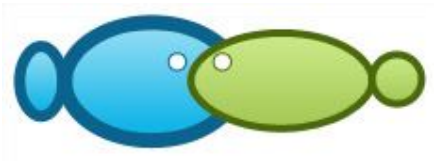

Current status of immunostimulant agents in tropical aquaculture: a review

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Abstract. Aquaculture is one of the fisheries possibilities for fulfilling the world's expanding demand for fish. However, the main problem is high mortality and disease spread in modern aquaculture, particularly bacterial infections that significantly drop farmed fish production. Antibiotic overuse to combat bacterial diseases in traditional aquaculture health management can harm the aquaculture ecosystem and farmed animals. These antibiotics misuse leads to antibiotic resistance to microorganisms and farmed animals, which eventually spread to humans. For reducing these side effects, there has been a global trend over the past years to investigate and apply numerous natural substances that have the power to fight diseases by boosting some immune response parameters in fish, commonly known as immunostimulants. This review aims to present the potential use of nucleotides and organic acids for immunostimulants in aquafeed as a critical research priority for sustainable and eco-friendly aquaculture, focusing on their ability to increase specific immune function for disease prevention in several tropical fish species.
Key Words: antibiotics, disease, fish, immunostimulant, nucleotides, organic acids.

Introduction. Aquaculture is one of the options in the realm of fisheries for meeting the world's growing demand for fish. Many countries such as India, China, Vietnam, Bangladesh and Egypt engage in more aquaculture than wild-caught fish. The issue with modern fish farming or aquaculture is the high density of fish and monoculture that allows the spread of disease and parasites (Heberer 2009). Bacterial infection in aquaculture hatcheries and farms cause a massive loss of productivity and remain a significant challenge (Jayaprakashvel & Subramani 2019). Treatment of animals is conducted for therapy, prophylactic or just for growth reasons. For growth reasons, antibiotics are used in sub-therapeutic doses that have been recognised to contribute to promoting resistance (Heberer 2009; Vaseeharan & Thaya 2014). A combination of some antibiotics was also found effective against fish pathogens such as *Aeromonas caviae, Vibrio anguillarum* and *Yersinia ruckeri* (Done et al 2015).

Antibiotics are the most successful and essential drug family to protect human health. Antibiotics are chemical substances, produced by microbes that can prevent or kill the growth of other microorganisms. The first era of existing antibiotics corresponds to the discovery of penicillin by Sir Alexander Fleming. At the end of the Second World War, penicillin was available for humans on a large scale (Vincent et al 2019). Some antibiotics administered to fight bacterial infections in human medicine are also used to treat animals such as fish (Heberer 2009).

Although antibiotics could be used against fish pathogens in aquaculture production, specific uses of antibiotics in a food-producing animal can lead to antibiotic resistance in intestinal bacteria (Done et al 2015). Such antibiotics can also create antibiotic resistance in non-pathogenic bacteria (Heberer 2009). Moreover, the utilisation of antibiotics in aquaculture also led to environmental pollution. Studies in China showed that fish ponds are a reservoir of antibiotics resistance genes (ARGs) and antibiotics residues accumulated in water and sediment (Xiong et al 2015; Lai et al 2018). Because

of the effects of sunlight, bacterial activity and temperature, certain antibiotics can change and become pollutant (Lei & Lai 2019). Antibiotics residues were also detected in catfish (Chuah et al 2016), and in freshwater fish and shrimp that were domestically sold in Vietnam (Pham et al 2015). All of these findings might imply the potential risk to human health such as hypersensitivity reaction, mutagenicity, carcinogenicity, bone marrow depression and disruption of normal intestinal flora (Moutou et al 1998).

To reduce the negative impact of antibiotics in aquaculture, investigation and utilisation of some natural compounds that have the ability to fight against pathogens by elevating some immune responses parameters in fish is the main research priority for better and eco-friendly aquaculture. Therefore, in this manuscript, we discuss the potential of some immunostimulants particularly nucleotides and organic acids in aquafeed to replace antibiotics used in aquaculture, with specific emphasis given to their potential to enhance specific immune function for prevention from disease-causing organisms in some tropical fish species.

Immune system in fish. The immune system is responsible for maintaining the organism's homeostasis and defence mechanism when invaded by a foreign organism (Mehana et al 2015). Foreign organisms such as bacteria, parasites, and viruses can be opportunistic microorganisms ordinarily present in the aquatic microflora (Tort et al 2003). Fish's immune system is divided into innate (nonspecific) immunity and adaptive (specific) immunity components. The innate immune system is the first-line defence mechanism of fish as well as plays a crucial role in activating an adaptive or specific immune response and maintaining cell homeostasis (Magnadóttir 2006; Uribe et al 2011; Rauta et al 2012). The innate immune system consists of humoral components, cellular parameters and epithelial/mucosal barriers. The humoral defence system in the innate immune system of fish includes various antimicrobial agents such as lysozyme, trypsin, antibacterial peptides, lectins, C-reactive protein/acute phase, complements, and transferrin. While, macrophages, neutrophils, T and B cells lymphocytes, natural killer (NK) cells, mast cells/eosinophilic granulocytes are the cellular defence system (Kiron 2012; Rauta et al 2012; Mehana et al 2015). Epithelial/mucosal barriers in the innate immune system provide protection associated with structure integuments and mucus, such as the mucosal barrier of skin, gills and intestine (Tort et al 2003).

