DOCTORAL THESIS

Culturing grass carp and grey mullet using food waste incorporated with traditional Chinese medicine, Baker's yeast and enzymes

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CULTURING GRASS CARP AND GREY MULLET USING FOOD WASTE INCORPORATED WITH TRADITIONAL CHINESE MEDICINE, BAKER'S YEAST AND ENZYMES

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Culturing Grass Carp and Grey Mullet Using Food Waste Incorporated with Traditional Chinese Medicine, Baker's Yeast and Enzymes

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A thesis submitted in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Principal Supervisor: Prof. WONG Ming Hung

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Declaration

I hereby declare that this thesis represents my own work which has been done after registration for the degree of PhD at Hong Kong Baptist University, and has not been previously included in a thesis or dissertation submitted to this or any other institution for a degree, diploma or other qualifications.

Signature: _____

Date: Sept 2013

Abstract

The present study focused on using food wastes and feed supplements, e.g. enzymes (bromelain and papain), baker's yeast and Traditional Chinese Medicines (TCMs) for rearing freshwater fish (grass carp and grey mullet) in Hong Kong. Different types of food wastes, e.g. meats, bones, cereals, fruits and vegetables were collected from local hotels, mixed in different ratios and processed into feed pellets for feeding trials.

The cereal dominant food waste feed (FW A) was more suitable for grass carp and grey mullet, with the best growth performance (e.g. feed conversion ratio (FCR), specific growth rate (SGR)) and higher protein digestibility (in grass carp), compared to FW B and FW C which contained higher proportions of meat products. The NBT (Nitroblue Tetrazolium) activities in blood and plasma protein levels were decreased in the grass carp, cultured with food waste feeds without any supplements, compared to the commercial feed, Jinfeng®, 613 formulation (Control).

Upgrading FW A by the addition of 1% and 2% mixtures of bromelain and papain significantly increased the feed protein solubility and subsequent to growth (SGR and relative weight gain (RWG)) and feed utilization (e.g. apparent net protein utilization (ANPU), protein efficiency ratio (PER)) in both fish species. The protein and feed utilizations by grass carp were also promoted by the yeast supplements with the optimal dose of 2.5% yeast (*S. cerevisiae*) added to FW A upgraded by enzymes. This showed that yeast could further enhance nutrient utilization contained in feeds after addition of bromelain and papain.

The *in vitro* study on the grass carp's plasma treated with TCM extracts also showed that TCM extracts could stimulate plasma bactericidal activity (on *Aeromonas hydrophila*), possibly through enhancing plasma complement activity. The

formulation with *Radix scutellaria*, *Rhizoma coptidis*, *Herba andrographis* and *Radix sophorae flavescentis* in the ratio of 1:1:2:3 was more effective in enhancing plasma bactericidal activity than single TCM extracts. Besides, *R. coptidis* and *R. scutellaria* possessed the strongest antimicrobial activity (*in vitro*) on fish pathogens (such as *A. hydrophila*, *Lactococcus garvieae* and *Vibrio cholerae*) among the 17 tested TCMs. In addition, TCMs were less likely for developing drug resistant pathogens than antibiotics.

Grass carp immunity (NBT activity in blood, plasma bactericidal activity and total immunoglobulin level) was boosted by the addition of TCM formulation and baker's yeast (*S. cerevisiae*). The disease resistance to pathogen (*A. hydrophila*) was also enhanced, with significantly lower mortalities observed in groups feeding with TCM (1 and 2% for 21 to 28 days) and baker's yeast (2.5 and 5% for 28-56 days).

The uses of yeast and TCMs led to positive effects on growth, immunity and disease resistance to pathogens in fish, but the effects (grass carp) were less effectual when both were supplemented in feed. The combined use of both supplements may impair the effects of TCM formulation or yeast in the modulation of gut mircoflora, and upset the balance of beneficial microbial communities.

The present study demonstrated the feasibility of using feed supplements (TCM and baker's yeast) to enhance fish immunity and enzymes upgraded food waste feeds for rearing fish, for the development of a more sustainable aquaculture in Hong Kong.

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Abbreviations and Acronyms

AFCD	Agriculture, Fisheries and Conservation Department
AFFS	Accredited Fish Farm Scheme
Ah	*Aeromonas hydrophila*
ANLU	Apparent net lipid utilization
ANPU	Apparent net protein utilization
CFS	Center for Food Safety
CFU	Colony Forming Units
CHO: L	Carbohydrates to Lipid ratio
CPI	Commodity Price Index
Cr_2O_3	Chromium Oxides
CSD	Census and Statistics Department
Ec	ATCC 25922 *Escherichia coli*
Ef	ATCC 29212 *Enterococcus faecalis*
EPD	Environmental Protection Department
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FCR	Feed Conversion Ratio
FDA	Food and Drug Administration
FIC	Fractional Inhibitory Concentration
FIN	Fishmeal Information Network
FM	Fish Meal
FW A	Food Waste Feed A
FW B	Food Waste Feed B
FW C	Food Waste Feed C
FW D	Food Waste Feed D
GDPBS	Guangdong Provincial Bureau of Statistics

GESAMP	Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection
Ha	*Herba andrographis*
IgI	Immunoglobulin
kg	kilogram
KOH	Potassium Hydroxide
Lg	*Lactococcus garvieae*
MDR	Multi-drug Resistance
mg	milligram
MIC	Minimum Inhibition Concentration
MPEDA	Marine Product Export Development Authority of India
NACA	Network of Aquaculture Centres in Asia
NaOH	Sodium Hydroxide
NBT	Nitroblue Tetrazolium
NCCLS	National Committee for Clinical Laboratory Standards
NRC	National Research Council
OIE	World Organisation for Animal Health
P/E	Protein to Energy Ratio
PER	Protein Efficiency Ratio
Rc	*Rhizoma coptidis*
Rs	*Radix scutellaria*
Rsf	*Radix sophorae flavescentis*
RWG	Relative Weight Gain
Sa	ATCC 35548 *Staphylococcus aureus*
Sm	ATCC 43861 *Serratia marcescens*
SGR	Specific Growth Rate
TCM	Traditional Chinese Medicine

Vc	*Vibrio cholerae*
WWF	World Wide Fund for Nature
WHO	World Health Organization
USD	US Dollar (United States of America)

Chapter 1

The necessity of feed alternatives with supplements in current and future aquaculture industry

1.1 Overview of world aquaculture status

Aquaculture is an important and fast growing industry around the world and the production expanded rapidly in recent 20 years. Excluding aquatic plants and non-food products, the total global food fish production in aquaculture was about 60 million tonnes at 2010 which was valued US$119 billion (FAO, 2012). At the same time, the annual production from capture fisheries was only remained at about 90 million tonnes in past 20 years (FAO, 2012); the fishery resource in nature is under tremendous pressure and becoming questionable food source in future. To cope with this, aquaculture has been gaining importance in the role of human food source in the past few decades.

The aquaculture industry in Asia is overwhelmingly important to global aquaculture, contributing over 90% global aquatic products and China alone contributed more than 60% of total world production (Fig. 1.1) (FAO, 2012). China dominated the global aquaculture production (exclude aquatic plant and non-food products) by 61.4% and 49% in terms of quantity and monetary value, respectively

(FAO, 2012). The freshwater finfish aquaculture production (33.9 million tonnes) dominated the world aquaculture industry, and mainly contributed by carps, 71.9% (~24.2 million tonnes) in 2010 (FAO, 2012). In the Guangdong Province of China, the economic value of aquaculture output reached 39.0 billion RMB in 2004 (GDPBS, 2005).

The needs of rapid increase in aquaculture productions can be predicted and maintained in the near future, but whether the growth rate can be sustained in an environmental friendly way is in doubt. The rapid explosion in aquaculture production raised two major concerns around the world: the prevalence of fish diseases and tremendous pressures on the demand of feeding materials, especially fish meal are crucial problems in future aquaculture development.

It is commonly known that fish diseases are the results of interactions between hosts, pathogens and environments. The fast growth of aquaculture industry during the past 20 years is attributed to the high stocking density. This resulted in the fish more susceptible to infections, and more nitrogenous sewage is generated due to intensive feeding and excretions from fish. As a consequence, fish diseases and the deteriorated environment have caused enormous economic loss. In 2010, China suffered 1.7 million tonnes of fish products owing to disease outbreaks, natural disasters and pollutions, which worth $3.3 billion US dollars (FAO, 2012).

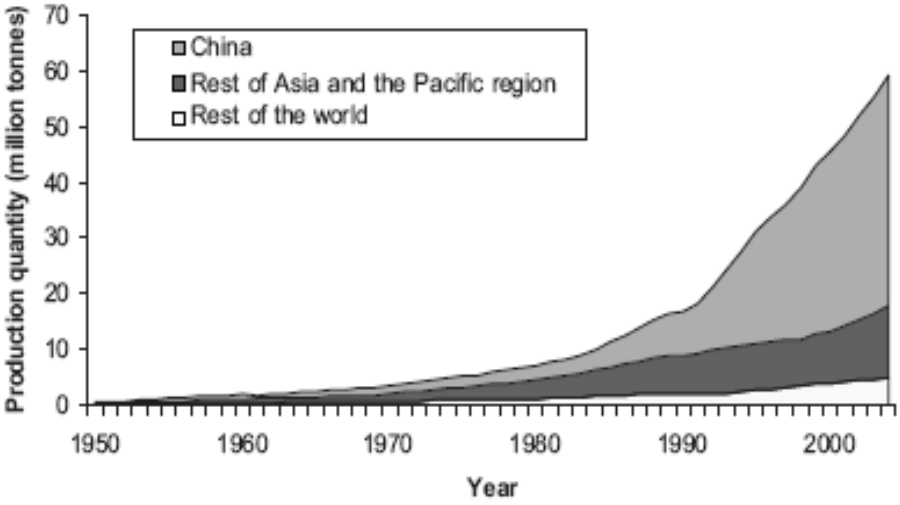

Fig. 1.1 World aquaculture production from 1950 to 2004 (showing China, rest of Asia and the Pacific region) (FAO, 2006)

According to the Food and Agriculture Organization (FAO), an additional 40 million tonnes of fish products which is 60% of the production in 2006, will be required to support the consumption in 2030 (FAO, 2006). Although the production increased sharply, the fish meal usage for aquaculture feed was steady at about 6 million tonnes in the past 10 years (FAO, 2012) and the prediction of demand was even lower due to decreased supply and higher prices (Tacon and Metian, 2008).

