EFFECTS OF DIETARY ORGANIC ACIDS ON GROWTH PERFORMANCE OF RED HYBRID TILAPIA, *Oreochromis* sp., AND INHIBITION OF SELECTED FISH PATHOGENS

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LIST OF ABBREVIATIONS

AGP	Antibiotic growth promotant
BHI	Brain heart infusion
CFU	Colony-forming units
CMC	Carboxymethyl cellulose
FCR	Feed conversion ratio
HSI	Hepatosomatic index
IPF	Intraperitoneal fat
KDF	Potassium diformate
Mt	Metric ton
NA	Nutrient agar
NaFish	National Fish Health Research Centre
NB	Nutrient broth
NFE	Nitrogen-free extract
OAB	Organic acids blend
OD	Optical density
OTC	Oxytetracycline dehydrate
PER	Protein efficiency ratio
rpm	Revolution per minute
SGR	Specific growth rate
sp. or spp.	Species (for singular or plural term)
ssp.	Subspecies
v/v	Volume/ volume
VSI	Viscerosomatic index
w/v	Weight/ volume

KESAN ASID ORGANIK DALAM DIET KE ATAS PRESTASI PERTUMBUHAN TILAPIA HIBRID MERAH, *Oreochromis* sp. DAN PERENCATAN PATOGEN IKAN TERPILIH

ABSTRAK

Asid organik yang berantai pendek berpotensi untuk menjadi pengganti antibiotik dengan berfungsi sebagai profilaktik kepada penyakit bakteria, dan juga meningkatkan pertumbuhan ikan. Tiga kajian yang berkaitan telah dijalankan. Kajian pertama direkabentuk untuk menilai kesan antibakteria bagi pelbagai asid organik yang berantai pendek terhadap *Aeromonas hydropilia* untuk menghasilkan satu campuran asid organik (OAB) yang unik bagi haiwan akuatik. Aktiviti *in vitro* antibakteria OAB terhadap *A. hydropilia* dan *Streptococcus agalactiae* telah dibandingkan dengan produk komersial asid organik, kalium diformate (KDF) dan antibiotik yang sering digunakan oksitetrasiklin (OTC). Semua asid organik yang dikaji dapat menghalang pertumbuhan *A. hydropilia* sepenuhnya pada kepekatan 0.3% dan ke bawah. Asid formik merupakan asid yang paling cekap dalam merencat pertumbuhan *A. hydrophila*, diikuti oleh asid tartarik, asid laktik, asid propionik, asid sitrik dan asid malik. Hasil kajian juga menunjukkan bahawa OAB mempunyai aktiviti antibakteria yang lebih kuat daripada KDF atau OTC apabila kepekatan 0.2% atau lebih tinggi digunakan. OAB juga memaparkan aktiviti bakterisid yang kuat terhadap *S. agalactiae*. Dalam bahagian kedua kajian ini, dua ujikaji telah dijalankan untuk menyelidik aplikasi praktikal asid organik dalam industri akuakultur. Dalam ujikaji kedua, ikan diberi diet ujikaji selama 14 minggu untuk menentukan kesan pemakanan asid organik. Diet ujikaji telah ditambah dengan 0, 1, 2 atau 3 g kg^{-1} OAB, atau dengan 2 g kg^{-1} KDF dan diberi makan kepada kumpulan triplikat ikan tilapia hibrid merah (*Oreochromis* sp.). Selepas 14 minggu, tilapia dicabar melalui perendaman dengan *S. agalactiae*. Tiada perbezaan yang signifikan ($P>0.05$) diperhatikan bagi pertumbuhan atau pencernaan nutrien, walaupun ikan yang diberi diet asid organik menunjukkan trend keputusan yang lebih baik. Asid organik menurunkan pH diet, mengakibatkan penurunan pH dalam digesta perut dan usus. Jumlah bakteria dalam najis telah dikurangkan secara signifikan ($P<0.05$) bagi ikan yang diberi diet asid organik. Trend yang sama diperhatikan bagi bakteria yang terikat pada usus. Tambahan pula, ikan yang diberi diet OAB menunjukkan pertahanan yang lebih baik terhadap jangkitan *S. agalactiae*. Dalam ujikaji ketiga, satu kajian berjangka 20 minggu dengan keadaan yang lebih merupai penternakan komersial telah dijalankan untuk mengenalpasti pertumbuhan dan percernaan nutrien tilapia dengan pemberian makanan yang ditambah dengan 0, 5 atau 10 g kg^{-1} OAB II, atau dengan 5 g kg^{-1} OTC, dan seterusnya rintangan ikan terhadap jangkitan *S. agalactiae*. Tambahan OAB II dalam makanan meningkatkan berat badan ikan tilapia hampir 12% serta meningkatkan kecekapan pemakanan walaupun tidak berbeza secara signifikan. Lebih-lebih lagi, pencernaan fosforus ikan meningkat dengan signifikan apabila 10 g kg^{-1} OAB II telah ditambah kepada diet ikan tilapia. Seperti yang diperhatikan dalam ujikaji kedua, jumlah bakteria dalam najis dan usus telah dikurangkan secara signifikan bagi ikan yang diberi diet OAB II. Penambahan OAB II sebanyak 5 g kg^{-1} dalam diet mempunyai kesan seperti 5 g kg^{-1} OTC bagi melindungi ikan daripada jangkitan streptokokus. Tiada sisa antibiotik dikesan pada tisu ikan yang diberi diet OAB II berlawanan dengan ikan yang diberi diet OTC. Kajian *in vitro* dan *in vivo* yang dijalankan telah membuktikan bahawa asid organik dapat memberi kesan antibakteria yang kuat dan mempunyai potensi untuk memberi kesan yang baik ke atas pertumbuhan, penggunaan nutrien dan rintangan penyakit tilapia.