The adaptive immune response plays a pivotal role to protect the host against reinfections by generating memory cells (cell-mediated immunity) as well as specific soluble and membrane-bound receptors (humoral immunity) to allow the fast and efficient elimination of the specific fish pathogens (Srivastava & Pandey 2015). Mechanisms of adaptive immune response involve specialised cells, proteins, genes, and biochemical messages. Those provide a means necessary for a host to respond, particularly to antigens, antibodies, and effector cells with high affinity. The adaptive or specific immune system components include antibodies, immunological memory, cellular cytotoxicity, and cytokines (Uribe et al 2011).

The components of the fish immune response can be generally divided into three factors: stimuli or input such as environment, pathogens, nutrition, antimicrobials, therapeutants, and vaccinations; recognition by the immunological system, and response mechanisms. These inputs are recognised by immune components such as innate, adaptive and mucosal as newly considered in a subsidiary branch based on some aspects. Outputs of response mechanisms provide pathogen clearance, memory response, and gut health regulatory (Bruce & Brown 2017). The cellular immune response involves cellular components within the plasma to combat foreign organisms. Phagocytic cells, granulocytes and macrophages, scan the inflammatory response and remove bacterial components. Furthermore, nonspecific cytotoxic cells degrade microbial through pattern recognition proteins (PRPs). The humoral immune response may diminish microbes via enzymatic degradation, such as lysozyme and prophenoloxidase (proPO) activating system (Srivastava & Pandey 2015; Bruce & Brown 2017).

Immunostimulants in aquaculture. Immunostimulant is a natural compound used commonly as an additive diet, and modulates the immune system by enhancing the

host's resistance against diseases. Natural immunostimulants are biodegradable, cost-effective, biocompatible, eco and human-friendly, safe for increasing diseases and stress resistance (Sakai 1999). Many natural compounds from various sources, such as bacterial components, chemical agents, animal or plant extracts, have been investigated as a prospective immunostimulant to combat infectious diseases in aquaculture (Srivastava & Pandey 2015). Recently, these compounds comprise a group of biological and synthetic compounds, may be chemical drugs or naturally extract compounds which improve immune response both the innate immunity (lysozyme, complement, phagocytic, bactericidal activity, respiratory burst activity, nitric oxide, total haemocytes, phenoloxidase) and adaptive immunity (antibodies and agglutination titre) as a defence mechanism against the infectious diseases (Mehana et al 2015; Srivastava & Pandey 2015). Immunostimulant increases immunocompetence and diseases resistance of fish as well as growth enhancement and increased survival rates under stress (Sahoo 2007; Mastan 2015; Wang et al 2016).

Nucleotides. Nucleotides are organic compounds that play vital roles in the various physiological functions (Carver & Walker 1995). As a non-protein nitrogen fraction, nucleotides consist of three main components including a heterocyclic nitrogenous bases either pyrimidine or purine, a pentose sugar (ribose or deoxyribose) and one or more phosphate groups (Hess & Greenberg 2012). Although nucleotides are categorised as a semi-essential nutrient, a high intake of nucleotides is required in certain conditions mainly during low protein intake or immunodeficiency conditions (Carver & Walker 1995; Carver 1999). Moreover, nucleotide could be obtained from all food sources as free-nucleotide and nucleic acid forms (Li & Gatlin 2006). However, the content of nucleotides in the food is influenced by the number of cell nuclei, and the number of cell nuclei in the animals were higher than in plant tissues (Jonas et al 2001). Thus, animal meats, fish meats and some shrimp contain high amounts of nucleotides, meanwhile, plant products such as cereal, beans, vegetables, mushroom and soybean products contain low amounts of nucleotide (Kaneko et al 2014).

In aquaculture, nucleotides are commonly supplemented onto fish diet in the forms of inosine (IMP), adenosine (AMP), guanosine (GMP), uridine (UMP), and cytidine (CMP) 5'-monophosphate disodium salts either singularly or in combination. It has long been used in the aquafeed as a feeding attractant to increase fish growth by elevating their feed intake and protein digestibility (Kubitza et al 1997; Lin et al 2009; Hossain et al 2016a). They are improving some digestive enzyme activities, such as amylase and lipase, which are also associated with the growth promotion effect of dietary nucleotides in fish (Selim et al 2020). In addition, dietary nucleotides also enhanced some immune parameters in fish either adaptive or innate immune responses, and thus, nucleotides are potential to replace antibiotics usage in aquaculture (Li & Gatlin 2006; Ringø et al 2012).

Investigation on the immuno-modulator effect of dietary nucleotides in the various tropical fish species have been reported previously as shown in Table 1. In details, administration of 240 mg kg^{-1} nucleotides in low fish meal-based diet increased lysozyme activity of hybrid tilapia (*Oreochromis niloticus* x *O. aureus*) (Shiau et al 2015). As a crucial component of the humoral innate immune response to prevent microbial infection in fish (Saurabh & Sahoo 2008), elevating lysozyme activity in hybrid tilapia by inclusion dietary of 0.15-0.30% nucleotides have also been reported by Xu et al (2015). Similarly, Reda et al (2018) found that supplementation of 0.05-0.25% nucleotides enhanced lysozyme activity of Nile tilapia (*O. niloticus*). Moreover, superoxide anion (O_2^-) production in fish was also influenced by dietary nucleotides (Lin et al 2009; Shiau et al 2015). Superoxide anion radical (O_2^-) has a critical function for immune defence due to its ability against pathogen organisms (Biller & Takahashi 2018). Supplementation of 0.15% either mixed-nucleotides or individual nucleotides i.e IMP, AMP, GMP, UMP and CMP improved superoxide anion (O_2^-) production in the head kidney of grouper (*Epinephelus malabaricus*) (Lin et al 2009). Shiau et al (2015) also reported that the production of superoxide anion (O_2^-) in the head kidney of hybrid tilapia fed 240 mg kg^{-1} nucleotides was higher than control diet without nucleotides supplementation.