The development of aquaculture industry has also suffered from the prevalence of diseases, other concerns such as environmental pollution and fish fry sources also endangered the industry. It is essential to investigate strategies for the sustainable development of fisheries, which is particularly true for Hong Kong fisheries as discussed below.

1.2 Hong Kong Inland Fisheries Status

Inland fishery in Hong Kong was vibrant in the 1960s, with an expansion of pond area during the 1960s to 1980s (Lau *et al.*, 2003), but then it declined due to shift in industry sectors and the development of new towns in the New Territories (Cheung, 1999; Lam, 1999). Pond area decreased by 40% from 1984 to 2004, while the number of inland fish farmers decreased from 1690 in 1991 to 637 in 2004. In

2010, 1,120 hectares of fish ponds are located mainly in the north-western New Territories of Hong Kong (HKSAR, 2012). Simultaneously, the annual production of pond fish decreased from 6,500 tonnes (HK$ 104 million) in 1984 to 2190 tonnes (HK$ 54 million) in 2010, reduced by 66 and 48% of the quantity and monetary value of total fish production (Fig. 1.2), respectively (Chan, 2005; AFCD, 2011).

The rapid development of aquaculture industry in the mainland China and the change of local policy also deeply impacted on the local industry. Traditionally, the "fish-cum-duck" practice with ducks simultaneously reared along the bunds of the carp polyculture ponds, utilized the duck manure as organic fertilizer to enhance pond productivity (Everitt and Cook 1997). Due to the "Livestock Waste Control Scheme" introduced in 1994, the "fish-cum-duck" practice ceased and fish farmers used other organic materials, mainly peanut cake, and also the infrequent use of pig manure and cattle manure instead of duck manure. The changed practice increased the production cost and reduced the income from selling edible ducks. Besides, the import of low-priced freshwater fish from the mainland further rendered the local fishery became less profitable and drove some fishermen away from the industry.

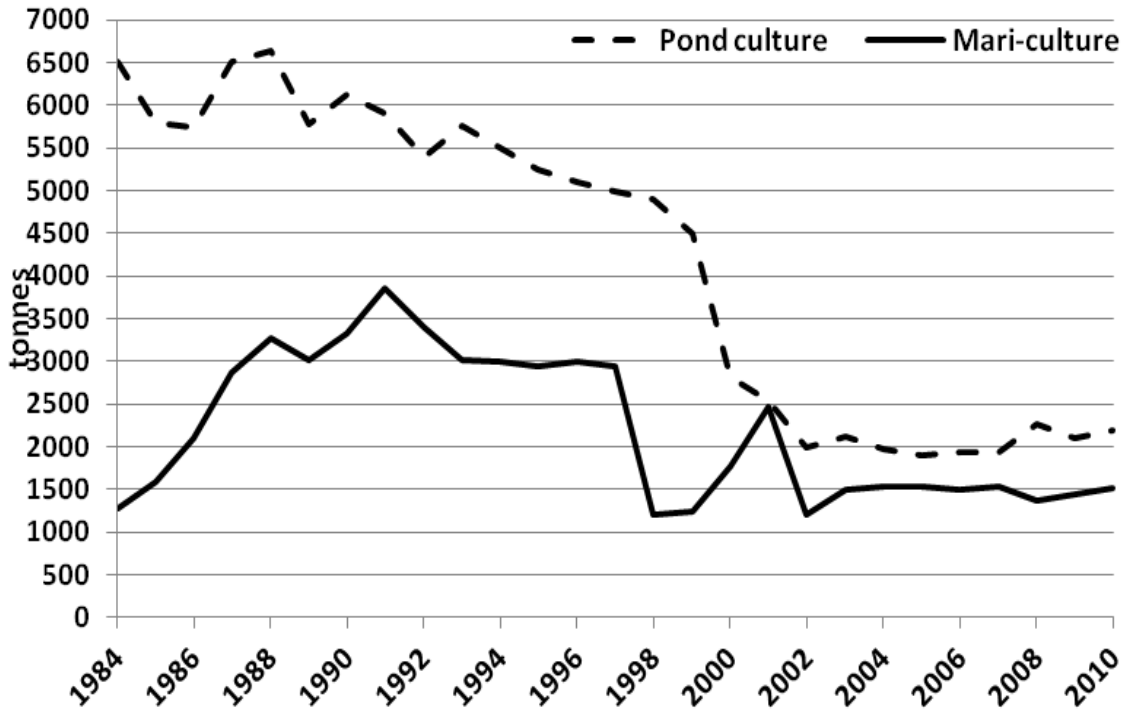

Fig. 1.2 The pond and mari-culture fish productions (tonnes) in Hong Kong aquaculture industry from 1984 to 2010 (Chan, 2005; AFCD, 2011)

Currently, the majority of fish farms use the polyculture mode which is similar to the past, but grey mullet became the major cultured species. The stocking composition included grey mullet (60-70%), grass carp (10%) and bighead (15%) in combination with small number of silver carp, common carp and tilapia (Lau *et al.*, 2003).

Grey mullet is the most popular species cultivated in the New Territories due to its relatively higher market value. In Hong Kong aquaculture industry, fish farmers (35%) usually start the fish culture cycle (except grey mullet) from fingerlings (~3-4" in length) to marketable size, but some farmers (~15%) start from juvenile (> 8"), this could reduce the grow-out period (Lau *et al.*, 2003; Pritchard, 2001). For example, the grass carp culture period is normally 10 to 12 months from fingerlings to martketable size (1.5-2 kg), but only 3-4 months are needed if started from juvenile (Lau *et al.*, 2003).

The decline in local aquaculture industry should be revitalized in the future due to the emerging food safety issues and better public awareness on the environment. In recent years, the Mainland's fish products quality has been challenged since a number of food safety issues have been reported and residents have re-evaluated the benefit of food produced locally. Various undesirable chemicals are detected in imported fish, such as malachite green which used for treating parasitic, fungal and protozoan diseases in fish and is probably carcinogenic to humans (Culp, 2004), was detected in

turbots, eel, freshwater grouper, and mud carp (CFS, 2006a, b & c). Nitrofurans are antimicrobial agents for veterinary use which may cause cancer in experimental animals, were detected in silver carp and freshwater grouper (CFS, 2006c). Endosulfan, an organochlorine pesticide which may cause chronic kidney damage, was found in live eels (CFS, 2006d). Although large scale food safety incidences on fish products were not found in recent few years, the occurrence of unsatisfactory aquatic products cannot be avoided (Fig. 1.3)

Due to the consecutive food safety incidences, local consumers have become more and more concerned over food safety, and some have higher preference on consuming local products. The consumers believed the local fish products are good in quality, although these products are usually sold at higher prices, especially when the products are labeled as “accredited” or “organic”. The Agriculture, Fisheries and Conservation Department (AFCD) implemented the voluntary Accredited Fish Farm Scheme (AFFS) to improve fish farms environmental hygiene standards and fish products quality. The fish products tagged with a specially designed mark were qualified from drug residues and heavy metals analyses before marketing.

Based on a survey sponsored by WWF, there are potentially 1.5 million households in Hong Kong who would be willing to consume “eco-fish” (fish labeled as chemical free). The “eco-fish” could be sold with a 30-40% price premium (about

HK$27.9/kg for grey mullet), and the profits could attract fishermen, making the industry economically sustainable (Boulanger *et al.*, 2008). The market share of local fishery products from quality farms are growing and could be sustained in future.

Moreover, edible marine and freshwater fishes for local consumption are mainly relied on the import from China and other countries. The marine fish culture production contributed about 0.2% (re-calculated from HKSAR's statistics) of total seafood consumption in Hong Kong, while pond fish farmers produced only ~2190 tonnes, about 4% of local consumed freshwater fish (HKSAR, 2012). However, the demand of local aquaculture products is expected to be promoted once other sources of fishery product supplies are limited. A remarkable decrease on the supply of capture fish products is foreseen in near future, as the Hong Kong Special Administrative Region (HKSAR) Government (hereafter "the Government") has issued the legislative amendments to ban trawling (including pair, stern, shrimp and hang trawling) in Hong Kong waters and the trawl ban became effective since 31 December 2012. The local fisheries should contribute more high quality fish products and reduce the demand for captured fish products.

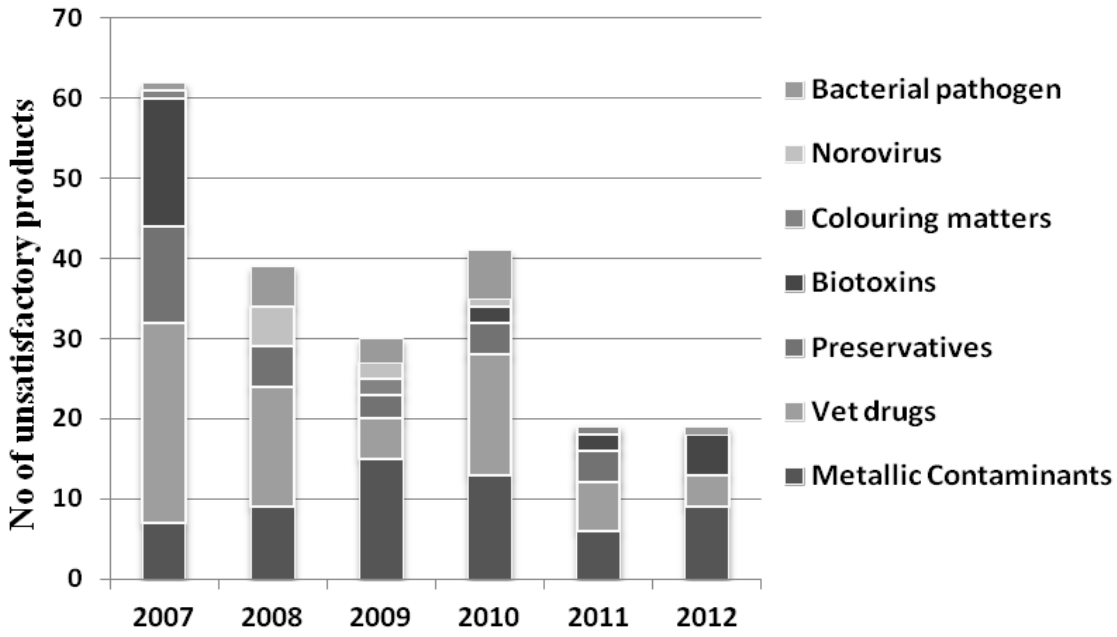

Fig. 1.3 The number of unsatisfactory aquatic products in Hong Kong from 2007-2012 (*Included data from Jan to Oct only for 2012) (Source: CFS Food Surveillance Programme)

