 2019). Furthermore, studies have revealed that dietary pomegranate peel positively impacted the growth and health of Nile tilapia and rainbow trout (Avazeh et al., 2021; Badawi and Gomaa, 2016; Monir et al., 2020). This agriculture by-product also effectively lowered the plasma cortisol glucose in the blood of common carp subjected to crowding stress (Yousefi et al., 2023 b).

Roles of probiotics in mitigating crowding stress in aquatic animals

Shewanella putrefaciens Pdp11 significantly (P<0.05) mitigated crowding stress by upregulating immune gene transcription, modulating gut microbiota, and stimulating disease resistance against *Vibrio harveyi* and *V. parahaemolyticus* in *Solea senegalensis* (Tapia-Paniagua et al., 2014) (see Table 4). Dietary *S. putrefaciens* Pdp11 also upregulated immune-related genes, such as C7, HP, NARS, HAMP1, TNFAIP9, and NCCRP1, responsible for producing antimicrobial peptides against pathogenic invasion. Likewise, these genes were upregulated in *S. senegalensis* lipopolysaccharide supplementation (Osuna-Jiménez et al., 2009; Prieto-Álamo et al., 2009). Meanwhile, *S. putrefaciens* Pdp11 potentially reduced the harmful bacteria family *Actinobacteria*, *Photobacterium,* and *Vibrio* spp. in the intestines of *S. senegalensis* (de La Banda et al., 2010; Tapia-Paniagua et al., 2010).

Roles of synthetic immune stimulants (SIS) in mitigating crowding stress in aquatic animals

Dietary gallic acid significantly reduced glucose and cortisol levels in common carp, *Cyprinus carpio,* to mitigate crowding stress (Ghafarifarsani et al., 2023) (see Table 5). A similar finding has been reported in broilers (Biswas et al., 2023). However, the mechanism of gallic acid in relieving crowding stress remains unclear. Dietary yeast, *Xanthophyllomyces dendrorhous* (sexual stage *Phaffia rhodozyma*) extract at a dose of 0.5 g/kg of feed for 40 days enhanced the antioxidant capacity and mitigated crowding stress in juvenile Atlantic salmon, *Salmo salar*.

Yeast extract contains bioactive compounds, primarily carotenoids. Carotenoids are vitamin A precursors acting as free radical scavengers to eliminate excess energy, preventing cellular oxidative damage (Amengual et al., 2011; Loto et al., 2012; Martinez-Moya et al., 2015). Astaxanthin is the main bioactive compound in yeast extract, a carotenoid that can inhibit lipid peroxidation activity (Contreras et al., 2015). Other carotenoids in yeast extract include lycopene, β-carotene, and phoenicoxanthin (Contreras et al., 2015; Córdova et al., 2016). The β-glucan is a primary bioactive compound in the yeast cell wall, known for the antioxidant properties and MDA inhibition (Lee et al., 2011). Therefore, astaxanthin and β-glucan in the yeast extract may be responsible for boosting the antioxidant capacity of the experimental fish by inhibiting lipid peroxidation, lowering MDA level, protecting cells from oxidative damage, and mitigating crowding stress.

The supplementation of dietary pennyroyal, *Mentha pulegium*, essential oil improved the growth performance, enhanced the immune system, increased antioxidant capacity, and mitigated crowding stress in common carp (Yousefi et al., 2023 a). Pennyroyal essential oil was found to activate digestive enzyme activity in the fish and promote gut health, enhancing the fish's growth performance (Kaur et al., 2018; Ringø et al., 2016). In addition, pulegone, an organic compound that is abundant in essential oil, was reported to enhance antioxidative response and ease stress due to crowding in the fish (Yousefi et al., 2023 a).

Chrysin is a bioactive compound present in various natural sources such as honey, propolis, blue passion, and mushrooms (Mani and Natesan, 2018). The medicinal properties of this compound have been widely reported, including immunostimulatory, anti-inflammatory, antimicrobial, and antioxidant activities (Stompor-Gorący et al., 2021; Zeinali et al., 2017). Thus, chrysin could be included as a feed additive to boost fish production and mitigate crowding stress in an aquaculture system. In a previous study, rainbow trout that received a diet containing chrysin for 56 days demonstrated improved antioxidative response and immune system (Yousefi et al., 2023 c).

Roles of feed additives combination in mitigating crowding stress in aquatic animals

A probiotic combination of *Lactobacillus acidophilus* (LAB) and resveratrol (RE) was found to mitigate crowding stress in common carp, *Cyprinus carpio* (Gabr et al., 2023) (Table 6). The functional roles of LAB include enhancing digestion and nutrient absorption, digestive enzyme activity, growth-related metabolite production, and eliminating harmful gut bacteria (Assan et al., 2022; Balcázar et al., 2006). Meanwhile, RE can ameliorate intestinal damage, reduce protein degradation, enhance antioxidant capacity, and improve lipid and glucose metabolism (Zhang et al., 2013; Salomão et al., 2019; Tan et al., 2019; Wilson et al., 2015). Furthermore, the LAB and RE combination enhanced the antioxidant capacity by increasing antioxidant enzymes to protect the antioxidant defence system, thus relieving crowding stress in common carp (Gabr et al., 2023). Previous studies also revealed that probiotics can protect the antioxidant mechanism against free radicals and oxidative stress in fish (Hoseinifar et al., 2020) and other animals (Heshmati et al., 2018).