Dietary nucleotides have also been reported to modulate antibody response of hybrid tilapia by increasing globulin concentration and reducing albumin-to-globulin ratio (A:G) in the serum (Xu et al 2015). Increasing total protein and globulin concentrations, and reducing A:G ratio was also recorded by Reda et al (2018) in Nile tilapia fed 0.25% nucleotides. Furthermore, total immunoglobulin (Ig) concentration in fish was also significantly influenced by dietary nucleotides (Lin et al 2009; Reda et al 2018). Lin et al (2009) found that Ig concentration in the plasma of grouper fed 0.15% nucleotides was higher compare to un-supplemented diet. A similar finding has also been reported by Reda et al (2018) who found that the IgM level of Nile tilapia fed 0.25% nucleotides was significantly higher than IgM level in fish fed nucleotide-free diet in the 15 days feeding period. However, changing IgM level in the serum was not detected after 30 days feeding trial. IgM is present in the most of fish species, and plays vital roles in both adaptive and innate immunity in fish (Mashoof & Criscitiello 2016). Thus, a significant effect of nucleotides on Ig concentration indicated that nucleotides effectively improved immune system in fish. Enhancing the immune system in fish by dietary nucleotides have also been reported to be associated with increasing gene expression of immunoglobulin M (IgM) and recombinase activating gene 1 (RAG-1) in the gill and spleen, and cytokine expression (interleukin-1β) (Low et al 2003).

A haematological profile is also an important tool that can be used for assessing and monitoring the physiological and health status of fish (Fazio 2019). Selim et al (2020) reported that the administration of dietary nucleotides significantly affected some haemato-immunological parameters such as red blood cells, haemoglobin, and mean corpuscular haemoglobin concentration in Nile tilapia. White blood cells, lymphocytes, and granulocyte also improved by administration dietary of 0.05-0.25% nucleotides (Reda et al 2018). A study by Hassaan et al (2018) found that dietary nucleotides also significantly elevated white and red blood cell contents in the plasma of Nile tilapia. However, haematocrit and haemoglobin contents were not affected by dietary nucleotides in their study.

In addition, increasing some immune parameters by dietary nucleotides was also in line with elevating the resistance of fish against various pathogens in some previous studies (Shiau et al 2015; Kader et al 2018; Reda et al 2018). In details, Shiau et al (2015) reported that survival rate of hybrid tilapia fed 120 mg kg^{-1} nucleotides had significantly 41.1% higher compared to an un-supplemented diet after being challenged with *Streptococcus iniae*. Reda et al (2018) also found that mortality of Nile tilapia after the challenge with *Aeromonas sobria* decreased by the inclusion of 0.25% nucleotides in the diet. Moreover, dietary administration of 1-2% nucleotides significantly improved the resistance of Nile tilapia to *S. iniae* (Kader et al 2018).

Nucleotides also affect antioxidant status by increasing superoxide dismutase (SOD) enzyme activity and decreased malondialdehyde (MDA) content in the liver of hybrid tilapia (Xu et al 2015). A similar finding has also been reported by Reda et al (2018) who found that dietary supplementation of 0.25% nucleotides increased SOD and MDA enzymes activities in the serum of Nile tilapia. A study by Zhao et al (2017) also found that supplementing 0.4% nucleotides in the diet significantly increased SOD and catalase (CAT) enzymes activities in the liver of yellow catfish (*Pelteobagrus fulvidraco*) comparing to fish fed control (0%) and 0.2% nucleotides-based diets. SOD, catalase, glutathione peroxidase (GPXs), and transferases are known as important enzymes for detoxification of reactive oxygen species that may cause damage to an organism (Slaninova et al 2009). Thus, the improvement of those enzymes by dietary nucleotides contributed to enhancing the health status of fish. In addition, besides improved liver status by decreasing alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) contents in the serum of fish fed nucleotides-enriched diet (Hassaan et al 2018; de Lima et al 2020), dietary nucleotides also prevent lipid oxidation by increasing thiobarbituric acid reactive substance (TBARS) in the yellow catfish (Zhao et al 2017).