1.3 Emerging problems in fast growing aquaculture industry in worldwide and China

The mass production and the densely cultured systems are vulnerable to disease once the environmental condition is deteriorated, and severe economic loss is suffered in the resulted disease outbreaks. In theoretical basis, fish disease is the closely related to the interactions between host (fish), environment and pathogen, the control of fish diseases should be based on multiple aspects i.e. maintaining good water quality and avoiding stressful condition for fishes. However, fish farmers are inclined culture fish at high stocking density due to economic factors, and applied antibiotics for combating fish diseases is commonly found in real situations. Apart from antibiotics, vitamins, hormones and other chemicals (e.g. fungicide) are also applied in aquaculture as antibacterial agent and growth promoters (Jayaprakas and Sambhu, 1996). All these production methods caused detrimental impacts on the environment and society. Another problem accompanied with the rapid growth of aquaculture industry would be the fish feed supply. Fish meal is an important and major ingredient and its availability is expected to decrease in near future because of the increased prices and raising concern on the sustainability of the ocean status (Tacon *et al.*, 2006). Currently, the supplies of other feeding ingredients e.g. soy bean, corn and wheat are tense as these materials are also consumed by animal husbandry sectors and humans

(Rana *et al.,* 2009). The development of aquaculture industry may not be environmental and economically sustainable and changes on feeding materials and drug applications are needed in the near future.

1.3.1 Disease prevalence and abuse of chemicals and antibiotics

With the rapid development of aquaculture, a wide range of naturally occurring and synthetic antibiotics have been frequently applied for curing the infections, the practice of using veterinary antibiotics and chemicals has been overwhelming and the demand on those drugs is on the rise. China ranked the first in terms of annual production of penicillin, terramycin and doxycycline in the world, which account for 60% of the world's total production (Grace, 2004). Various antibiotic such as sulfonamide, terramycin, penicillin, streptomycin, erythromycin, chloramphenicol and oxolinic acid were commonly used through oral administration, bath treatment and injection in China (Southeast Asian Fisheries Development Center, 2000). Most of these are used for aquaculture as well as livestock and poultry farming. Antibiotics are very effective in combating bacterial diseases and saved salmon farming in Norway, which suffered from serious infections from *Vibrio* spp. during the 1980s (Grave *et al.*, 1990). The application of antibiotics has also become the inevitable practice in China

aquaculture (Xu *et al., 2006*), and various types of antibiotics are detected in the coastal area in China, which is believed that aquaculture discharge is one of the major sources (Zou *et al.*, 2011)

Despite the imperative contributions of antibiotics in controlling fish diseases, the adverse effects caused by the abuse of antibiotics and their environmental fates have stimulated public attentions. Most of the veterinary drugs are only partially metabolized in the fish body before entering the water environment via urine and excreta (Hamscher *et al.*, 2000), accumulation of substantial amounts of antibiotics in the river or fishpond sediment are resulted. They may also enter into the soil together with the fishpond mud which is commonly used as fertilizer for crop production (Boxall *et al.*, 2006; Zhu *et al.*, 2007).

Consequently, resistance genes have been developed against antimicrobial agents and are commonly found in aquatic bacteria e.g. *Vibrio* spp. and *Aeromonas* spp. (Kim *et al.*, 2004; Sørum, 2006). The abuse of antibiotics endangered the natural environments, numerous studies showed antibiotics resistant bacteria are frequently detected around aquaculture sites and closely related to the aquaculture use of antibiotics (Karunasagar *et al.*, 1994; Chelossi *et al.*, 2003; Sahul Hameed *et al.*, 2003; Alcaide *et al.*, 2005). Not only therapeutic doses of veterinary antibiotics, sub-

therapeutic doses in aquaculture which is also generally used in fish farms for prophylactic purposes and both applications caused the development of antibiotic resistant bacteria (Smith *et al.*, 2002). Even worse, terrestrial veterinary pathogens and human pathogens may obtain the antibiotic resistance through horizontal transmission from bacteria in the aquatic environment (Angulo and Griffin, 2000). The recent appearance of super-bacteria has opened the "Pandora's box" for a wide array of upcoming problems. The situation has raised social and environmental concern due to potential risks for human health (Cabello, 2006).

The antibiotic residues may also contaminate the aquaculture products and also exert profound impacts on the economic development. The European Union (EU) banned the shrimps and prawns imported from China in 2002 due to their high residual antibiotics (Hernández, 2005). The prohibition was only ceased after intensified monitoring and testing for drug residues on aquatic products (EU, 2002). In recent years, the incidence of "poison fish" in the Hong Kong market is no longer a rare occurrence, some aqua-products contaminated with antibiotics (e.g. furazolidone) and fungicide (e.g. malachite green) are directly imported from the Mainland as previous mentioned. Consumption of those contaminated products may assist the development of antibiotic resistance bacteria in human intestine (Salyers *et al.*, 2004), a higher risk to human in front of bacterial infections could be resulted (Sørum, 2006).

The safeguarding of food supply has become a serious public issue. There is a noticeable demand to seek an alternative to antibiotics via environmentally-sustainable and ethically-acceptable approaches.

1.3.2 Rising feed materials prices

Another challenging issue in sustaining the rapid growing aquaculture industry would be the source of protein in fish feed. In the aquaculture industry, fish feeds are made from different natural plant materials and related by-products, such as soybean meal, cotton seed, corn gluten and rice bran and animal products e.g. fish meal and fish oil. However, the prices of these materials are rising due to competitions with animal husbandry sectors, and human consumption and increased fuel cost when transported from production countries (Chile, Brazil, Argentina and U.S.) to major markets i.e. Asia (Rana *et al.*, 2009).

Fish meal (FM) and fish oil are the important and expensive ingredients as they are major protein and lipid sources in aquaculture feeds. Fish meal is usually processed from by caught or non food fish captured by fishery industry and by-products from fish processing industry i.e. fish offal or fish trimmings (FIN, 2006; FAO, 2012), about 1/3 (30 million tonnes) of total capture fishery productions are

used to manufacture fish oil and FM (Anon, 2002).

Approximately 68% of FM in the world was used for aquaculture feed productions (Tacon and Metian, 2008). The FM production raised from 5 million tonnes in 1970s to 7.5 million tonnes in mid 1990s, then dropped to about 6 million tonnes in 2009, while the demand on FM was froze in the past 10 years but the aquatic resource captured from environment has been leveled off in the past 20 years (FAO, 2012). Same trend was also observed in the supply of fish oil. The diminishing supplies and increasing market price of FM and fish oil also forced a decreasing usage in aquaculture in future (Rana *et al.*, 2009; Tacon and Metian, 2008). The global price for fishmeal was just US$500 to $700 per tonne in 2000–2005, but the price was doubled to US$1,210 per tonne in 2008 (Rana *et al.*, 2009).

In fact, the reliance on FM based feed was also disputed as inefficient and uneconomical, as the conversion from wild fish (for producing FM) to farmed fish (fish product) is about 6 to 1 in ratio, especially on carnivorous fish species, i.e. salmon, trout and various groupers (Staniford, 2002; Milewski, 2002). Consequently, alternative feeding materials especially for replacing FM are advocated for production animal feeds.

Besides, low FM consuming fish species (herbivores and omnivores) e.g. tilapia and carps are preferred over carnivorous species like trout and salmon in aquaculture

industry nowadays. The nutritional demand of herbivores and omnivores is also easier to be fulfilled than carnivores (Hardy and Tacon, 2002), and those fish feed are less costly.

1.4 Using feed supplement as immuno-stimulants

As mentioned before, fish disease is the consequential interactions between pathogen, host and environment. A multiple approach involving inhibiting and eliminating pathogens, reducing host infections and improving environmental quality is essential for combating fish diseases. However, the pathogens especially bacteria are becoming more resistant to drugs and the environment like aquaculture zones are getting more polluted due to human activities. The enhancement on host immunity through feed supplement could be an advanced progress in aquaculture research, while herbal medicine such as Traditional Chinese Medicine (TCM) and baker's yeast are the novel aquaculture applications. Feed supplements have been widely used in aquaculture industry nowadays, including probiotics e.g. yeasts, but the use of herbal medicine is relatively a new topic in the industry. Herbal supplement as immunostimulants, is preferred because it is natural, biocompatible, biodegradable,

cost effective, and more importantly it is safe for the environment (Ortuno *et al.*, 2002).

1.4.1 Herbal supplement: Traditional Chinese Medicine (TCMs)

Traditional Chinese medicine (TCM) and herbs have been used as immunostimulants in human for thousands of years (Tan and Vanitha, 2004), but its use in aquaculture have been only explored in recent years, The herbal medicines can serve as antimicrobial agents or immunostimulants to prevent fish disease and could be a potential alternative to replace vaccines (Anderson, 1992; Secombes, 1994) and antibiotics (Galina *et al.*, 2009).

The principle of TCM is quite different from the Western pharmacological and therapeutic principles which targets on diseases or pathogens directly. The rationale of TCM is mainly based on few theories, such as the five elements theory and the Yin-Yang balance, which are considering the overall balance of human body (Cheng, 2000). TCM compound formulation contained various constituents which played different roles in disease treatments. The major constituent showed chief therapeutic effects e.g. anti-inflammation and balanced the disharmony in the body, while the adjuvant and minister constituents (second component) assisted the therapeutic action

of major components. The messenger constituent (component making the formula prescription targeting pathological tissues) functioned as a “guider” to modulate the formulation to targeted organ or to eliminate the disharmony caused by other medicines in the formulation (Cheng 2000; Gao and Wu 2008). In other words, TCM is aimed at maintaining and restoring the balance of body or enhancing the immunity to defence diseases. Therefore, TCM formulas are not just an addition of herbs but involve complex interactions between the herbs. Investigation on the compound formulations of TCMs could be beneficial to aquaculture industry in combating fish diseases.