Combining selenium and garlic extract as feed additives positively impacted grass carp, *Ctenopharyngodon Idella,* exhibiting improved growth performance, antioxidative response, and mitigated crowding stress (Adineh et al., 2021 a). Selenium was reported as an essential component of glutathione peroxide enzyme, which protects the cellular membrane from oxidative impairment (Rotruck et al., 1973). Meanwhile, garlic is a widely used phytobiotic that is utilised as a feed additive to enhance the growth and health of various aquatic species (Adineh et al., 2021 a). Flavonoids and sulphur are two primary components of garlic that are rich in antioxidants and can eliminate free radicals in fish (Chung, 2006; Sharma et al., 2010). Therefore, the selenium and garlic combination is ideal in fish farming.

Table 5. Roles of synthetic immune stimulants (SIS) in mitigating crowding stress in aquatic animals

SIS – Dosage	Application/Duration	Aquatic animals/stage	Impacts	References
Gallic acid – 150, 300, 450 and 600 mg/kg of feed	Feed additive – 60 days	Common carp, *Cyprinus carpio,* juvenile	Improve growth, enhance immune system, increase antioxidant capacity, mitigate crowding stress	(Ghafarifarsani et al., 2023)
Yeast, *Xanthophyllomyces dendrorhous* (sexual stage *Phaffia rhodozyma*) extract – 0.5 g/kg of feed	Feed additive – 40 days	Atlantic salmon, *Salmo salar*, juvenile (4.83 g)	Enhance antioxidant capacity Mitigate crowding stress	(Reyes-Cerpa et al., 2018)
Pennyroyal essential oil – 250 mg/kg of feed	Feed additive – 56 days	Common carp, *Cyprinus carpio,* juvenile (13 g)	Improve growth, enhance immune system, increase antioxidant capacity, mitigate crowding stress	(Yousefi et al., 2023 a)
Chrysin – 100–700 mg/kg of feed	Feed additive – 56 days	Rainbow trout, *Oncorhynchus mykiss* Juvenile (51 g)	Improve growth, enhance immune system, increase antioxidant capacity, mitigate crowding stress	(Yousefi et al., 2023 c)

Table 6. Roles of feed additives combination in mitigating crowding stress in aquatic animals

Feed additives – Dosage	Application/Duration	Aquatic animals – stages (weight)	Impacts	References
Lactobacillus acidophilus (LAB) + resveratrol (RE) – 1.5 × 10^7 CFU/g + 300 mg/kg	Feed additive 60 days	Common carp, *Cyprinus carpio* Juvenile (30.16 g)	Mitigate crowding stress	(Gabr et al., 2023)
Nano selenium + garlic extract – 1–2 mg + 1–2 g	Feed additive 60 days	Grass carp, *Ctenopharyngodon idella* Juvenile (2.1 g)	Improve growth, increase antioxidant capacity, mitigate crowding stress	(Adineh et al., 2021 a)

Conclusion

High stocking density in aquaculture causes crowding stress in farmed species, impairing their growth performance and health status. Researchers have proposed using feed additives, namely probiotics, phytobiotics, and synthetic immune stimulants, to mitigate this issue by modulating the immune system, antioxidant capacity, and intestinal condition of aquatic animals. Furthermore, feed additives have exhibited protective effects against crowding stress, but the mechanism of these components has yet to be fully elucidated. Moreover, limited studies have explored the use of probiotics as feed additives to mitigate crowding stress in fish farming.

Currently, studies have revealed that feed additives can potentially control cortisol and glucose levels in fish after being exposed to crowding stress. Most studies incorporated phytobiotic as feed additives between 0.5% and 5% of the diet to manage the effects of crowding stress in fish farming. Alternatively, synthetic immune stimulants have demonstrated the potential as a feed additive and reducing stress due to crowding in fish farming at an even lower inclusion level (≤0.5% of diet). A combination of synthetic immune stimulants and probiotics or phytobiotics also demonstrated positive outcomes in mitigating stress in fish farming. Future studies should investigate the appropriate dosage and optimise feed additives administration to identify the best practices for sustainable aquaculture production. Furthermore, future studies should continue to explore the application of the biofloc technology in fish farming, which was found to reduce crowding stress due to high stocking density.

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Author contributions

All authors contributed to the study conception and design. Lee Seong Wei, Martina Irwan Khoo, Ramasamy Harikrishnan, Ümit Acar, Md Eilious Hosain, Azra MN, Ahmad Syazni Kamarudin, Lau Kia Kian, and Wendy Wee performed material preparation, data collection, and analysis. Lee Seong Wei and Wendy Wee wrote the first draft of the manuscript, while Martina Irwan Khoo commented on previous versions. All authors have read and approved the final manuscript.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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