Table 1

Nucleotide supplementation in the tropical aquaculture

Species	*Nucleotides*	*Dosage*	*Initial weight; rearing period*	*Results*	*References*
Nile tilapia *Oreochromis niloticus*	AccelerAid®	0, 0.5, 1.0, 2.0 and 4.0%	4.74 g; 60 days	Enhanced survival to *Aeromonas hydrophila*.	Barros et al (2015)
	NuPro®	0, 0.1, 0.2, 0.4, 0.6 and 0.8%	2.63 g; 75 days	Increased growth, thrombocyte, leucocyte and monocyte in blood.	Berto et al (2016)
	IMP	0, 0.1, 0.2, 0.4 and 0.8%	0.59 g; 60 days	Increased growth, hyperplastic and hypertrophic fiber frequencies; upregulated the expression of *GH, GHR-1, IGF-1, MyoD, Pax7* and *myostatin* genes.	Asaduzzaman et al (2017)
	Nucleoforce™	0, 0.05, 0.15 and 0.25%	42.9 g; 30 days	Enhanced TP, total globulin, WBC, lymphocyte, granulocyte; decreased albumin, ratio albumin to globulin; increased lysozyme, nitric oxide, IgM, antiprotease, SOD, GPX, glutathionine peroxidase, MDA; upregulated the relative expression of intestinal cytokine *TGF-β, IL-1β, IL-10β* and *TNF-α* genes; decreased mortality to *Aeromonas sobria*.	Reda et al (2018)
	Nucleoforce™	0, 0.05, 0.15 and 0.25%	42.9 g; 60 days	Increased growth and feed utilization, RBC, Hb, platelets, MCHC, AST, ALP, urea, lipase, amylase and growth hormone; upregulate the relative expression of *ghrelin* and *Insulin-like growth factor* genes.	Selim et al (2020)
	IMP	0, 0.01, 0.02, 0.04 and 0.08%	0.59 g; 60 days	Increased growth, Hct, total serum protein, lysozyme, SOD, bacterial activities, and survival to *Streptococcus agalactiae*.	Kader et al (2018)
	Ascogen®	0, 0.25, 0.5, 0.75 and 1%	286 g female; 536 g male; 150 days	Increased growth and feed utilization, Hb, albumin, Gly, SOD and GST; decreased ALT, ALP, calcium, TG and ovary oogonia.	de Lima et al (2020)
Hybrid tilapia (*Oreochromis niloticus* × *O. aureus*)	Rovimax NX	0, 120, 240, 360, 480 and 600 mg NT kg^{-1}	0.15 g; 10 weeks	Enhanced lysozyme, superoxide anion and survival to *Streptococcus iniae*.	Shiau et al (2015)
	Biotogether	0, 0.15, 0.30, 0.6 and 1.2%	8 g; 8 weeks	Increased growth, SOD and lysozyme; decreased MDA and putative butyrate-production.	Xu et al (2015)
Channel catfish *Ictalurus punctatus*	Mixed-nucleotide	0, 0.1, 0.3, 0.9 and 2.7%	14.4 g; 8 weeks	Enhanced antibody titre, alternative complement (SH_{50}), bacterial activity; decreased cortisol level.	Welker et al (2011)
Yellow catfish *Pelteobagrus fulvidraco*	Mixed-nucleotide	0, 0.02, 0.04, 0.06, 0.1 and 0.15%	1.53 g; 56 days	Increased growth, cholesterol, SOD; decreased TBARS	Zhao et al (2017)

Grass carp *Ctenopharyngodon idella*	Mixed-nucleotide	0, 0.2, 0.4, 0.6, 0.8 and 1.0%	200 g; 60 days	Increased growth, umami and sweetness amino acid, water holding capacity, tenderness, antioxidant enzyme, EPA and DHA.	Tie et al (2019)
Grouper *Epinephelus malabaricus*	Mixed-nucleotide; IMP, AMP, GMP, UMP, CMP	0, 0.05, 0.1, 0.15 and 0.2%; 0.15%	5.90 and 10.33g; 8 weeks	Increased growth, superoxide anion and Ig concentration.	Lin et al (2009)
Amberjack *Seriola dumerili*	NBP, Ajinomoto	0, 0.3 and 0.9%	26 g; 50 days	Increased feed intake, blood urea nitrogen, enterocyte height of anterior intestine; decreased TG and CAT activities.	Hossain et al (2016b)
	Inosine	0, 0.1, 0.3, 0.6 and 0.9%	26 g; 50 days	Increased growth, Hct, blood urea nitrogen, lysozyme, peroxidase and bacterial activities; increased enterocyte height of anterior intestine.	Hossain et al (2017)
	Inosine	0 and 0.6%	25 g; 56 days	Improved digestibility, immune responses and intestinal morphology.	Hossain et al (2018)
Whiteleg shrimp *Litopenaeus vannamei*	Nucleoforce™	0, 0.05 and 0.1%	4.24 g; 70 days	Enhanced lysozyme activity and survival to *Vibrio harveyi*.	Novriadi et al (2021)
	Nucleotides-rich yeast	0, 1.0, 3.0, and 5.0%	1.86 g; 8 weeks	Increased growth performance and feed utilization, crude protein content in the whole body, TP and TG; decreased AST and ALT; increased phenol oxidase and lysozyme activity, fold height, fold width and microvillus height; upregulated of the relative expression of ALP and lysozyme genes.	Xiong et al (2018)
	Rovimax NX	0, 60, 90, 120 and 1200 mg kg^{-1}	0.39 g; 10 weeks	Increased the activities of acid phosphatase, total nitric oxide synthase, SOD, lysozyme, and respiratory burst; increased THC; increased jejunum wall thickness and villus height.	Guo et al (2016)
	Optimun	0, 0.2 and 0.5%	2.92 g; 30 days	Increased final weight, survival, respiratory burst and THC.	Murthy et al (2009)
	Nucleoforce™	0 and 0.05%	12.05 gr; 56 days	Increased growth and feed utilization, crude protein and fiber contents, but decreased crude lipid and ash content in the whole body; increased THC, phagocytosis, phagocytic index, total protein, acid phosphatase, ALP, lysozyme, phenoloxidase, SOD, and total nitric oxide activities; upregulated the relative expression of *cMnSOD*, *penaeidin4, HSP70* genes, and downregulated the relative expression of *TCTP* gene.	Abdel-Rahim et al (2021)

	IMP, GMP, mixed IMP and GMP, mixed IMP, GMP, UMP and CMP	0, 0.025, 0.05 and 0.1%	0.99 g; 8 weeks.	IMP and GMP increased final body weight and body weight gain; IMP and GMP also increased GLU and TG after challenged at 70 mg L^{-1} ammonia.	Yong et al (2020)

ALP, alkaline phosphatase; ALT, alanine aminotransferase; AMP, adenosine 5'-monophosphate; AST, aspartate aminotransferase; CAT, catalase; CMP, cytidine 5'-monophosphate; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GLU, glucose; Gly, glycogen; GMP, guanosine 5'-monophosphate; GPx, glutathionine peroxidase; GST, glutathione s-transferase; Hb, haemoglobin; Hct, hematocrit; IMP, inosine 5'-monophosphate; MCHC, mean corpuscular haemoglobin concentration; MDA, malondialdehyde; RBC, red blood cells; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substances; TG, triglyceride; UMP, uridine 5'-monophosphate; THC, total hemocyte count; TP, total protein; WBC, white blood cells.