EFFECTS OF DIETARY ORGANIC ACIDS ON GROWTH PERFORMANCE OF RED HYBRID TILAPIA, *Oreochromis* sp., AND INHIBITION OF SELECTED FISH PATHOGENS

ABSTRACT

Short-chain organic acids have the potential to be viable alternatives to antibiotics by acting as a prophylatic to bacterial disease and enhance fish performance. To test this, three related experiments were set up. The first part of the research was designed to determine the antibacterial effects of various organic acids on *Aeromonas hydropilia*, in order to develop a tailor-made organic acid blend (OAB). The OAB was then compared with a commercial organic acid product, potassium diformate (KDF) and commonly used antibiotic, oxytetracycline (OTC) on *in vitro* antibacterial activities to *A. hydrophila* and *Streptococcus agalactiae*. All the tested organic acids could completely inhibit *A. hydrophila* at a concentration of 0.3% or lower. Among the acids, formic acid was the most effective followed by tartaric acid, lactic acid, propionic acid, citric acid and malic acid. The OAB had stronger antimicrobial activity than KDF or OTC when used at 0.2% or higher. It also showed strong bactericidal activity against *S. agalactiae*. In the second part of the research, two feeding trials were conducted to investigate the practical applications of dietary organic acids in the aquaculture industry. In the second experiment, a 14-week feeding trial was conducted to determine the effects of dietary OAB. The experimental diets were added with 0, 1, 2 or 3 g kg^{-1} of OAB, or with 2 g kg^{-1} of KDF and fed to triplicate groups of red hybrid tilapia (*Oreochromis* sp.). Upon completion, tilapia were challenged by immersion with *S. agalactiae*. Results showed no significant differences (P>0.05) to growth or nutrient utilization, although a slight improvement was observed for tilapia fed the acidified diets. Organic acids decreased the pH of the diets, causing a reduction in the digesta pH of stomach and gut. Total bacteria per g of feces were significantly (*P*<0.05) reduced in fish fed organic acid diets. A similar trend was observed for adherent gut bacteria. Further, there was an improvement to the resistance of tilapia to *S. agalactiae* challenge when fed the organic acids-supplemented diets. In the third experiment, a long-term (20-week) feeding trial under more commercial culture conditions was conducted to further elucidate the growth and nutrient utilization of tilapia fed diets supplemented with 0 (control), 5 or 10 g kg^{-1} of OAB II, or 5 g kg^{-1} of OTC and their subsequent resistance to *S. agalactiae*. Tilapia growth was improved by almost 12% while feeding efficiency slightly improved when fed OAB II diets, but these were not significant. Moreover, dietary OAB II at 10 g kg^{-1} led to significantly higher phosphorus availability for the fish. Significantly lower total bacterial counts in the feces and intestine were detected for fish fed the OAB II diets than the control diet. The inclusion of 5 g kg^{-1} OAB II was as effective as 5 g kg^{-1} OTC in protecting the fish against streptococcal infections. Unlike fish fed the OTC-based diet, no antibiotic residues were detected in tissue of fish fed the OAB-based diets. These *in vitro* and in *vivo* experiments showed that organic acids can exert strong antibacterial effects and have the potential to impart beneficial effects on growth, nutrient utilization and disease resistance in farmed tilapia.

CHAPTER 1

INTRODUCTION

Fish are an excellent source of animal protein that contains a full range of essential amino acids required by the human body for growth and maintenance. Unlike livestock meat, fish are high in unsaturated fatty acids, especially omega-3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which can lead to good human general health (von Schacky, 2003). In 2009, fish accounted for 16.6% of the total animal protein intake of the human population and 6.5% of all protein consumed (FAO, 2012). Due to the growing knowledge of the beneficial effects of fish consumption along with the global scarcity of capture fisheries, the demand of cultured food fish has become increasingly important in the world's food supply.

Over the last three decades, global aquaculture production has risen greatly with an average annual growth rate of 8.8%, reaching a plateau of 59.9 million metric tons (Mt) valued at USD 119.4 billion in 2010 (FAO, 2012). Approximately 3.5 million Mt were tilapia, making this species the second most important farmed fish in the world after the carps. The total aquatic food supplies for human consumption reached 128 million Mt in 2010, of which aquaculture contributed 47% (FAO, 2012). As one of the fastest growing animal food-producing sector worldwide, aquaculture is anticipated to continue growing rapidly in the future. The rapid growth in modern aquaculture is fuelled by a variety of factors, which include the increasing use of formulated aquafeeds and intensification of culture systems. Total compound aquafeed production increased over 600% from 4 to 29.3 million Mt in the past 14 years (1994 - 2008) and is estimated to increase to a value of 70.8 million Mt by

2020 (Tacon, 2010). However with the increasing intensification of fish-farming practices worldwide, one of the most limiting problems in the aquaculture sector is disease outbreaks (Bondad-Reantaso et al., 2005).

Infectious diseases are caused by microorganisms and bacterial pathogens are by far the most significant constraints faced by the aquaculture industry (Thune et al, 1993), which causes severe economical losses to farmers (Meyer, 1991). For example, *Aeromonas hydrophila* is one of the most common disease-causing bacterial pathogen of freshwater fish worldwide that causes haemorrhagic septicaemia in a variety of freshwater fish species, including tilapia (Austin and Austin, 1993). Similar to terrestrial livestock production, large amount of antibiotics are often used in the aquaculture industry to prevent and/ or control the infectious diseases caused by bacterial pathogens, following the discovery of the growth promoting and disease fighting capabilities of antibiotics (Hernández-Serrano, 2005). The extensive use of a wide variety of antibiotics in the aquaculture industry, both as therapeutic and growth-promoting agents, has increased the potential harmful effects on the human and animal health as well as the aquatic environment (Cabello, 2006). This practice has resulted in the development of antibiotic-resistant in fish pathogens that can be transmitted by horizontal gene transfer to bacterial pathogens of animal and human (Cabello, 2006). The emergence of antibiotic resistance in various bacterial pathogens associated with fish disease had been documented (De Paola et al., 1995; Schmidt et al., 2000; Miranda and Zemelman, 2002; Agersø et al., 2007). In addition, the extensive use of antibiotics in aquaculture also has the potential to threaten public health issues due to the residue bio-accumulation in consumer-ready aquaculture products.

Intensive public awareness regarding to the prophylactic use of antibiotics in animal feeds, which may lead to the transfer of bacterial immunity of species pathogenic to humans, has led to their ban in animal feed formulations. A worldwide effort to minimize and eventually eliminate the use of antibiotics for growth promoting purposes in the aquaculture and livestock industry started with the ban of sub-therapeutic antibiotics in 01 January 2006 in the European Union (European Parliament and Council Regulation (EC) No 1831/2003). The development of effective non-antibiotic compounds as an alternative to prophylactic antibiotics to control infectious disease and enhance growth performance is therefore paramount for the further development of the aquaculture industry.