In modern pharmacologicalogy, the therapeutic effects of TCM active ingredients have been validated in some animal models such as mice, fish and human cells. S*cutellaria* extracts exhibited good antibacterial and antiviral effects, and boosted phagocytic activity in mice at low dose (Cai *et al.*, 1994), and with similar activity found in fish at low dose (Yin *et al.*, 2006). *Andrographis paniculata* extracts were especially important for treating inflammation by modulating macrophage and neutrophil activity in mouse (Chiou *et al.*, 2000) and human blood cells (Shen *et al.*, 2002). *Rhizoma coptidis* exhibited superoxide and hydroxyl radical scavenging properties in rat kidney and brain tissues homogenates (Liu and Ng, 2000).

The medicated values of TCMs in fish mainly focused on the non-specific immune responses and disease resistances of fish to bacterial infections. Rohu, *Labeo rohita* fed with *Withania somnifera* enhanced its phagocytic activity, lysozyme activity, total immunoglobulin level and Nitro blue tetrazolium (NBT) activity (Sharma *et al.*, 2010). The disease resistance to *Aeromonas hydrophila* in rohu (*L. rohita*) was also enhanced when fed mango kernel (*Magnifera indica*) added feed (Sahu *et al.*, 2007b). Common carp (*Cyprinus carpio*) fed with *Astragalus* root extract showed better survival rate after challenged with *A. hydrophila* (Yin *et al.*, 2009).

The use of compound formulation may have better effect on immune-stimulation or improvement on disease resistance of fish, compared with the use of single herb. Nile tilapia (*Oreochromis niloticus*) fed with mixed *Astragalus* and *Lonicera* extracts enhanced phagocytic and respiratory burst activity of blood phagocytic cells and its disease resistance against *A. hydrophila* (Ardó *et al.*, 2008). Mixed *Scutellaria radix* and *Herba euphorbiae* also showed a remarkable prevention and treatment rate up to 85% against bacterial septicemia, the increase of phagocytic activity was also elevated accompanied with higher herbal concentration and longer feeding period to goldfish, *Carassius auratus auratus* (Zheng *et al.*, 2006). All these basic medical values of herbs showed in fish advocating the uses of TCMs in fish farming for replacing drugs like antibiotics.

1.4.2 Probiotics: Baker‘s yeast

Probiotics is the feed supplement defined as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance” (Fuller, 1989). The probiotics is used in the form of feed additives or directly introduced into the water body, resulted enzymatic contribution in digestion, inhibition of pathogenic microorganisms, antimutagenic and anti-carcinogenic activity, growth promoting factors, and increased immune response (Ziaei-Nejad *et al.,* 2006; Wang *et al.,* 2005a; Wang and Xu, 2004).

The bacteria *Bacillus* sp. is reported to improve water quality, survival and growth rates and the health conditions of juvenile shrimp (*Penaeus monodon)* (Dalmin *et al.*, 2001). *Saccharomyces cerevisiae*, the yeast applied in baking and fermenting alcoholic beverages industries has been used to improve feed utilization and growth performance in various carp species (Mohanty *et al.,* 1996; Swain *et al.*, 1996) and Nile tilapia (*O. niloticus*) (Abdel-Tawwab *et al,* 2008). These positive effects may be due to the adherence of yeast cells to the fish gut and secretions of amylase enzymes which increased digestibility of diets (Scholz *et al.* 1999). Furthermore, the cell wall constituents of yeast could stimulate the innate immune responses and protect fish against infections (Esteban *et al.,* 2001).

1.5 The necessity of alternative protein sources in fish feed

In modern aquaculture industry, fish feed is the most important and the major cost for production and it should contain balanced nutrients and sufficient energy for fish growth. The feeds are mainly made from different ingredients from plant and animal derivatives, e.g. soy bean meal, rapeseed meal, rice bran, wheat bran, corn gluten from plants and fish meal, blood meal, meat meal, bone meal, and fish oil from animals, which provided different nutrients (e.g. amino acids, fatty acids) and essential elements (e.g. vitamins, trace metals) for fish growth. Different fish diets are formulated by adjusting the mixture of ingredients as requirements on nutrients and energy are varied between fish species and different growth stages (larvae, fingerling, juvenile and broodstock). In general, fish meal is the major protein source used in aquaculture in the past due to the low-cost, abundance, good palatability and high protein quality; but the preference of FM as feeding materials no longer exists as described before. As a result, alternative sources of protein for fish feeding are essential to support the industry in the coming future and it is an important topic in aquaculture industry.

1.5.1 Utilization of food waste as fish feed in worldwide

In past decades, shifting from animal protein to plant based protein was major development in fish feed producing industry, the fish meal in aquaculture feed has been gradually and partially replaced by other protein sources, such as soy bean meal and rapeseed meal. The inclusion levels of fish meal were decreased in feeds for various species e.g. trout, Salmon and carps (Table 1.1) (Tacon *et al.*, 2011). Various studies focused on the reduction of fish meal in feeds and the less demand of fish meal is predicted. It has been shown that salmon fed with a diet containing 20% soybean protein achieved a similar growth rate to those fed with high-quality fish meal only (Olli *et al*, 1995*)*.

However, the previous motives of using low cost and high availability of plant based protein sources no longer exist due to the stress on food supply and competition from livestock sector and human consumption (Fasakin *et al.*, 1999; Rana *et al.*, 2009). When the feeding materials are less available and the production costs increased, the industry tried to use food wastes and industrial by-products e.g. soy bean residue and papaya waste for rearing fish. Some categories of food wastes, such as meat waste, fruit and vegetable waste and fish waste are rich in nutrients, they could be considered as substitutes for original raw materials for animal feed productions (García *et al.*, 2005). Various types of food and industrial wastes from poultry, soy sauce producing, rice wine and papaya processing industries, are

incorporated into aquaculture feed production in recent years as alternative for fish meal are performed (Erturk and Sevgili, 2003; Bake *et al.*, 2009; Kang *et al.*, 2010; Vechklang *et al.*, 2011).

In general, meat and fish waste are good protein and lipid sources for fish feed, meat waste was rich in ether extract and fish waste contained a high level of crude protein, while fruit and vegetable waste contained more nitrogen free extract (digestible carbohydrates) (García *et al.*, 2005). In Hawaii, fermented papaya processing wastes by yeast are implemented as fish meal substitute in shrimp feed, the result showed that 50% of fish meal can be replaced by papaya wastes based on growth rate, feed conversion ratio (FCR) and survival rate after 8 weeks of feeding (Kang *et al.*, 2010). Similar results, comparable growth, survival rate and feed efficiency to control feed were also found in soybean poultry by-product feed with egg supplemented in Pacific white shrimp (Samocha *et al.*, 2004).

Brewery wastes which contain about 20% of crude protein and possess a very good amino acids profile such as lysine, arginine and methionine (NRC, 1983). The brewery waste could replace the rice bran which is another major ingredients in fish feed, but different growth performance were observed in 3 carp spcies, *Catla catla (Ham.), Labeo rohita (Ham.) and Cirrhina mrigala (Ham.). C. catla* and *L. rohita*

Table 1.1 Reduced fish meal inclusions in aqua feed of different aquaculture species (FAO, 2012)

Species/ Species group	Fishmeal inclusion in compound aquafeed (%)		
	1995	2008	2020*
fed carp	10	3	1
Tilapias	10	5	1
Catfishes	5	7	2
Milkfish	15	5	2
Miscellaneous freshwater fishes	55	30	8
Salmons	45	25	12
Trouts	40	25	12
Eels	65	48	30
Marine fishes	50	29	12
Marine shrimps	28	20	8
Freshwater crustaceans	25	18	8

*Predicted value in Tacon *et al.* (2011)

(Ham.) also showed best growth performance and FCR in 30% brewery waste incorporated feed, *C. mrigala (Ham.)* showed retarded growth on the contrary (Kaur and Saxena, 2004).

Poultry by products are other alternative protein source which has been studied in past 30 years (Higgs *et al.*, 1979; Rawles *et al.*, 2009), however, the results were varied in carnivorous and omnivorous fishes. Rawles *et al.* (2006; 2009) showed the poultry by products could replace the fish meal by 40% or entirely in hybrid striped bass (carnivorous) diet, while poor growth in common carp, *Cyprinus carpio* fingerlings was observed in all tested feed with 33, 67 and 100% replacement of fish meal by poultry by-product meal (Emre *et al.*, 2003).

1.5.2 Using enzyme for enhancing feed conversion in fish feed

Enzyme supplementation on animal feed materials has been studied to enhance feed conversion and utilization which can be found in poultry and swine industry (Cowan *et al.*, 1996; Kitchen, 1997). In fish processing industry, the wastes contained heads, viscera, skin, and skeleton are dumped without further utilization in the past, and the quantity is believed to be more than 20 million tonnes, or 25% of fish products (Rustad, 2003). However, these by-products are rich in protein and can be

utilized as alternative protein source in the form of fish protein hydrolysate, which is a soluble product of fish wastes treated with enzymes (Guerard *et al.*, 2002) and it is a potential ingredient producing aquaculture feeds (Bhaskar and Mahendrakar, 2008; Nilsang *et al.*, 2005). A commercial enzyme, Ronozyme^{TM} VP enhanced growth performance and net protein utilization in tilapia fed with enzyme supplemented palm kernel meal based feed (Boonyaratpalin *et al.* 2000).

Other enzymes like bromelain and papain could be utilized in aquaculture, these proteases may enhance the digestibility of protein through hydrolyzing connective tissue and skin in raw materials in food wastes. Bromelain is extracted from the stem and fruit of pineapple and it could hydrolyse the feed proteins into smaller protein peptides with higher digestiblity (Fennema, 1996). Previous study by Wong and his colleagues (1996) also showed that pretreatment of soybean residue by papain could enhance the feed digestibility and conversion of common carp (*Cyprinus carpio*).

1.6 Potential uses of drug alternatives and food waste as fish feed in Hong Kong aquaculture industry

The reactivation of local inland fisheries is needed as increasing demands on high quality fish products and preserving wild fisheries resources. Hence, more

technical support is preferred for enhancing the aquacultural productivity by environmental friendly and sustainable means, ideally reducing production costs at the same time.