Organic acids. Organic acids and their salts or blend have been used for feed additives in aquaculture that might increase nutrient digestibility, growth performance and feed utilisation efficiency (Lim et al 2015). It is also potential as a stimulatory immune system, improve disease prevention, and affects microbial composition on the digestive tract feed utilisation efficiency in aquaculture production in a non antibiotic way (Lückstädt 2006, 2008; Lim et al 2015; Mendoza Rodriguez et al 2017). Thus, organic acids and/or salts are a potential substitute for antibotic promoters in aquafeeds (Ng & Koh 2017).

Additives like acidifiers play signifcant role in aquaculture diets (Lückstädt 2008). Fish culture performance, particularly in feeding treatment that used organic acid as diet supplementary, was influenced by species, size, age, level of organic acids and their salts, composition of nutrient, culture condition and feeding management (Lim et al 2015). Growth promoting of dietary oganic acid affected by the type and level of organic acids, diet composition, species, physiological age and culture condition (Ng et al 2009). Organic acids and/or salts are commonly used in aquaculture feed including formic acid, acetic acid, propionic acid, and butyric acid as shown in Table 2.

Formic acid. The formula of formic acid is H-COOH or CH_2O_2 in liquid form with 46.03 g mol^{-1} of molecular mass, 1.22 g mL^{-1} of density, 3.75 of pK-value, 48.0 g of molecular weight, 5.8 KJ g^{-1} of gross energy and odour is pungent (Mroz 2005; Freitag 2007; Lückstädt 2008). Formic acid is a small fatty acid with excellent solubility in water (Freitag 2007).

Formic acid is one of the short-chain fatty acids that have antimicrobial effects in some species of bacteria or fungi and potential to become alternatives to antibiotics and dietary in aquaculture (Freitag 2007; Adams & Boopathy 2013; Elala & Ragaa 2015). The valuable impacts of formic acid are specifically for growth-promoting and improving protein digestibility (Tran-Ngoc et al 2019). Salts of formic acid, potassium diformate (KDF), are used for non-antibiotic growth promoters and could be utilised in tropical fish (Zhou et al 2009; Elala & Ragaa 2015). Komar (2008) indicated that the first substance used as a non-antibiotic growth promoter includes 35% free formic acid, 35% formate and 30% potassium which approved by the European Union.

Some studies reported dietary salts of formic acid such as KDF in tropical fish. Elala & Ragaa (2015) reported that supplementation of 0.2% and 0.3% KDF in the diet of Nile tilapia raised growth performance, feed intake, live weight gain, feed conversion ratio (FCR), the protein efficiency ratio (PER) and protein digestibility. This experiment has a crucial effect on fish's cellular and humoral non-specific immunity, and improved immune response stimulation. Furthermore, Zhou et al (2009) also found that the addition of 3.0 and 6.0 g kg^{-1} DF in the diet of hybrid tilapia indicated that final body weight and specific growth rate (SGR) were higher than other diet levels. Moreover, there was no impact on survival, growth performance and FCR. In another study, Ng et al (2009) concluded that dietary of 2 g kg^{-1} KDF in red hybrid tilapia improved growth, feed utilisation and nutrient digestibility. In addition, Ramli et al (2005) studied that supplementation of 0.2 and 0.5% potassium diformate in hybrid tilapia cultured in 85 days experiment which fish were challenged orally with *V. anguillarum* on day 10 for 20 days showed that feed intake, live weight gain, FCR and PER were significantly increased.

Lim et al (2010) also investigated the different levels of KDF in Nile tilapia diets were 0.25, 0.50, 0.75, 1.00, 1.25 and 1.50% respectively. The result showed that there were no effects on survival, immunological responses, haematological parameters and resistance against to *S. iniae* bacteria during 12 weeks of treatment. On the other hand, the dietary increased weight gain and feed efficiency with up to 10 g kg^{-1} inclusion. In addition, Cuvin-Aralar et al (2011) reported supplementation of 0.3% KDF increased growth performance and feed conversion of male Nile Tilapia.

Dietary KDF has also been reported to decrease bacterial growth population in the gut of hybrid tilapia (Zhou et al 2009). It has been known that organic acids have specific roles. For instance, reducing the pH level in the small intestine and stomach, preventing the growth of gram-negative bacteria through cell wall penetration and inhibiting microbial activity in the intestinal tract of aquatic animals (Freitag 2007; Lückstädt 2008; Bai et al 2015), diminishing potential microbial contamination such as pathogenic bacteria and fungi for feed storage (Lim et al 2015).