Among other compounds, short-chain organic acids (C1-C6) and their salts or mixtures appear to be the most promising alternatives for antibiotic growth promotants (AGP), and have been receiving growing attention from aquaculture researchers (Lückstädt, 2008a; Ng and Koh, 2011). Organic acids, such as benzoic, formic, lactic and propionic acids, have traditionally been used as storage preservatives in food and feed ingredients for preventing deterioration caused by fungi and microbes (Ricke, 2003; Van Dam, 2006). Commercial mixtures of organic acids are widely used to control pathogenic bacteria such as *Salmonella* spp. and *Escherichia coli* in feed ingredients and terrestrial livestock feeds (Partanen and Mroz, 1999; Van Immerseel et al., 2003, 2006; Franco, et al., 2005). The mechanism of action of these weak acids in limiting microbial growth has been reviewed by Booth and Stratford (2003). The un-dissociated form of an organic acid is lipophilic and can passively diffuse through the cell wall of bacteria. Once inside the more alkaline cytoplasm, it dissociates and causes the internal pH to decrease, causing an inhibition of bacterial cell metabolism. Categories of bacteria that cannot tolerate changes in trans-membranous pH gradients will undergo cellular stress and eventually die. Additionally, the subsequent accumulation of weak acid anions within the cytoplasm may also has detrimental effects, which eventually results in the cells death.

Some organic acids have been shown to have strong antibacterial effects against important foodborne pathogens such as *Listeria monocytogenes*, *E. coli* and *Salmonella* spp. (Cherrington et al., 1991b; Vasseur et al., 1999; Van Immerseel et al., 2003; Skrivanova et al., 2006), and their mixtures are currently employed in terrestrial animal feeds to control bacterial pathogens (Van Immerseel et al., 2002, 2003). Although many organic acids appear to have the potential to prevent bacterial outbreaks when incorporated into aquafeeds, first implementing a comprehensive screening process to quantify their antibacterial activities is important. Thus, Experiment 1 (*Chapter 4*) was designed to screen the *in vitro* antibacterial effects of various water-soluble weak organic acids, namely, formic, propionic, lactic, malic, tartaric, and citric acid at different concentrations, on *A. hydrophila* and thereby identify the most appropriate alternative to replace traditional antibiotics in aquaculture feeds.

Since each organic acid has its own spectrum of antibacterial activity and it is generally accepted that one type of organic acid is not completely effective against all disease causing micro-organisms. In fact, combinations of organic acids are typically believed to be more effective against pathogenic bacteria compared to their single acids, and can have synergistic effects (Stonerock, 2007; Thompson and Hinton, 1997). Therefore, in order to maximise the antimicrobial effect of organic acids, a combination of various organic acids having different modes of action is necessary. Based on the data from the first *in vitro* study and comprehensive literature review of organic acids in aquaculture, the most effective and cost-effective organic acid blend (OAB) was developed and tested *in vitro* for their antimicrobial activity. This novel OAB was also directly compared with a commercially available antibiotic and organic acid against bacterial pathogens which can be commonly encountered in commercial tilapia aquaculture.

Even though the anti-microbial effects of organic acids are well understood, the explanation for the growth promoting effects of these compounds remains to be elucidated (De Wet, 2005). Nevertheless, poultry and swine fed organic acid-supplemented diets have been reported to show improved feed intake, growth, feed utilization efficiency and health (Alp et al., 1999; Partanen and Mroz, 1999; Partanen et al., 2002; Kluge et al., 2006). There is currently great interest in the commercial use of organic acids in fish feeds, both to control disease and to enhance growth performance. Recently, several researchers have reported that some organic acids, their salts and/ or mixtures thereof can improve the growth, feed utilization, mineral availability and disease resistance in fish (Baruah et al., 2007; Hossain et al., 2007; Sarker et al., 2007; Lückstädt, 2008a). Despite the reported improvement in nutrient availability of organic acid-supplemented diets, contradictory results have been reported for the growth-promoting effects of dietary organic acids, which seems to depend on the fish species, physiology, age and/ or the type of organic acid used (as reviewed by Lückstädt, 2008a; Ng and Koh, 2011).

Ramli et al. (2005) reported that hybrid tilapia (*Oreochromis* sp.) fed potassium diformate-added (KDF) diets showed significantly better weight gain, feed utilization efficiency and survival when challenged with *Vibrio anguillarum*. In contrast, Petkam et al. (2008) and Zhou et al. (2009) reported no significant improvement in growth performance of tilapia fed an organic acid/ salt blend or KDF, respectively, at various dietary levels. However, the studies on the use of organic acid, particularly their blends, in tilapia diets have not been thoroughly investigated to date. The more time consuming and costly *in vivo* experiments were therefore conducted to assess the practical dietary use of the novel OAB in aquaculture using red hybrid tilapia, *Oreochromis* sp. as the experimental animal model. Thus, the objectives of Experiment 2 (*Chapter 5*) are:

1) To evaluate the effect of dietary OAB and a commercially available organic acid product, potassium diformate (KDF) on growth performance and feed utilization of red hybrid tilapia.
2) To investigate the apparent nutrient digestibility (dry matter, protein and lipid), and phosphorus (P) availability of red hybrid tilapia fed diets supplemented with various levels of OAB or KDF.
3) To determine the impact of dietary OAB or KDF on the quantity and composition of the microbiota in fish gut (before and after challenge) and feces (before challenge).
4) To determine the effect of dietary OAB or KDF on the resistance of red hybrid tilapia to *Streptococcus agalactiae* challenge.

Antibiotics profoundly impart beneficial effects particularly when the immunity of animals are compromised during periods of stress triggered by intensive husbandry or transportation (Halverson, 2000). Considering the antimicrobial mechanisms of organic acids, it is anticipated that under stressful, crowded and unhygienic culture conditions, a greater positive growth response in tilapia may be observed when they are fed organic acid-supplemented diets. A longer-term feeding trial (Experiment 3; *Chapter 6*) under less-controlled commercial-like culture conditions was thus conducted and the aim was as follows:

1) To further elucidate the mechanism(s) of the potential growth-promoting effects of dietary organic acids in tilapia aquaculture.
2) To determine whether increased levels of the dietary OAB could significantly influence the growth performance, bioavailability of phosphorus and other major minerals in soybean meal-based diets.
3) To compare the efficacy of long-term administration of dietary OAB and oxytetracycline (OTC) with respect to health and growth promotion, as well as their ability to withstand *A. hydrophila* and *S. agalactiae* challenge.