Local pond fish farmers usually applied wheat bran, soybean and peanut cake as major fish feed, but the fish also fed with corn meal, bread, noodles, flour and biscuits (Lau *et al.*, 2003). In those feed types, soybean, bread, noodles and biscuits can be regarded as industry by-products or food wastes. Only a very small amount of fish farmers adopted fish feed pellets as regular feed. As cost is the dominant consideration for farmers (Lau *et al.*, 2003), the cost of food waste fish feed is therefore a crucial point when attracting farmers' attentions as well as the fish grow rates.

The recycle of food wastes could also be one of the waste management strategies in Hong Kong. According to the Government, food wastes comprised of 38.4% of the domestic waste in 2007 (Leung *et al.*, 2008). The Government's policy framework (2005-2015) mainly focused on the municipal solid waste management through landfill disposal bans, waste charging and producer responsibility schemes. More alternatives for food waste treatment would be important for easing landfill disposal pressure. Recycling of food wastes can utilize these valuable organic wastes and

therefore alleviate the loading of local landfills. A pilot plant, the Waste Recycling Centre at Kowloon Bay, with a 4 tonne/day capacity, was commissioned in 2008 to handle food waste from the Olympic equestrian events. A trial operation for the recycling of source-separated food wastes generated from selected commercial and industrial sectors including restaurants, hotels, wholesale markets and generators of the catering, food production, bakery and bean curd industries was subsequently commenced (EPD, 2009). Based on the results obtained from these trials, larger facilities (Siu Ho Wan, Lantau Island and Shaling, North District operated in the late 2010s) could handle total 400 tonnes (mostly food waste) for the production of biogas and compost daily, but the capacity is just about 10% of daily produced food wastes (EPD and ENB, 2008).

The use of food wastes for rearing fish could be a sustainable strategy to support fish feed and aquaculture production ecologically and economically. According to Kowloon Biotechnology Limited's website, the only food waste recycling factory for animal feed production in Hong Kong, could handle 50 tonnes of food waste daily (2012). The factory sold their feed to local fish farms, nonetheless the animal feed products were not endorsed by the Agricultural, Fisheries and Conservation Department (AFCD) (Asia City Network, 2012), which is the local department managing local capture and aquaculture fisheries industry.

As a result, more scientific research to formulate quality food waste feeds, and the feasibility of application in Hong Kong is needed. Incorporating of food wastes into fish feeds would be a sound technology for easing food waste problem and reducing economic costs of aquaculture industries.

1.7 Aims and objectives

This thesis focused on using food wastes and feed supplements e.g. enzymes, baker's yeast and TCM for rearing freshwater fish (grass carp and grey mullet) (Fig. 1.4) with the following aims:

1) To investigate the appropriate food waste composition, mainly meat, cereal, vegetable and fruit wastes as viable protein sources in the diets of grass carp and grey mullet diets based on feeding trials (Chapter 2). The feed utilization and fish growth of both species and the dietary protein and lipid digestibility by grass carp was also determined were evaluated;

2) To investigate the effects of growth and immunity of both species by food waste feed supplemented with bromelain and papain mixture based on feeding trials (Chapter 3). Different parameters to indicate fish growth performance e.g. specific

growth rate, feed utilization e.g. protein efficiency ratio and some hematological parameters e.g. total plasma immunoglobulin were measured;

3) To investigate effects of growth and immunity of grass carp with the dietary supplementations of yeast (Chapter 4), Traditional Chinese Medicine (TCM) (Chapter 6) and their combined form (Chapter 7). The hematological parameters e.g. Nitro-Blue tetrazolium (NBT) activity, total plasma immunoglobulin and plasma bactericidal activity and the disease resistance to pathogen through *Aeromona hydrophila* injection were determined;

4) To investigate the antimicrobial activities of various TCM extracts on several field isolated fish pathogens, e.g. *A. hydrophila*, *Vibrio cholerae* and *Lactococcus garvieae*, and the development of drugs resistant pathogens through broth microdilution method (Chapter 5); and

5) To investigate the effects of single and combined TCM extracts on plasma bacteriacidal activity of grass carp by *in vitro* study (Chapter 6)

It was hypothesized that the use of enzymes and yeast could improve feed conversion of food wastes feed, a low feed conversion ratio (FCR) and a faster growth rate are observed in fish. It was also hypothesized that the Traditional Chinese

Medicine (TCM) could enhance the fish immunity and disease resistance to pathogens. Finally, using food waste as feed material, and enzymes, baker's yeast and TCM as feed supplements for rearing fish were generally discussed, and an overall conclusion was drawn based on the results generated in this study.

1.8 Contributions and significance of the present research

On the whole, this study is the first investigation focused on food waste with feed supplements (e.g. enzymes, baker's yeast and TCM) for Hong Kong aquaculture industry. The results provided preliminary but important information on using feed supplements (TCM and baker's yeast) to enhance fish immunity and enzymes upgraded food waste feeds for rearing fish.

Food wastes contained notable protein levels and other nutritional contents which became a controversial issue for waste disposal, shortening the lifespan of local landfills. Utilization of food wastes could diminish the volume of dumped waste and extend the lifespan of landfills, while the protein rich food waste is a valuable and useful resource which could be incorporated into fish feeds as raw materials, and should not be simply disposed as waste.

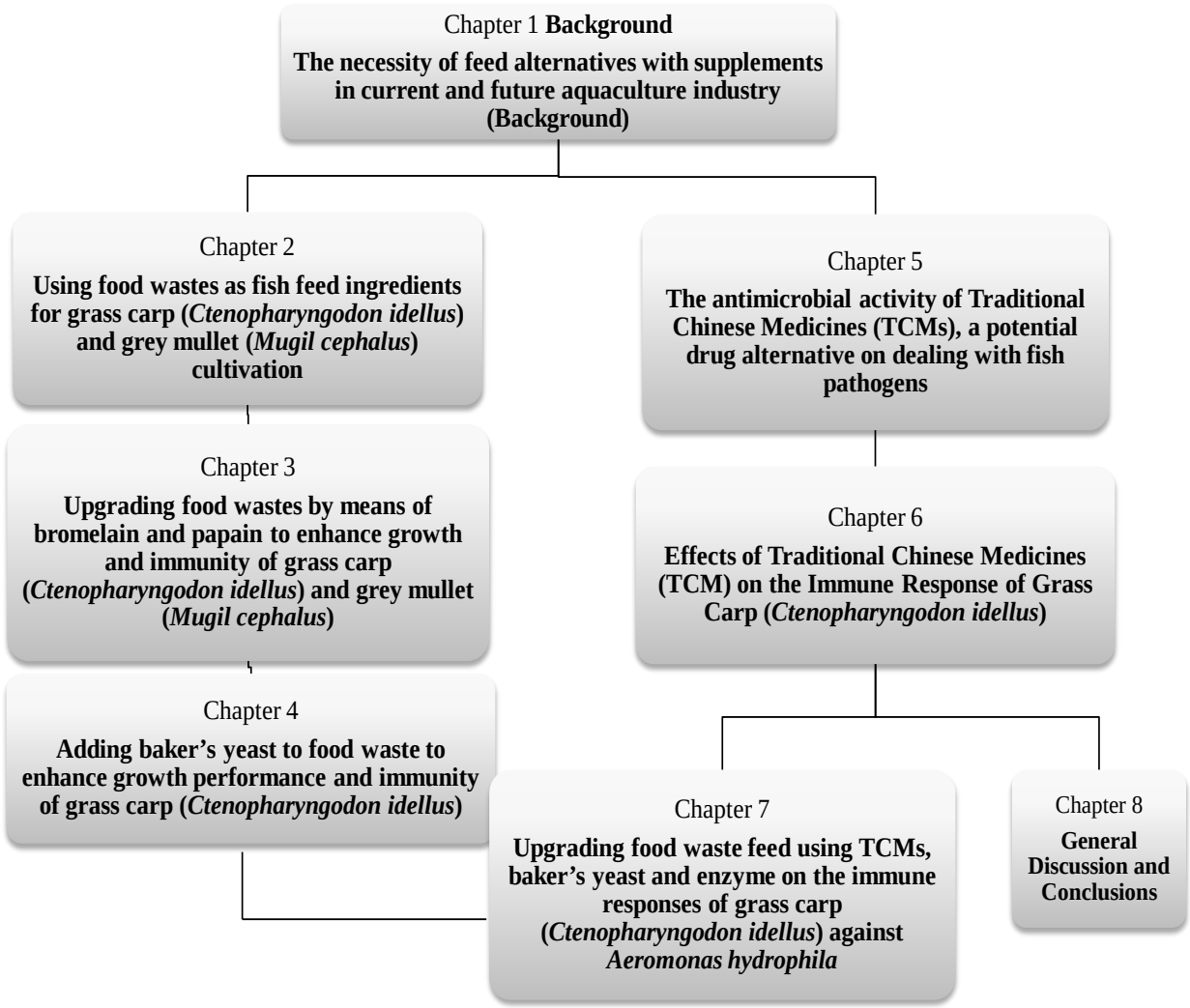

Fig. 1.4 Framework of research

The feed supplements (TCM and baker's yeast) could enhance fish immunity and reduce mortality due to infections, in other words the yield could be increased. These supplements may also reduce the use of antibiotics which has been applied in aquaculture as antibacterial agents for many years (Jayaprakas and Sambhu, 1996).

The ultimate goal of this study is to activate local inland fisheries through applying low-cost and reliable food waste feeds supplemented with environmental friendly feed supplements for rearing fish. The same notion could be applied in marine aquaculture, for the development of both sustainable marine and freshwater aquaculture in Hong Kong.

Last but not least, the general public could also consume those high quality and green fish products, and enjoy the better preserved fish ponds habitats for leisure. The better managed fish ponds could also provide shelter and foods for both local and migratory birds. All these outcomes could contribute to the better development of the sustainable aquaculture industry, economically and environmental friendly and these positive impacts on the environment, society and the inland fisheries industry are precious and intimately interrelated.

Chapter 2

Using food wastes as fish feed ingredients for grass carp (*Ctenopharyngodon idellus*) and grey mullet (*Mugil cephalus*) cultivation

2.1 Introduction

Aquaculture is growing in its importance as one of the most important food supplies in the world. The industry contributed only 4% of global fishery production in 1970 and the expanded production accounted for 36% of total production in 2006 (Rana *et al.*, 2009). The current production is only 60 million tonnes (FAO, 2012), but it has been estimated that 120 million tonnes will be needed by the year 2020 (Delgado *et al.*, 2003).