Formic acid has an inhibitory effect on the growth of pathogenic bacteria *Vibrio harveyi* (Mine & Boopathy 2011). Furthermore, growth of other marine pathogenic Vibrionaceae including *V. alginolyticus, V. cholerae, V. parahaemolyticus* and *V. vulnificus* have also been inhibited by dietary formic acid (Adams & Boopathy 2013). According to their results, the effective concentration (EC_{50}) values of formic acid for those species were at low concentration with less than 0.039%. The addition of KDF in the diet of Nile tilapia may reduce total bacterial in faeces and increased resistance against of *Aeromonas hydrophila* (Elala & Ragaa 2015). Supplementation of 3.0 and 6.0 g kg^{-1} KDF in hybrid tilapia dietary diets also increased the relative richness of intestinal bacteria such as *Mycobacterium* sp. and *Pseudomonas* sp. (Zhou et al 2009). Ramli et al (2005) determined that a 0.2% dose of KDF may be an effective tool to manage bacteria in tilapia culture and may enhanced resistance against of *V. anguillarum.* Ng et al (2009) stated that bacteria *A. hydrophila* in the gut of red hybrid tilapia were significantly declined in a 0.2% KDF containing diet. Komar (2008) used *S. agalactiae* for bacterial challenge test in tilapia. This bacteria is indicated as one of the primary bacterial pathogens influencing fish culture.

Formic acid in diet had also significant impacted intestine pH (Vielma & Lall 1997). They observed a higher value of gut pH caused by alteration of supplemented feed of formic acid to hydrochloric acid and bile accretion. Dietary of KDF declined diet pH, leading to a reduction in the digesta pH of the gut and stomach (Ng et al 2009). Lim et al (2015) studied that low pH as a natural barrier could diminish growth rates of gram-negative bacteria. Furthermore, hydrochloric acid concentrations in the stomach decrease when the diet contains high protein. This depletion negatively affects pepsin activation, pancreatic enzyme secretion and impairs digestion. Tran-Ngoc et al (2019) also reported that inclusion of 1.4 g kg^{-1} formic acid in the Nile tilapia diet decreased the concentration of cyme pH in the stomach during the normoxic condition (from 6.8 to 6.2).

Javid et al (2021) studied organic acids combination of formic acid and lactic acid (1:1) for diet supplementation of carp (*Labeo rohita*). The result exhibited that feed supplemented with organic acids blend showed significantly enhanced growth parameters such as final weight, weight gain, feed intake and SGR, and reduced FCR. The addition of organic acids blend in the diet might increase the digestibility of nutrients by lowering the gastrointestinal pH. Those diets also rose significantly in minerals absorption (P, Ca, Na and Cu).

Another salt of formic acid used in aquaculture is Na-diformate (NaDF) (Liebert et al 2010; Ng & Koh 2017). Liebert et al (2010) used 0.3% and 0.5% dietary NaDF in male Nile tilapia fingerlings. The result indicated that the addition of 0.3% NaDF in the diet enhanced growth, feeding efficiency, protein efficiency ratio and protein retention efficiency.

Acetic acid. The formula of acetic acid is CH_3COOH or $C_2H_4O_2$ in liquid form with 60.05 g mol^{-1} of molecular mass, 1.05 g mL^{-1} of density, 4.76 of pK-value, 60.1 g of molecular weight, 14.8 KJ g^{-1} of gross energy and solubility in water is very good and odour is pungent (Mroz 2005; Freitag 2007; Lückstädt 2008).

Acetic acid is one of the organic acids commonly used as feed additive or dietary organic particularly in aquaculture. This substance is produced by lactic acid bacteria (LAB), which have an inhibitory impact on the growth of several fish pathogens (Planas et al 2004; Elala & Ragaa 2015). According to Lim et al (2015), acetic acid can be used as a mould inhibitor and are effective in a minimum concentration of 0.5%. Mine & Boopathy (2011) also reported that acetic acid has an inhibitory effect on the growth of pathogenic bacteria *V. harveyi*.

Table 2

Organic acids supplementation in the tropical aquaculture

Species	*Organic acids*	*Dosage*	*Initial weight; rearing period*	*Results*	*References*
Nile tilapia *Oreochromis niloticus*	Potassium diformate	0, 0.1, 0.2 and 0.3%	53.5 g; 60 days	Increased growth and feed utilization, total lactic acid bacteria, phagocytic activity and index, lysozyme in the serum and intestinal mucus; decreased stomach pH, mortality to with *Aeromonas hydrophila*.	Elala & Ragaa (2015)
	Potassium diformate	0 and 0.3%	7.84 g; 78 days	Increased growth and feed utilization.	Cuvin-Aralar et al (2011)
	Organic acid blend and organic salts blend	0, 0.5, 1.0 and 1.5%	7.05 g; 12 weeks	Increased growth and feed utilization, Hb, Hct, TP, total lipid, digestibility of dry matter and crude protein; decreased ALT, AST, digestibility of crude lipid and ash.	Soltan et al (2017)
	Organic acid blend	0, 0.1 and 0.2%	28.8 g; 60 days for growth trial and 30 days for immunity test	Increased growth and feed utilization, RBC, Hb, platelets, MCV, MCHC, WBC, lymphocyte and neutrophil, lysozyme and nitric oxide, crude protein and crude lipid contents in the whole body of fish; decreased total bacterial intestinal population and mortality to *Aeromonas sobria*; and upregulated the relative expression of interleukin-1β (*il-1β*) and tumor necrosis factor-alpha (*tnf-α*) genes in the liver and kidney.	Reda et al (2016)
	Propionic acid	0 and 20 mg kg^{-1}	52 g; 2 weeks for antibacterial test, and 2 weeks for immunomodulatory test	Enhanced Hb, RBCs, thrombocyte, WBC, lymphocyte, ALT, AST, urea and creatine; increased TP, globulin, phagocytic activity, phagocytic index, lysozyme and serum IgM; decreased mortality to *Aeromonas hydrophila*.	El-Adawy et al (2018)
	Sodium butyrate	0, 0.5, 1.0, 2.0 and 3.0%	1.0 g; 45 days	Increased growth and feed utilization; decreased crude protein and ash contents in the whole body of fish.	Ali et al (2018)
	Butyric acid	0, 0.5, 1.0, 1.5 and 2.0%	25.5 g; 12 weeks	Improved growth and feed utilization.	Omosowone et al (2018)
Hybrid tilapia (*Oreochromis niloticus* × *O. aureus*)	Potassium diformate	0, 0.3, 0.6, 0.9 and 1.2%	2.67 g; 8 weeks	Increased growth and abundance of intestinal bacteria such as *Mycobacterium* sp., *Mycobacterium peregrinum*-like and *Pseudomonas*-like.	Zhou et al (2009)