CHAPTER 2

LITERATURE REVIEW

2.1 Farming of tilapia

Tilapias are one of the most important cultured freshwater fish species with commercial production in more than 100 countries around the world. They are members of the *Cichlidae* family and native to Africa. The fish occur mainly in freshwater and occasionally brackish water environments in tropical and subtropical climates (Nandlal and Pickering, 2004). Because of their rapid growth rates, palatability, hardiness and adaptability to a wide range of culture systems, tilapia has become one of the most widely grown group of any farmed fish in the world (FAO, 2012). Currently, Nile tilapia, *O. niloticus* is the most widely farmed tilapia species worldwide. Other commercially important species of tilapia include Mozambique tilapia (*O. mossambicus*), blue tilapia (*O. aureus*), three spotted tilapia (*O. andersonii*), Sabaki tilapia (*O. spilurus*), and their hybrids.

Tilapia farming is a major global industry and are currently the second most important farmed fish in the world after the carps (FAO, 2012). In 1995, global tilapia aquaculture production (Figure 2.1) was only 703 thousand tonnes and not even in the top 10 farmed species. However in recent years, intensive farming of tilapia is growing vigorously with a global tilapia production of about 3.5 million Mt valued at USD 5.7 billion in 2010 (FAO, 2011) and is forecasted to reach 8.9 million Mt by the end of year 2020 (Tacon and Metian, 2008).

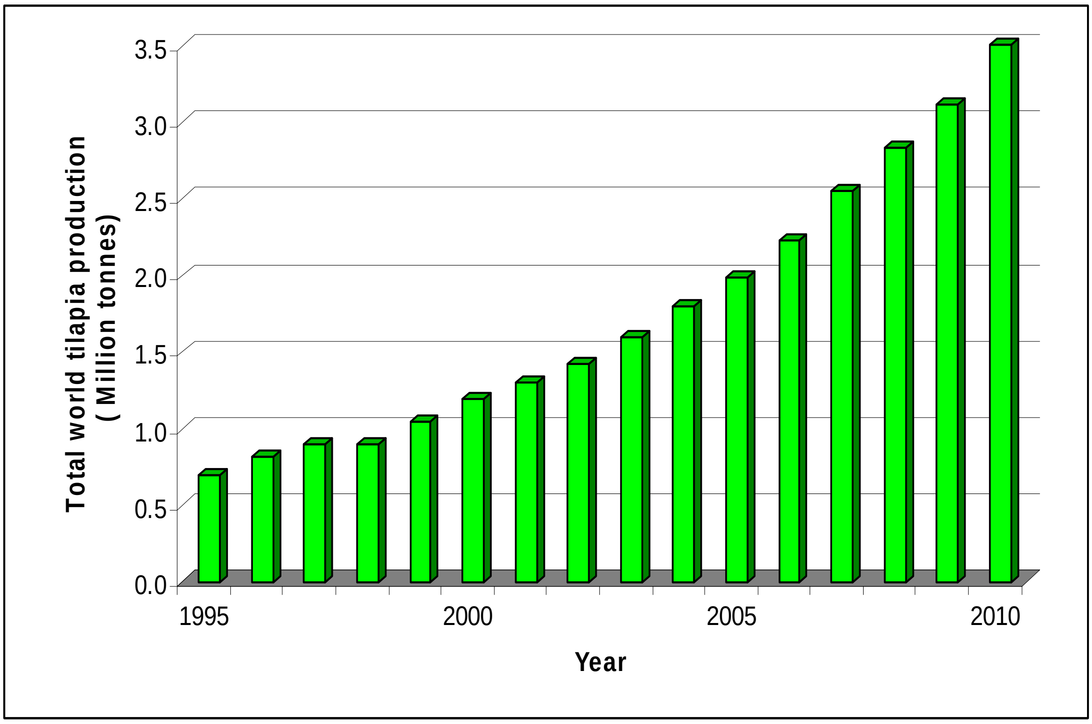

Figure 2.1 Total world production of farmed tilapia (million tonnes) from 1995 to 2010 (FAO, 2011).

In Malaysia, the total tilapia aquaculture production has risen by approximately 300% from 8,866 to 38,886 Mt per year in the past 15 years (Figure 2.2). In contrast to other tilapia farming countries in the Asian region, where Nile tilapia (~ 89%) is the major cultured species, the red tilapia (*Oreochromis* spp.) is the dominant species cultured in Malaysia accounting about 85% of the total tilapia aquaculture production, due to its attractive red color and high marketability (Ng and Hanim, 2007).

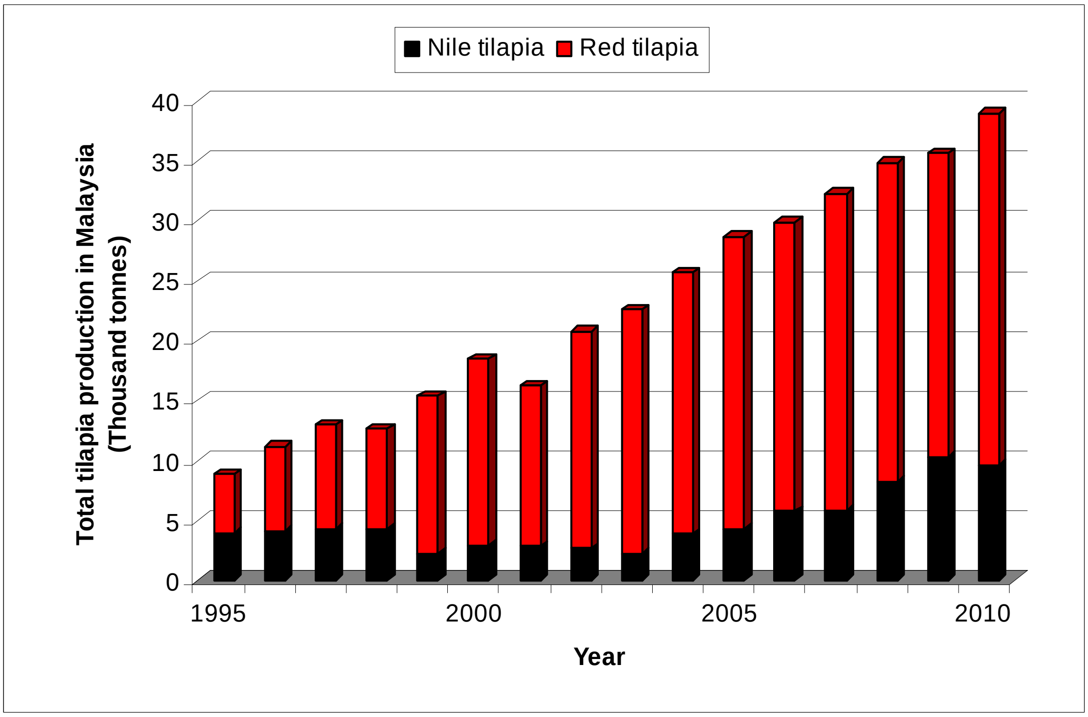

Figure 2.2 Total production of farmed tilapia (tonnes) in Malaysia between 1995–2010 (FAO, 2011).