Fish feed accounted for more than 50% of the total cost in the aquaculture industry (Rana *et al.*, 2009). The major feed raw materials are fish meal and cereal grains e.g. soy bean, rice bran, wheat, corn etc., which are the important sources of protein and carbohydrate, respectively. The prices of these raw materials are affected by various factors such as climate, weather conditions, global economic growth and fuel costs. From 2000-2009, the prices of fishmeal, soybean meal, corn and wheat rose by 55, 67, 124, 130 and 250 %, respectively (Rana *et al.*, 2009) (Fig. 2.1). This was due to the increasing energy cost (e.g. oils, gas) and demands, and also higher

feed cost from increasing manufacturing and transportation cost. The supply and cost of the raw materials were also affected by weather, e.g. drought, flooding and even global warming. Approximately 68% of fish meal in the world was used for aquaculture feed productions (Tacon and Metian, 2008) and the rest mainly consumed by pig and poultry sectors (FIN, 2007). Therefore, there is a great pressure on the production cost of fish meal due to competition between the two sectors.

The tremendous demand of aquaculture feed would also provoke the price in the future. In 2020, the global production of aquaculture feed is predicted reach 71 million tonnes, which is doubled the quantity in 2008 (29.2 million tonnes) and almost ten-fold of 1995 production (7.6 million tonnes) (FAO, 2012). It is estimated that the aquaculture feed is mainly consumed by carps, which accounted for 31.3 % (9.1 million tonnes) of the total feed production (FAO, 2012). As a result, seeking alternatives for aquaculture feed are urgently needed; and food wastes are one of the low-cost and nutritious sources of feeding materials which could be beneficial to the industry.

Based on government statistics, food wastes contributed 42.3% and 34.9% of the total domestic waste and commercial and industrial (C&I) waste in 2011 respectively (EPD, 2012). More food wastes from C&I sectors have been found in recent years, with only 400 tonnes daily in 2001, but reached 1,056 tonnes in 2011 (EPD, 2012).

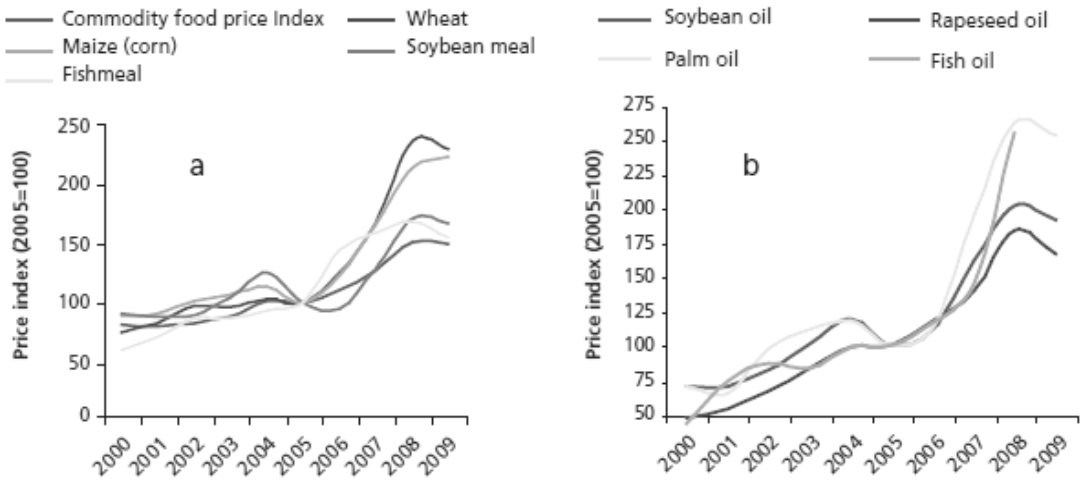

Fig. 2.1 The price index (2005=100) of a) food grains and fish meal and b) plant and fish oils, used for animal feeds production and human consumption (Rana *et al.*, 2009)

The utilization of food waste could diminish the volume of dumped waste and extend the lifespan of landfills, while the protein rich food waste could be incorporated into fish feed as raw materials.

Different types of food wastes from poultry, soy sauce producing, rice wine and papaya processing industries, have been used as alternative protein to replace fish meal in several investigations (Erturk and Sevgili, 2003; Bake *et al.*, 2009; Kang *et al.*, 2010; Vechklang *et al.*, 2011). It has been shown that salmon fed with a diet containing 20% soybean protein achieved a similar growth rate to those fed with high-quality fish meal (Olli *et al*, 1995*)*. Rawles *et al.* (2009) showed the poultry by products could replace the fish meal completely in the diet of hybrid striped bass (*Morone chrysops* ♀ x *M. saxatilis* ♂). In the present study, various types of food wastes including fruit peel and vegetables, meat, bone meal and cereal wastes were collected from hotels and food processing plants and transferred to Kowloon Biotechnology Limited in Lau Fau Shan for manufacturing fish feed. Formulated feeds with different proportions of each category of wastes subsequently used for rearing grass carp *(Ctenopharyngodon idellus)* and grey mullet (*Mugil cephalus*).

Herbivorous and omnivorous fishes have fewer requirements on dietary protein than carnivorous species (NRC, 1993). Grass carp is a herbivorous cyprinid, which consumes aquatic macroflora and converts into meat protein as nutritional food source

(Gîlcă, 2010). Grey mullet is an omnivore and feed on algae, detritus and microscopic invertebrates (Olukolajo, 2008). Polyculture is commonly adopted by local fish farmers with the following stocking composition: grey mullet (60-70%), grass carp (10%) and bighead (15%) in combination with small numbers of silver carp, common carp and tilapia (Lau *et al.*, 2003). Grass carp and grey mullet are the major components, and therefore, they are the ideal species for the present investigation to use food waste as fish feeds.

The major objectives of the present study were 1) to determine the optimum formulation comprising various categories of food wastes for rearing grass carp and grey mullet 2) to evaluate the effects of food waste on the growth performance and body composition of both species. It is hypothesized that food wastes could be used as alternative protein source for rearing fish. It could be a sustainable means of aquaculture with both ecological and economical significance. This approach could also promote recycling of food wastes to ease part of the waste disposal pressure faced in Hong Kong.

2.2 Materials and Methods

2.2.1 Food waste fish feed preparation

Food wastes mainly consisted of food processing waste and partially post

consumed waste, collected from local hotels and food processing plants, grouped into 4 major categories: (1) fruit peel and vegetables, (2) meat, (3) bone meal and (4) cereal wastes. Fruit waste mainly contained peels with some flesh of various fruits, about 25% of pineapple, 25% watermelon, 15% Cantaloupe and 35% other fruits, e.g. strawberry, banana, apple. Meat wastes included 60 to 70% of beef, pork and chicken, 30 to 40% of fish such as salmon and groupers. Vegetables contained various types of leaf vegetables, such as lettuce and spinach. Cereals usually included rice bran, soy bean meal, rice grain and spaghetti (Table 2.1).

All the food wastes were collected daily from the hotels and food processing plant and transferred to the factory of Kowloon Biotechnology Limited (at Lau Fau Shan) for further processing within 2 h. They were diced into small pieces (~1cm diameter), with excessive water squeezed out, and dried at 80ºC for 6 h. Each type of food wastes was grinded into powder to form different food waste products, under different ratio and mixed with other raw materials (non-food waste), such as fish meals, and corn starch for pelletizing fish feed.

Table 2.2 shows the different types of food wastes prepared for laboratory experiments. They included Food wastes A (FWA- without meat), B (FWB- with 25% meat) and C (FWC- control), A commercial feed: Jinfeng®, 613 formulated feed (~30% protein), which mainly contained wheat middling, flour, bean pulp, rapeseed meal, fish meal, bean oil and fish oil used as the control feed. The proximate

composition (ash, moisture, protein, fibre, lipid and nitrogen free extracts), amino acid profile, and the feed protein solubility in KOH were determined based on the methods described in Section 2.2.5 and the results are shown in Tables 2.3 and 2.4.

In general, FWA mainly comprised of cereal food wastes e.g. rice bran, soy bean meal, rice grain and spaghetti, which would be ideal protein sources for herbivores and omnivores, i.e. grass carp and grey mullet, with some meat products used to replace parts of cereals in FWB and FWC. The control feed contained mainly wheat middling, flour, bean pulp, rapeseed meal, and the formula was similar to FWA.

2.2.2 Feed digestibility of different food wastes formulations in grass carp

Three hundred fingerlings of grass carp *Ctenopharyngodon idella* (herbivore) were used for testing different fish feeds, with 12 individuals stocked in each tank (~60 L water) in triplicates. 0.5% by mass (dry weight) of the chromium oxides (Cr_2O_3) (Sigma Aldrich) were extruded with the experimental feed. Each type of experimental feed pellet (3mm) was fed to the experimental fish at a feeding rate of 3% body weight wet weight diet per day for 35 days. Feeding with the control feed was carried out at a rate of 3% body weight daily in each experimental feeding group and the fish were acclimated for 3 weeks before the start of experiment. The water temperature, pH and dissolved oxygen were measured three times a week using a portable Hanna

Table 2.1 Food waste categories collected from hotels

Categories	Food		Amount (%)	Non-cooked or cooked
fruit peel and vegetables	-	pineapple	20	Non-cooked
	-	watermelon	20	
	-	Cantaloupe	15	
	-	strawberry, banana, apple	30	
	-	leaf vegetables, such as lettuce and spinach	15	
Meat	-	beef, pork and chicken	60-70	Cooked
	-	salmon	35	Non-cooked
	-	groupers	5	Cooked
Cereals	-	rice bran	50	Non-cooked
	-	soy bean meal	20	Cooked
	-	rice grain	20	Cooked
	-	spaghetti	10	Non-cooked
Bone	-	beef, pork and chicken	60-70	Cooked
	-	salmon	30	Non-cooked
	-	groupers	10	Cooked

Table 2.2 Food waste feed formulations (containing 75% food waste)

Formulation	Food waste products					Non-food waste products		Total (%)
	fruit/ vegetables	meat products	cereals	bone meal	other	fish meal	corn starch	
Food waste A	10	0	53	8	4	10	15	100
Food waste B	10	25	28	8	4	10	15	100
Food waste C	10	10	43	8	4	10	15	100