	Organic acid blend	0, 0.1, 0.2 and 0.3%	6.90 g; 14 weeks	Decreased total bacterial count in the faeces and mortality to *Streptococcus agalactiae*.	Ng et al (2009)
	Organic acid belnd	0 and 0.092%	7.0 g; 9 weeks	Improved villus height; decreased digestibility of dry matter, crude protein, calcium, phosphorus, iron, and copper.	Huan et al (2018)
Carp *Labeo rohita*	Organic acid blend	0, 0.15 and 0.3%	5.21 g; 8 weeks	Increased growth and feed utilization, digestibility of dry matter, crude protein, crude fiber, phosphorus, calcium, magnesium, natrium, kalium, zinc, cuprum, iron, manganese and lipase activity.	Javid et al (2021)
Catfish *Clarias gariepinus*	Organic acid blend, butyric acid	0, 0.05 and 0.1%	8.78 g; 56 days	Increased growth and feed utilization.	Asriqah et al (2018)
	Butyric acid	0, 0.5, 1.0, 1.5 and 2.0%	42.39 g; 12 weeks	Improved growth and feed utilization.	Omosowone et al (2018)
Whiteleg shrimp *Litopenaeus vannamei*	Nutrifarma	0 and 0.5%	1.1 g; 30 days	Increased GLU, monocyte, height of villi, number of goblet cells in the anterior intestine and survival rate; decreased the concentration of total heterotrophic bacteria and *Pseudomonas* sp.	Addam et al (2019)
	Propionic acid	0 and 0.5%	10.2 g; 60 days	Upregulated the relative expression of lysozyme (*Lys*), crustin (*Cru*) and prophenoloxidase (*proPro*) genes, and down-regulated the relative expression of penaeidin-3a (*Pen-3a*) gene.	Pourmozaffar et al (2017)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; GLU, glucose; Hb, haemoglobin; Hct, hematocrit; MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; RBC, red blood cells; TP, total protein; WBC, white blood cells.

Addam et al (2019) determined effects of supplementation dietary of organic acids blend 0.5% including ammonium formate (126.5 g kg^{-1}), formic acid (115.5 g kg^{-1}), vegetable fatty acids (82.5 g kg^{-1}), propionic acid (66.0 g kg^{-1}) and acetic acid (55.0 g kg^{-1}) in Nile tilapia. Their result showed no improvement in growth and feed conversion ratio (FCR). Furthermore, pH decreased to 0.92, and the concentration of total heterotrophic bacteria and *Pseudomonas* sp. in the intestine declined significantly. Nevertheless, survival and concentration of glucose were enhanced. They concluded that the diet led to alteration of the morpho-structure of the gut tract and decreased the number of lymphocytic infiltrates in the liver.

Improvement in growth, digestibility of dry matter, crude protein, minerals and protein retention of hybrid tilapia were also observed by a combination of alkaline protease and a mixture of organic acid salts i.e calcium-propionate, calcium-formate and natrium-acetate in the diet (Huan et al 2018). Similarly, Soltan et al (2017) determined organic salts blend with composition 1:1 calcium lactate and sodium acetate at different doses (0.5, 1.0, and 1.5%) as dietary supplementation of Nile tilapia. The result exhibited significant improvements in final body weight, body length, weight gain, and specific growth rate of Nile tilapia. In addition, feed intake, FCR and PER were also increased. Valencia & Chavez (2002) concluded that acetic acid blended with phytase could promote mineral digestibility particularly in phosphorus and calcium.

The effective concentration for rearing the milk fish (*Chanos chanos*) larvae have also been investigated at 0.05, 0.05, 0.025 and 0.075% for acetic acid, malic acid, formic acid, and citric acid, respectively. The addition of organic acid increased survival rates of larvae, and reduced the pathogenic *Vibrio* spp. (Kumar et al 2018).

Propionic acid. Propionic acid (CH_3CH_2COOH) is corrosive, colourless, with a rancid odour as well as soluble in alcohol, chloroform, and ether. In high concentration, it irritates the mucous membrane and skin. Propionic acid can be obtained by chemical synthesis (Reppe process, Larson process, Fisher-Tropsch process) and microbial biosynthesis, a fermentation process using gram-positive bacteria (*Clostridium* spp. and *Propionibacterium* spp.) and gram-negative bacteria (*Veillonella* spp., *Fusobacterium* spp. and *Selenomonas ruminantium*) (Eş et al 2017).