2.2 The prophylactic use of antibiotics in aquaculture and its potential hazards

In intensive farming production, infectious diseases are a major problem that causes high economic losses to fish culturists (Bondad-Reantaso et al., 2005). Bacteria such as *Streptococcus* and *Aeromonas* are ubiquitous opportunistic pathogens that, when present in high numbers, are a major disease problem where mortality rates of over 50% in a matter of days are not unheard of (Yanong and Francis-Floyn, 2006). Thus, the inclusion of large amounts of various antibiotics in aquafeeds to control infectious diseases are not uncommon in the aquaculture industry, particularly in developing countries, following the discovery of the growth promoting and disease fighting capabilities of antibiotics (Hernandez-Serrano, 2005; Cabello, 2006). According to the World Health Organization (WHO), approximately 2 to 700 g of antimicrobials have been used per ton of aquaculture product, depending on the countries (WHO, 2006). In Malaysia, erythromycin and OTC are directly sprayed onto commercial tilapia pellets and fed to infected fish until mortality rates decrease (Musa *et al.*, 2009). The major classes of antibiotics used in aquaculture worldwide are summarized in Table 2.1.

Table 2.1 The major classes of antibiotics used in aquaculture worldwide (adapted from WHO 2006).

Antibiotic class	Example	Administration
Aminopenicillins	Amoxicillin Ampicillin	Oral Oral
Amphenicols	Chloramphenicol Florfenicol	Oral/injection/bath Oral
Macrolides	Erythromycin	Oral/injection/bath
Aminoglycosides	Streotomycin, neomycin	Bath
Nitrofurans	Furazolidone Nitrofurantoin	Oral/bath Oral
Quinolones	Oxolinic acid	Oral
Fluoroquinolones	Enrofloxacin Flumequine	Oral/bath Oral
Tetracyclines	Oxytetracycline, chlortetracycline, tetracycline	Oral/injection/bath
Sulphonamides	Sulphonamides	Oral

The prophylactic use of antibiotics is also rampant in the livestock industry and these compounds are collectively called AGPs. The over-use of prophylactic antibiotics in aquaculture can eventually be detrimental to the health of the fish, but also that of animals, human consumers and the aquatic environment. This practice encourages bacterial resistance which could lead to increased antibiotic resistance in the pathogens of fish and can also be transferred to bacteria of terrestrial animals and to human pathogens, leading to overall increased in infectious diseases that become harder to treat (Hernandez-Serrano, 2005; Cabello, 2006). Moreover, some antibiotics are non-biodegradable and can therefore remain in aquaculture systems for long periods of time, thereby further encouraging the growth of antibiotic-resistant bacteria strains (Cabello, 2006). The emergence of antibiotic resistance in various bacterial diseases of fish has been documented for *A. hydrophila*, *A. salmonicida*, *Edwardsiella tarda*, *V. anguillarum*, and *Pasteurella piscida*, among others (De Paola *et al.*, 1995; Schmidt *et al.*, 2000; Miranda and Zemelman, 2002; Alcaide et al., 2005; Agersø *et al.*, 2007). In addition, when antibiotics are mixed into fish feeds, residual antibiotics are often found in seafood products compromising the health of the human consumers (Nawaz *et al.*, 2001).

2.3 Organic acids

Organic acids are organic compounds with one or more carboxyl groups (-COOH) in their structure. These include saturated straight-chain monocarboxylic acids (chain lengths C1-C18) and their respective derivatives, such as unsaturated (cinnamic, sorbic), hydroxylic (citric, lactic), phenolic (benzoic, cinnamic, salicylic) and multicarboxylic (azelaic, citric, succinic) acids (Cherrington et al., 1991b), with a general molecular structure of R-COOH, where R represents the monovalent functional group. These acids are commonly referred to as short-chain fatty acids, volatile fatty acids or weak carboxylic acids.

Organic acids are produced through the microbial fermentation of carbohydrates by various bacterial species under different metabolic pathways and conditions. Some lower-molecular-weight organic acids, for example, acetic, propionic and butyric acids are also formed within the large intestine of humans and animals at high concentrations by anaerobic microbial communities (Cummings et al., 1987; Macfarlane and Macfarlane 2003). Many of these, particularly, short-chain organic acids (C1-C7), are naturally present as normal constituents of plants or animal tissue. However, most of these substances commerically used in the food industry are produced synthetically. Organic acids may also form into single or double salts of their acid through combining with potassium, sodium, calcium, etc.

Weak lipophilic organic acids and their salts are widely known as "Generally Regarded as Safe" (GRAS) substances, and have been used for centuries as food preservatives in foods and beverages (Russell and Gould 2003). They also have long been listed in the EU regulations as permissible feed additives in food animal production. For example, organic acids, their salts or combinations thereof, have been used in swine feeds for decades as potential alternatives to AGP's to prevent diarrhoea and improve the performance of weaned piglets, fattening pigs and reproductive sows as reviewed by Partanen and Mroz (1999). These acids, particularly, formic, fumaric and citric acid, seem to effectively enhance animal growth performance and feed efficiency (Partanen and Mroz, 1999). Moreover, commercial mixtures of organic acids are widely used to control pathogenic bacteria, such as *Salmonella* species, in feed ingredients and terrestrial livestock feeds (Van Immerseel et al., 2002).

The main advantages of using weak organic acids to control microbial growth are the absence of harmful residues in animal products (Chaveerach et al., 2002; Castillo et al., 2004), or cross-resistances to humans (Swick, 2011). For these reasons, among many others, explains why research efforts on weak organic acids as the alternative to AGP's are crucial. Table 2.2 presents a list of organic acids that are currently being used in feeds for terrestrial livestock and typically, a blend of these acids or their salts are used as commercial livestock feed additives. Each organic acid has its own spectrum of antimicrobial activity due to their specific physical and chemical properties (Dibner and Buttin, 2002). Therefore, the advantage of using organic acids blend in animal feed is that it may have synergistic effects (Chaveerach et al., 2002), leading to a broad spectrum of antimicrobial activity against a wider range of disease-causing bacteria. Moreover organic acids blend allows an even further reduction of the dose used in animal feeds and reducing feed costs.

Table 2.2 List of organic acids and their physicochemical properties (adapted from Mroz, 2005).