Table 2.3 Composite analysis of experimental feeds

Formulation	Control	FW A	FW B	FW C
Dry matter (%)	93.68±0.20a	95.69±0.02a	93.20±0.05a	92.62±0.09a
Ash (%)	8.24±0.09a	9.18±0.46a	18.89±0.03b	17.04±0.15b
Protein (%)	30.16±1.55a	31.44±0.44a	31.13±3.36a	30.86±1.64a
Lipid (%)	5.17±0.94a	6.12±1.66a	13.25±1.81b	19.04±1.64c
Fibre (%)	9.57±0.21 a	9.62±0.63 a	5.72±0.87 b	5.85±0.30 b
Carbohydrates (%)[1]	40.53	39.34	24.21	19.82
Energy (kJ/g diet)[2]	16.17	16.64	16.77	18.23
CHO/L ratio [3]	7.84	6.43	1.83	1.04
P/E (mg/kJ)[4]	1865.0	1889.1	1856.5	1692.5
Protein solubility (%)[5]	60.57±2.64a	51.81±1.43b	38.74±1.35c	39.93±2.25c

*Different superscripts (a, b, c) among feeding groups are significantly different ($p<0.05$)

[1]Carbohydrates (%) = 100 − (crude protein % + crude lipid % + moisture % + ash %+ fibre %) (Castell and Tiews, 1980)

[2]Energy (kJ/g diet) = (% crude protein × 23.6) + (% crude lipids × 39.5) + (%carbohydrates × 17.3) (Chatzifotis *et al.*, 2010)

[3] Carbohydrates to Lipid (CHO: L) ratio = % wt. in CHO/ % wt. in lipid

[4] Protein to energy (P/E) **(**mg/kJ) = crude protein (%) / energy

[5] Protein solubility (%) = Protein in KOH / protein in sample x 100 % (Araba and Dale, 1990)

Table 2.4 Amino acid composition (% dry weight basis) of the four experimental feeds (Control, FW A, B and C)

	Control	FW A	FW B	FW C
Essential amino acid				
Threonine	1.3	1.35	1.35	1.2
Valine	1.57	1.75	1.74	1.51
Methionine	0.65	0.63	0.63	0.41
Isoleucine	1.34	1.46	1.46	1.29
Leucine	2.5	2.77	2.77	2.26
Phenylalanine	1.49	1.66	1.67	1.48
Lysine	1.76	1.81	1.8	1.7
Histidine	0.77	0.88	0.88	0.81
Arginine	2.09	2.37	2.36	2.25
Sub-total	13.47	14.68	14.66	12.91
Non-essential amino acid				
Aspartic Acid	2.9	3.17	3.17	2.82
Serine	1.41	1.56	1.56	1.46
Glutamic Acid	5.64	6.4	6.39	6.01
Glycine	1.7	1.74	1.73	1.49
Alanine	1.8	1.92	1.91	1.4
Tyrosine	1.06	1.19	1.18	1.04
Proline	1.72	1.87	1.89	1.79
Sub-total	16.23	17.85	17.83	16.01
Total	29.7	32.53	32.49	28.92

pH meter and a YSI digital Dissolved Oxygen (DO) meter and the values ranged from 22.2-23.5℃, 6.5-7.2 and 6.0-7.5 mg/mL respectively.

Feces were collected 7 days after the experiment started in order to allow for adaptation to the experimental diet. Subsequently, feces were removed daily for 5 consecutive days per week by siphoning out from the bottom of the aquariums. The feces were recovered in the afternoon (3-4 h after feeding) as soon as they were voided by the fish. The feces were filtered through fine nets, immediately transferred to flasks, dried in an oven at 50℃ for 48 h and stored in desiccators until analysis. The feces recovered on different days, but coming from the same aquarium, were pooled.

After 30 days, the weight of fish was recorded and the fish were starved for 24 h before collecting carcass (3 fish per tank) for chemical analyses. The fish were killed by MS-222, weighed, freeze dried, ground into a homogeneous sample and kept at -20ºC. Five fish were collected at the beginning of the experiment for comparison. The ash, dry matter, protein and lipid content of fish carcass and fish feces were analysed, with detail procedures stated in Section 2.2.5.

Different parameters: apparent digestibility on protein, specific growth rate, relative weight gain, feed conversion ratio, protein efficiency ratio, apparent net protein utilization and fat deposition rate were calculated for monitoring the growth performance based on different treatments (Section 2.2.4).

2.2.3 Feed conversion of different food wastes formulations in grey mullet

Wild caught fries of grey mullet (omnivore) (4000, ~0.5 g each) were imported

from Taiwan for testing different fish feeds (formulation A to C) with 400 individuals stocked in a tank (~1000 L water each, 10 tanks in total) with continuous aeration and water flow-through, under a 12 h-light:12 h-dark cycle. The experiment was conducted in Ta Kwu Ling Operation Centre of Agriculture, Fisheries and Conservation Department (AFCD). Three food waste formulations (FW A, B and C) (Table 2.3) and a commercial feed Jinfeng®, 613 formulated feed (~30 % protein) were also used to feed the grey mullet as control, each with 2 replicate tanks accordingly. Fish mortality and fish weight were recorded daily and at the start and end of experiment, respectively. The water temperature, pH and dissolved oxygen were measured three times a week using a portable Hanna pH meter and a YSI digital Dissolved Oxygen (DO) meter (maintained at 20.1-23.1, pH 7.2-7.8, 6.9-7.8mg/L respectively).

Thirty individuals (5 fish were grouped as a sample and 6 samples in total representing the initial fish composition) were randomly sampled, and weighed at the beginning of the experiment. After 30 days of the feeding experiment, the fish were starved for 24 h before collecting the carcass for chemical analyses, with 30 individuals (same as the initial sampling) collected from each tank. They were killed by MS-222, weighed, freeze-dried, grounded into a homogeneous sample by a mechanical blender and kept at -20ºC until analysis. Experimental diets were dried to a constant weight at 50°C and ground prior to analysis. The ash, dry matter, protein and lipid content of fish carcass were analysed, and the detailed procedures were stated in Section 2.2.5.

Various parameters: specific growth rate, relative weight gain, feed conversion

ratio, protein efficiency ratio, apparent net protein utilization, fat deposition rate were calculated for monitoring the growth performance based on different treatments (Section 2.2.4)

2.2.4 Fish growth performance parameters

Growth rates were calculated for each aquarium as a specific growth coefficient resulting from the following expression:

a) Daily feeding rate (% body weight/day) = Daily feed intake (g/fish) / initial average body weight (g)

b) Specific growth rate, SGR (%/day) = 100 (Ln Wf- Ln Wi) / t

c) Relative Weight Gain, RWG (%) = (Wf – Wi) x 100 / Wi

where:

Wf is the mean final body weight (g) for the fish in each aquarium, Wi is the mean initial body weight of the fish in the same aquarium, and t is time in days.

d) Feed conversion ratio, FCR = feed intake (g) / (Final biomass – Initial biomass (g))

e) Protein Efficiency Ratio, PER = weight gain (g) / protein intake (g).

f) Apparent Net Protein Utilization, ANPU (%) = 100 × (final fish body protein (g) - initial fish body protein (g)) / total crude protein intake (g)

g) Apparent Net Lipid Utilization, ANLU (%) = 100 × (final fish body lipid (g) - initial fish body lipid (g)) / total lipid intake (g)

h) Apparent digestion coefficient (ADC) of protein and lipid of diet (%)

= 100 x [1-(dietary Cr_2O_3/fecal Cr_2O_3) x (fecal nutrient / dietary nutrient)]

2.2.5 Chemical analysis on fish carcass and feed

Feed protein solubility in potassium hydroxide (KOH)

The protein solubility was determined according to the procedure described in Araba and Dale (1990). Feed sample (~1.5g) was added to a 0.2% KOH solution (75 mL) with a magnetic stirrer for 20 min (in duplicate). The sample was then centrifuged at 3000 rpm for 15 min and the supernatant was filtered and the total nitrogen (15 mL of extract each, determined in triplicate) determined by the Kjeldahl method. The solubility of protein, expressed as a percentage, was calculated by dividing the protein content of the KOH extracted solution by the protein content of the fish feed sample.

Chromium analysis

Chromium (Cr) content in fish feed and feces were analysed by spectrometer (UV-1601, Shimadzu, Tokyo, Japan) after acid digestion of the samples according to Furukawa and Tsukahara (1966).

Protein analysis

Samples of fish feed and feces were analyzed for nitrogen content with a CHN analyzer (Carlo Erba NA 1500, CE Instruments, Thermoquest Italia, Milan, Italy), the protein content (%) was based on the nitrogen content of sample (%) multiplied by 6.25 (McKinney *et al.*, 2004).

Amino acid determination

The amino acid profile in the fish feeds was determined by the methods by GB/T 18246-2000 of Standardization Administration of China (2001). In brief, the feeds were hydrolyzed with 6N HCl at 110℃ for 24 h. The reactant was filtered through a 0.45 μm filter membrane and then the amino acid profile analysed by an amino acid analyzer (Hitachi, 835-50). The chemical analysis was performed in duplicate samples.

Moisture and ash content determination

The moisture and ash contents of the experimental diets and fish carcass were determined according to the procedures of the Association of Official Analytical Chemist (AOAC) (1990a, b). For the moisture determination (AOAC, 1990a), the sample (fish feed or carcass) (~0.5g) was weighed ($W_{\text{sample initial}}$) and dried at pre-weighed porcelain crucible with a cover at 100 ± 2° C for 24 h. Then the crucible was dried in a desiccators for 2 h and weighed to the nearest 0.1 mg immediately ($W_{\text{sample final}}$). The crucible was previously dried at 100 ± 2° C for 2 h and weighed (W_c) as mentioned above. The Percent Total Dry Matter (Total DM, %) was calculated as the following:

% Total DM = 100 x ($W_{\text{sample final}} - W_c$) / ($W_{\text{sample initial}} - W_c$)

$W_{\text{sample final}}$ = sample and crucible weight after drying

$W_{\text{sample initial}}$ = sample and crucible weight before drying

W_c = dried crucible weight

For the ash determination (AOAC, 1990b), the sample (fish feed or carcass) (~0.5g) was weighed ($W_{\text{ash initial}}$) and combusted at pre-weighed porcelain crucible

with a cover at 600 ± 2° C for 2 h. Then the crucible was dried in a desiccator for 2 h and weighed to the nearest 0.1 mg immediately ($W_{\text{sample final}}$). The crucible was previously dried at 100 ± 2° C for 2 h and weighed (W_c) as mentioned.