Propionic acid has beneficial effects for aquacultural production due to improved feed utilisation, growth performance, immune response, and diseases resistance. Previous research reported that dietary supplementation of organic acids, including propionic acid, improved feed-biting behaviour and the growth performance in tilapia (Ng et al 2009; Reda et al 2016), as well as the growth of various fish and shrimp species (Mine & Boopathy 2011; Pourmozaffar et al 2017). Supplementation with propionic acid increases protein and fat in the body of Nile tilapia, and it may be due to the improved digestibility of nutrients with these supplementations. It also improved immune status in Nile tilapia, evidenced by high serum bactericidal percentages, lysozyme activities and nitric acid assay (Reda et al 2016), as well as enhanced white blood cell counts, particularly lymphocytes (El-Adawy et al 2018). Previous studies reported that propionic acid has antimicrobial effects to combat pathogenic microorganisms in tilapia challenged with *S. agalactiae* (Ng et al 2009; Ng & Koh 2017), *Aeromonas sobria* (Reda et al 2016), *A. hydrophila* (El-Adawy et al 2018), in shrimp challenged with *V. harveyi* (Mine & Boopathy 2011). This evidence revealed that propionic acid enhanced resistance against pathogens in farmed aquatic animals.

Butyric acid. Butyric acid is a saturated short-fatty acid with a 4-carbon backbone (Załęski et al 2013). The molecular weight from butyric acid is 88.11 g mol^{-1}. Butyric acid appears as a colourless liquid with a penetrating and unpleasant odour. Butyric acid is also known as butanoic acid, magnesium butyrate or sodium butyrate. Butyric acid has several potential applications in industry (Zigova & Šturdík 2000) such as fuel industry (Dwidar et al 2012), foods and beverages (Annunziata et al 2020) and aquaculture (Robles et al 2013; Estensoro et al 2016; Hoseinifar et al 2017; Ali et al 2018; Asriqah et al 2018; Omosowone et al 2018).

Butyric acid is thought to play several beneficial roles in the gastrointestinal tract (Borycka-Kiciak et al 2017). Several functions from butyric acid are its anion used as a source of cell energy, play as regulator colonocyte proliferation and apoptosis, gastrointestinal tract motility and bacterial microflora composition in addition to its involvement in many other processes including immunoregulation and anti-inflammatory activity (Załęski et al 2013; Miguel et al 2018). In animal nutrition, butyrate is used since it promotes increased in body weight and benefits the intestinal tract. A study on juvenile sea bream (*Sparus aurata*) showed a significant increase in the weight of fish receiving butyrate (Robles et al 2013). Butyrate might increase transmethylation activity, reducing fat deposition, apoptosis and accumulation of damaged protein (Kharbanda 2007). It also increases the availability of several essential amino acids and nucleotide derivatives. Thus, the energy provision for enteric cells might have been enhanced by a decrease in glucose and amino acid oxidation related to the use of butyrate as fuel (Robles et al 2013). However, the addition of butyrate as a supplement diet can give positive results influenced by several factors such as duration of trials, fish age/or size, diet formulation, and dosage (Estensoro et al 2016). Positive results on the use of butyrate as feed additive also occurred in freshwater fish such as *Clarias gariepinus* and *O. niloticus*. (Ali et al 2018; Omosowone et al 2018).

Adding butyrate to the fish diet affects the gut microbiome. Butyrate is a bacterial product that converts indigestible carbohydrates into short-chain fatty acids, including butyrate. When the host sense butyrate, it will trigger some responses such as strengthening the epithelial barrier, reducing inflammation and increasing the production of mucins (Onrust et al 2015). It also increases intestinal microbiota diversity as well as restores gut integrity and function (Piazzon et al 2017). The Firmicutes phylum includes different genera of lactic acid bacteria such as *Streptococcus, Lactobacillus* and

Leuconostoc increased in the intestinal gut. They are thought to be beneficial microorganisms associated with a healthy intestinal epithelium (Rimoldi et al 2018).

Furthermore, Piazzon et al (2017) explained that butyrate supplementation helps to enhance fish survival rate that has been infected by *Photobacterium damselae*, a gram-negative bacterium that cause photobacteriosis. It restored the expression of genes related to epithelial permeability, structure, mucus production and restored transepithelial electric resistance. Most genes whose expression was modulated at gut anterior are related to immune function (cytokines and PRRs or Pattern Recognition Receptors) (Estensoro et al 2016).

Conclusions and future research. Immunostimulants are considered alternative antibiotics that will boost the immune system in aquaculture organism. Among available immunostimulant agents, nucleotides and organic acids can prevent disease outbreaks by enhancing some immune responses in fish. The immunomodulator effect of those components was influenced by several factors, including species, size, age, the dosage of those immunostimulants, the composition of nutrients, culture condition and feeding management.

In addition, selecting suitable immunostimulants to increase the protection of fish is also a difficult task and competitive research field. For instance, the source of immunostimulants can be obtained from bacteria, animals and plants. Then, discovering those immunostimulant agents from various sources and their application in fisheries is needed mainly for immunostimulant agents that have potential against a broad spectrum of pathogens, are competitive in price and are efficient in preparation. Moreover, utilisation of natural immunostimulant compounds in the fish diet is also intriguing to be more developed in order to support sustainable and eco-friendly aquaculture.

Acknowledgements. This work was fully funded by the Indonesian Institute of Sciences (LIPI) through COREMAP-CTI 2021–2022 (17/A/DK/2021) program.

Conflict of interest. The authors declare that there is no conflict of interest.

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Received: 24 February 2022. Accepted: 26 May 2022. Published online: 01 August 2022.
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How to cite this article:
Ridwanudin A., Anggorowati D. A., Indriana L. F., Fahmi V., 2022 Current status of immunostimulant agents in tropical aquaculture: a review. AACL Bioflux 15(4):1908-1925.