Organic acid	Mol. formula	pKa value	Mol. wt (g/mol)	Physical form	Odour	CR[1]
Formic	CH_2O_2	3.75	46.03	Liquid	Pungent	+++
Acetic	$C_2H_4O_2$	4.6	60.05	Liquid	Pungent	+++
Propionic	$C_3H_6O_2$	4.88	74.08	Oily liquid	Pungent	++
Butyric	$C_4H_8O_2$	4.81	88.12	Oily liquid	Rancid	+
Lactic	$C_3H_6O_3$	3.86	90.08	Liquid	Sour milk	+
Sorbic	$C_6H_8O_2$	4.76	112.1	Solid	Mildly acrid	+
Fumaric	$C_4H_4O_4$	3.02, 4.76	116.1	Solid	Odourless	0 to +
Malic	$C_4H_6O_5$	3.40, 5.1	134.1	Solid	Apple	+
Citric	$C_6H_8O_7$	3.13, 4.76, 6.49	192.1	Solid	Odourless	0 to ++

[1] CR= Corrosiveness rate: high (+++), medium (++), low (+), negligible (0).

The strength/ acidity of an acid in solution is represented in its logarithmic constant (pKa) value, which is equal to $-\log_{10}$ (K_a = acid dissociation constant = $[H^+][A^-]/[HA]$). Therefore, the smaller the value of pKa, the stronger the acid. In general, since most organic acids are considered as weak acids, and only partially dissociates/ ionizes into their ions in aqueous solutions, the majority of organic acids will remain in their undissociated form (*i.e.* free organic acid). In an aqueous solution, weak organic acids dissociate and form a pH-dependent dynamic equilibria between undissociated acid molecules and dissociated anions as in the chemical equilibrium below:

$$\mathbf{HA + H_2O \leftrightarrow A^- + H_3O^+}$$

where HA represents a weak acid, A^- is a conjugate base (acid anion), and H_3O^+ is a hydroxonium ion. Organic acids change from undissociated forms to dissociated forms depending on the environmental pH and its pKa value. The proportion of the free acid molecules could be theoretically calculated by the Henderson-Hasselbalch equation ($pH = pKa + \log [A^-]/[HA]$).

The pH at which 50% of the acid is dissociated is called its pKa value and is unique for each individual acid (Table 2.2). At a pH below their individual pKa values, organic acids will mostly be present in an undissociated form, thus, increasing the proportion of free acids that readily enter the bacterial cells by simple diffusion. Lower-molecular-weight organic acids, for example, formic, acetic and lactic acids are miscible in water, whereas higher-molecular-weight organic acids such as benzoic acid are insoluble in water due to their hydrophobicity. The physical and chemical characteristics of some organic acids that are commonly used in the feeds for monogastric animals are given in Table 2.2.

2.4 Antimicrobials activities of organic acids

The antimicrobial activity of lipophilic weak acids was traditionally explained by the perturbation of membrane function, which blocks the transport of substrate molecules (amino acids, organic acids, phosphate etc) into cells (Freese et al., 1973; Stratford and Anslow, 1998). The cause to such an inhibition of necessary substrates into cells is due to the partition of undissociated lipophilic weak acids into the cell membrane. However, there are other mechanisms believed to be responsible for the ability of organic acids to limit microbial growth and have been reviewed by Cherrington et al. (1991b) and Booth and Stratford (2003). The most obvious mode of action of these lipid-soluble weak acids is via direct acidification of the extra-cellular pH through its ability to dissociate into ions and release hydrogen ions (protons) to the surrounding medium. However, it is now currently accepted that the predominant mode of action of these acidifiers is mainly based on their ability to lower the cytoplasmic pH once they traverse across the cell membranes.

2.4.1 Acidification of the external medium by releasing hydrogen ions

The majority of bacterial species have specific pH requirements for optimal growth and are unable to grow under extreme acidic conditions ($pH < 4.5$). Thus, outside the bacterial cells, a significant amount of organic acids can exert their antimicrobial activity on microbes by directly lowering the pH of the environment via releasing hydrogen ions and thus preventing/ impeding the growth and proliferation of acid-sensitive bacteria. Weak organic acids such as acetic, citric, benzoic, sorbic and lactic acids, for example, have been employed for many years to lower the pH of foods or beverages in order to limit microbial growth (Stratford and Eklund, 2003).

2.4.2. Acidification of the cytoplasm and its consequences

It is now generally accepted that the antimicrobial efficacy, as both bacteriostatic and bacetericidal effects of these acidifiers, is mainly based on their ability to traverse across the semi-permeable membrane of bacteria and to dissociate in the near neutral cytoplasm (Cherrington et al., 1991b; Booth and Stratford, 2003) as shown in Figure 2.3. Despite their various molecular structures as shown in Table 2.2, all organic acids seem to have a similar mode of action against micro-organisms (Stratford and Anslow, 1998). Organic acids are believed to be more effective at low pH when the majority of these are present in the undissociated form, and are thus the most effective form in killing microorganisms (Salmond et al., 1984; Brul and Coote, 1999; Lambert and Stratford, 1999; Thomas et al., 2002). This is because the undissociated form of an organic acid is lipophilic and can therefore passively diffuse through a bacterium's cell membrane. Once inside the nearly neutral cytoplasm, it dissociates, releasing charged acid anions and protons that are impermeable back across the cell's membrane (Eklund, 1985; Cherrington et al., 1991b; Warth, 1991; Lambert and Stratford, 1999).

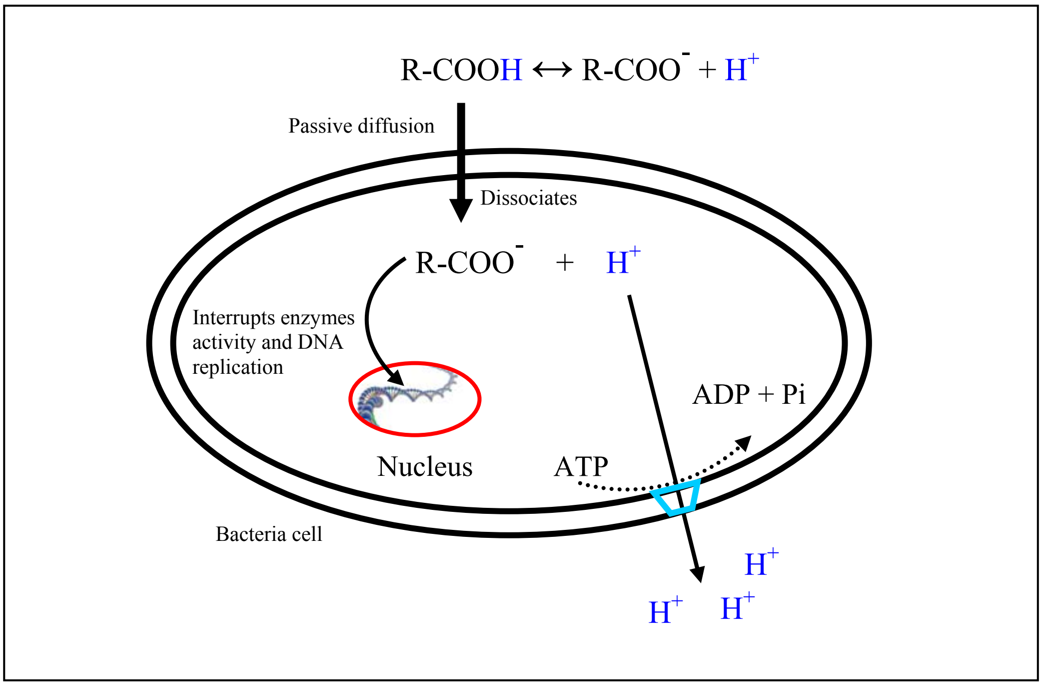

Figure 2.3 Mode of action of organic acids against microorganisms (Lambert and Stratford, 1999). The undissociated organic acids traverse across the cell wall of bacteria via passive diffusion and dissociate inside the cytoplasm, causing the cytoplasmic pH to decrease. Eventually the cell enzymes and nutrient transport systems are suppressed resulting in irreversible damage to the microbial cell, often causing death.

Such an accumulation of excess protons within the cell will lower the cytoplasmic pH, thereby causing an inhibition of bacterial cell metabolism through the suppression of cell enzymes (Warth, 1991), particularly, the pyruvate decarboxylase enzyme which contributes to energy metabolism (Sava, 2011). Cell death may occur when the cytoplasmic pH drops below the physiological optimal range for growth (Smigic et al., 2009). Lowering the cytoplasmic pH might also be expected to neutralize the electrochemical gradient, for example the pH gradient (ΔpH) across the plasma membrane, which is required for the active transport of necessary nutrients. Bacteria that do not tolerate changes in trans-membranous pH gradients such as *E. coli*, *Salmonella* and *Campylobacter* (Dibner and Buttin, 2002) will undergo cellular stress and eventually die (Jensen, 2001). The acidification within the cytoplasm of the cell may also inhibit the central metabolism at the expense of anionic metabolites from the cell. To restore the intracellular pH within a physiological optimal range for growth and sustain functional macromolecules, the cell is forced to pump out the excess protons, released by the acids, via the membrane-bound H^+-ATPase. Proton pumping by the H^+-ATPase requires substantial metabolic energy in the form of adenosine triphosphate (ATP) (Holyoak et al., 1996) and could therefore lead to depletion of cellular ATP, and the cells will eventually die of exhaustion (Warth, 1991; Ricke, 2003).

ATP depletion is not the sole mechanism for inhibiting celluar growth or inducing death since proton removal also leads to an accumulation of weak acid anions within the cytoplasm, which is responsible for the growth inhibition mechanisms of organic acids at low pH (Russell, 1992; Brul and Coote 1999; Lambert and Stratford, 1999). This accumulation in turn inhibits the synthesis of macromolecules, *e.g.*, nucleic acids (Cherrington et al., 1990), proteins, lipids and carbohydrates (Jensen, 2001) as well as enzyme activity (decarboxylases and catalases) and nutrient transport systems within the cytoplasm (Roth and Kirchgessner, 1998; Russell and Diez-Gonzalez, 1998; Partanen and Mroz, 1999), and eventually results in cell death. A summary of the inhibitory mechanisms of some organic acid anions are shown in Table 2.3.

Table 2.3 Inhibitory mechanisms of some organic acid anions (adapted from Van Dam, 2006).

Acid anion	Mode of action
Formate	- inhibits enzyme activity, specially decarboxylase and catalase
Acetate	- inhibits enzyme activity
	- increases heat sensitivity
Propionate	- influences membrane transport
	- inhibition on synthesis of some amino acids
Lactate	- inhibits enzyme activity
Sorbate	- inhibits a series of enzymes, amino acid uptake, and synthesis of RNA/ DNA
	- cell membrane-damage
Benzoate	- inhibits a series of enzymes and amino acid uptake
	- cell membrane-damage
	- changes membrane fluidity

The substantial accumulation of weak acid anions in the cytoplasm may also cause hyper-osmotic stress on the cell, which may also contribute to growth inhibitory effects (Roe et al., 1998). The high anion concentration within the cytoplasm has the potential to increase the turgor pressure on the cell via increasing the osmotic pressure (Kroll and Booth, 1983). To maintain the turgor pressure at a constant level, the cell appears to reduce other anion pools, for example, intracellular glutamates to compensate this accumulation (Roe et al., 1998). Thus, perturbation of anion balance may contribute to inhibitory effects on growth and eventually results in cell death (Roe et al., 1998).

2.5 Effects of organic acids on nutrient availability

Various hypotheses have been proposed to explain the effects of organic acids on enhancing nutrient utilization in terrestrial livestock which includes, among others, (1) lowering gastric pH leading to increased pepsin activation, (2) lowering diet and intestinal pH, which may increase mineral solubilization, (3) acting as chelating agents binding to various cations within the intestine, which results in increased mineral absorption or (4) inhibiting the colonization of harmful microbes in the intestine which may otherwise utilize nutrients and thus becomes spared for the host animal (Schöner, 2001; De Wet, 2005). In animal nutrition, organic acids and their salts can act as a growth promotant primarily in the feed and gastrointestinal tract of the animal (Freitag, 2007).

2.5.1 Effects of organic acids in feed

In the food animal production, organic acids have been used for decades as additives or preservatives to help prevent some diseases and protecting feeds and feed ingredients from the deterioration caused by bacteria, molds and yeasts (Thompson and Hinton, 1997; Ricke, 2003; Skrivanova et al., 2006; Van Dam, 2006). Even under favourable storage conditions, bacterial proliferation seems inevitable in the feeds, but particularly under high humidity conditions (≥ 14%). Inclusion of organic acids reduces the pH value of the feed and therefore prevents the growth of undesired microbes during storage that could lead to potentially harmful bacteria and/ or toxic metabolites (especially mycotoxins) produced by fungi (Schöner, 2001;

Freitag, 2007). The ingestion of even small amounts of mycotoxins from contaminated feedstuffs may cause serious nutritional and health problems during animal production (Müschen and Frank, 1989). Therefore, acidification enhances the hygienic quality of the feed, which prevents the loss of its nutritional value via a decomposition of proteins and carbohydrates, In addition to improving food hygiene, organic acids reduce the buffering capacity of the dietary feed ingredients. This is important since lowering the buffering capacity of feed containing organic acids ensures optimal intestinal pH, which results in better feed digestion and health status of livestock, especially in young animals (Metzler and Mosenthin, 2007).

2.5.2 Effects of organic acids in gastrointestinal tract

In the gastrointestinal tract, organic acids exert their effects on performance via two main mechanisms. Firstly, it reduces the pH within the stomach and possibly small intestine of farm animals (Schöner, 2001; Lückstädt, 2009), and secondly, through their ability to inhibit and kill harmful bacteria by disturbing their metabolisms, as described earlier.

The addition of organic acids reduces the gastric pH, allowing a faster acidification of the digesta in the stomach, which favours proteolytic enzyme activity and thus stimulates protein digestibility and animal performance (Kirchgessner and Roth, 1988; Roth and Kirchgessner, 1998; Dibner and Buttin, 2002). Moreover, organic acids added in feeds may slow down the emptying rate of the stomach, therefore allowing more efficient hydrolysis of proteins in the stomach and absorption of nutrients in the small intestine. These effects are generally more pronounced in younger animals, where the pancreatic enzyme secretion and the hydrochloric acid production are inadequate in the digestive tract compared to adults (Freitag, 2007).

Another beneficial effect of lowering gastric acidity is an improvement of P bioavailability from phytate-P in the plant feed ingredients (Dibner and Buttin, 2002). The majority of organic P in plant feed ingredients exists in the form of phytic acid or phytate (salts of phytic acid) and it is generally not digestible to monogastric animals due to lack of phytase activity in the digestive tract (Hughes and Soares Jr, 1998). The addition of organic acids can increase the phytate-P utilization by inducing microbial phytase activity, which is more efficient in lower pH values (Dibner and Buttin, 2002; De Wet, 2005). Lowering the intestinal pH by weak organic acids may also increase mineral solubility leading to improved absorption of minerals such as calcium and P. Furthermore, the anions of weak acids can act as chelating agents by binding up various cations to form mineral and acid complexes along the intestine, which results in increased mineral absorption by the intestinal cells (Ravindran and Kornegay, 1993).

A further positive effect of organic acids on animal performance is through a direct stimulation of mucosa proliferation activity in the gastrointestinal tract. In rats, organic acids (acetate, propionate, and butyrate) stimulate the proliferation of gastrointestinal mucosal cells by inducing the expression of plasma glucagon-like peptide (GLP-2), ileal proglucano mRNA, glucose transporter (GLUT2), and c-*myc*, c-*jun*, and c-*fos*, which can potentially mediate mucosal proliferation (Tappenden and McBurney, 1998). It has been suggested that the positive effects of organic acids, particularly butyrate, in stimulating the growth of intestinal epithelium cells, leads to increased nutrient absorptive capacity (Topping and Clifton, 2001). Recently, Adil et al*.*, (2010) reported that the dietary supplementation of organic acids showed increases in villus height in the duodenum and jejunum of broiler chicken. Besides, the thickness of muscularis on the intestinal mucosa was also decreased in all the segments of small intestine. These alterations in structure of the gastrointestinal may facilitate the nutrient absorption in the small intestine, and thus improve the growth performance (Adil et al., 2010; Samanta, 2010).

Finally, it is also thought that the improved nutrient utilization may be attributed to the strong antimicrobial activity of organic acids that inhibit the colonization of harmful microbes within the digestive tract (Kluge et al., 2006). The result of reducing harmful microbial counts is a healthier gut, and the energy or nutrients, which may otherwise be utilized by the microbes, are now spared for the host animal. Such a reduced competition for nutrients between microbes and the host animal is one of the mechanisms responsible for improved nutrient utilization (Partanen and Mroz, 1999; Dibner and Buttin, 2002; Adil et al., 2010).

2.6 The use of organic acids as growth promotants and antimicrobials in aquaculture

The potential benefits of organic acids in improving feed intake, growth, feed utilization efficiency and health of both poultry and swine have been well documented for decades (Alp et al., 1999; Partanen et al., 2002; Kluge et al., 2006). Despite the reported beneficial effects on improving the performance parameters and health of terrestrial livestock, limited comprehensive research has been done to elucidate the use of organic acids or their salts in farming of aquatic animals, until recently. Only a few studies have been published regarding the use of organic acids in aquafeeds prior to the ban of AGP use in livestock production (Bjerkeng and Storebakken, 1991; Ringø, 1991; Gislason et al., 1994, 1996; Vielma et al., 1999).

One of the earliest studies on the application of organic acids in aquaculture was about 31 years ago by Rungruangsak and Utne (1981). However, in this 140 day trial, moist diets acidified with formic acid-preserved fish silage seemed to depress growth, feed utilization and proteolytic activities in the digestive tract of rainbow trout, *Oncorhynchus mykiss*. More recently, several studies have also been conducted to determine the effects of organic acids and their salts on growth performance, nutrient utilization and disease resistance in several commercially important farmed fish species, such as rainbow trout (Pandey and Satoh, 2008; Gao et al., 2011), salmon (Christiansen and Lückstädt, 2008), carp (Baruah et al., 2005, 2007) and tilapia (Ramli et al., 2005; Zhou et al., 2009; Liebert et al., 2010; Lim et al., 2010). The major organic acids and their salts tested in aquafeeds to date are presented in Table 2.4 – 2.8.

2.6.1 Citric acid and its salt

Citric acid or its salts are by far the most investigated organic acid in aquaculture. Numerous studies have reported that citric acid can improve growth and feed utilization in various fish species (Sarker et al., 2005, 2007; Baruah et al., 2007; Pandey and Satoh, 2008), while some showed contradictory findings (Fauconneau, 1988; Vielma et al, 1999). The earliest study regarding the use of citric acid was conducted on *O. mykiss* by Fauconneau (1988). In this study, citric acid at 120 g kg^{-1} was supplemented in a fishmeal-based diet to partially replace the protein content in trout diets. While acidification of the diets appeared to lower the voluntary feed intake, this did not affect the efficiencies of protein and energy utilization. Since then, several studies with more promising results have been reported in *O. mykiss*, red sea bream, *Pagrus major* and Indian major carp, *Labeo rohita* (Table 2.4).