% Total ash = $100 \times (W_{\text{ash final}} - W_c) / (W_{\text{ash initial}} - W_c)$

$W_{\text{ash final}}$ = sample and crucible weight after combustion

$W_{\text{ash initial}}$ = sample and crucible weight before combustion

W_c = dried crucible weight

Lipid determination

Ultrasound-assisted extraction with modifications was adopted for lipid content determination in fish feed and fish carcass (Metherel *et al.*, 2009). 0.1 g of sample was extracted with 3:2 v/v hexane: isopropanol mixture (12 mL) for 20 min with 40KHz sonication bath (Branson Bransonic 5510). The organic layer was collected after centrifuged, the extraction was then repeated for 2 times and the organic layer were pooled and dried in a pre-weighed glass tube under nitrogen and the tube was keep at 102°C for 30 min. The tubes were weighed again after keeping in desiccators for 3 h.

Crude Fibre determination

Crude fibre was determined by the loss on ignition (AOAC, 1984) of defatted feeds (2g) after digestion with 200 ml of 0.255N H_2SO_4 and then 0.313 NaOH for 30 min each. The residues were filtered and washed with hot distilled water at least twice. The residue was washed with 10ml of acetone twice after all digestions. The weight

of residue was determined after 105ºC (24 h) and 550ºC (3 h).

The percentage fibre was determined as the following:

% Fibre= Weight of residue after 105ºC - Weight of ash after 550ºC x 100 / Weight of sample

Nitrogen-free extract determination

Nitrogen-free extract (non-fibrous carbohydrate) was calculated by subtracting the sum of (moisture % + crude protein % + crude fat % + crude fibre % + ash %) from 100 (Castell and Tiews, 1980).

2.2.6 Statistical analysis

The ADC of experiment feed of grass carp and effects of different feed types on the feed compositions, fish growth and fish carcass compositions and in two fish species were analyzed by one-way ANOVA, comparing the mean value with Duncan's multiple range tests ($p<0.05$) (SPSS Statistics 17.0, Chicago, Illinosis, USA).

2.3 Results

2.3.1 Results of feed digestibility of different food waste formulations in grass carp

2.3.1.1 Feed digestibility of different food wastes formulations and growth performance of grass carp

The grass carp fed with FW A, the cereals dominant (53%) feed showed the

most comparable growth performance to the control, with no significant difference was found between the two groups ($p>0.05$) (Table 2.5). SGR, RWG and PER of the control group were significantly higher than FW B and FW C ($p<0.05$), while FCR of FW C was significantly higher than the control group ($p<0.05$).

The protein and lipid digestibility in the control group (97.05% and 95.60% respectively) were significantly higher than all food waste feed groups (FW A, B and C). The protein digestibility in FW A and FW B were significantly higher than FW C ($p<0.05$). The ANPU and protein digestibility in FW A were significantly higher than FW C ($p<0.05$), but there were no significant differences on the ANLU and lipid digestibility between two groups.

In general, grass carp fed with control diet showed the best growth which was superior to that of FW B and C significantly in terms of APNU, protein digestibility, PER, SGR and RWG ($p<0.05$), while FW C had the lowest growth performance.

2.3.1.2 Carcass composition of grass carp fed with different food waste formulations

The carcass moisture was significantly higher in FW A than other groups ($p<0.05$). Significantly lower levels of protein (%) in grass carp carcass were found in FW C than other groups ($p<0.05$). The lipid content in carcass were significantly lower in FW A than all other groups ($p<0.05$) (Table 2.6).

Table 2.5 Growth performance of grass carp fed with different food waste feeds

Measurement	Control	FW A	FW B	FW C
Initial body weight (g)	18.69±1.0a	18.04±0.8a	18.80±0.8a	18.07±1.3a
Final body weight (g)	32.17±2.5a	27.20±2.7ab	25.97±1.2b	24.2±0.8b
Feeding rate (% b.w./day)	3.03±0.01a	2.91±0.1a	3.11±0.4a	2.93±0.1a
SGR[1] (% b.w./day)	1.94±0.29a	1.46±0.24ab	1.15±0.28b	1.05±0.23b
Relative weight gain (%)	72.34±13.7a	50.53±10.3b	38.36±10.6b	34.38±8.5b
Feed conversion ratio	1.34±0.24a	1.88±0.13ab	2.22±0.64ab	3.03±1.47b
Protein efficiency ratio	2.07±0.36a	1.35±0.13b	1.16±0.25b	0.94±0.22b
ANPU[2] (%)	48.39±4.3a	43.39±1.7ab	37.14±5.4bc	34.68±4.7c
ANLU[3] (%)	118.98±7.0a	39.14±6.9b	54.75±12.2b	55.26±19.7b
Protein Digestibility (%)	97.05±1.0a	83.29±3.5b	78.64±4.0b	73.05±2.4c
Lipid digestibility (%)	95.60±1.5a	86.15±3.6b	75.50±3.3c	80.64±6.8bc

*Different superscripts (a, b) among feeding groups are significantly different ($p<0.05$)
[1] Specific growth rate, [2] Apparent net protein utilization, [3] Apparent net lipid utilization

Table 2.6 Proximate composition (wet weight basis) of grass carp carcass fed with different food waste feeds

Measurement	Control	FWA	FWB	FWC
Moisture (%)	73.06±0.84a	77.58±1.32b	73.81±0.93a	73.92±1.32a
Ash (%)	3.58±0.55a	3.18±0.22a	3.18±0.15a	3.17±0.23a
Protein (%)	15.18±1.23a	14.45±0.47a	14.59±0.74a	12.78±0.64b
Lipid (%)	5.56±0.40a	2.83±0.78b	5.37±1.12a	6.03±1.39a

*Different superscripts (a,b) among feeding groups are significantly different ($p<0.05$)

2.3.2 Results of feed conversion of different food waste formulations in grey mullet

2.3.2.1 Growth performance of grey mullet fed with different food waste formulations

The PER, RWG and SGR were the highest in the control, followed by FW A and FWC, while FW B was the lowest (Table 2.7). The same pattern was observed in terms of survival rate of the control (89.63%) and FW A (83.75%), compared with FW B (56.0%). The FCR was the lowest in the control and the highest in FW B. The ANPU and ANLU were also the highest in the control (20.11% and 60.73% respectively) and lowest in FW B (8.04% and 20.43% respectively).

2.3.2.2 Carcass composition of grey mullet fed with different food waste formulations

No significant difference was found in carcass moisture and protein levels between all treatments ($p>0.05$) (Table 2.8). A significantly lower ash content was found in the control than all other groups ($p<0.05$), while lipid content in the control was significantly lower than FW A and FW C ($p<0.05$).

Table 2.7 Growth performance of grey mullet fed with different food waste feeds (experiment in duplicate)

Measurement	Control	FW A	FW B	FW C
Initial weight (g)		0.455		
Final weight (g)	0.850	0.730	0.546	0.623
Feeding rate (% b.w./day)	4.69	6.13	5.70	5.36
SGR[1] (% of b.w./day)	2.48	1.86	0.73	1.26
Survival rate (%)	89.63	83.75	56.00	70.00
Relative weight gain (%)	86.95	60.68	20.11	37.06
Feed conversion ratio	2.01	3.442	8.91	4.27
Protein efficiency ratio	1.73	1.07	0.42	0.76
ANPU [2] (%)	20.11	14.45	8.04	9.74
ANLU [3] (%)	60.73	41.39	20.43	22.48

[1] Specific growth rate, [2] Apparent net protein utilization, [3] Apparent net lipid utilization

Table 2.8 Proximate composition (wet weight basis) of grey mullet carcass fed with different food waste feeds

Measurement	Control	FW A	FW B	FW C
Moisture (%)	69.78±0.35a	69.43±0.52a	68.81±0.76a	68.83±2.43a
Ash (%)	4.55±0.32a	5.75±0.83b	5.99±0.63b	5.48±0.52b
Protein (%)	12.43±0.63a	13.60±2.07a	12.90±1.43a	12.96±1.34a
Lipid (%)	4.10±0.40a	5.48±0.53bc	4.89±0.97ab	6.50±1.31c

*Different superscripts (a,b,c) among feeding groups are significantly different ($p<0.05$)

2.4 Discussion

2.4.1 Growth of grass carp and grey mullet fed with different food waste feeds

The two fish species, grass carp and grey mullet showed similar growth performances (specific growth rate, relative body weight gain and feed conversion ratio) when fed with different feed formulations, in which the best growth performance was observed in the control feed. The cereals dominant feed, i.e. FW A showed the best growth performance among all food waste feeds, but it was not satisfactory when compared to the control. The FW B and FW C containing 25% and 10% of meat products showed significantly inferior growth (in terms of RWG, SGR, FCR and PER) in grass carp, compared to the control group ($p<0.05$). The FCR in the control group was the lowest among all feeding groups, while PER in the control group of grass carp was significantly higher than other food waste groups. It is known that PER can reveal the relationship between weight gain of fish and protein consumed (Zeiotoun *et al.*, 1973). The significantly lower PER also indicated that the proteins in food wastes were less effectively utilized by grass carp.

FWA contained mainly cereals (~53% in total), which may be more suitable for both grey mullet (omnivore) and grass carp (herbivore). Food wastes could provide nutrients and energy for fish metabolism. In general, meat and fish waste are good protein and lipid sources for fish growth, while fruit and vegetable waste contained high nitrogen free extract (non-fibrous carbohydrate) (García *et al.*, 2005). Herbivorous fish possessed a higher retention of plant than animal proteins (Javed and Watanabe, 2000), which may be related to the enzyme system in the long gut of these

species such as grass carp (Smith, 1989). The digestion of cyprinids fish relied on the buccal cavity and pharynx for replacing the absented stomach function, and higher trypsin activity and lower pepsin activity were found in grass carp (Vasile and Ciornea, 2009). Similar adaptation of intestine was also observed in grey mullet, and the proximal intestine is highly folded, with enhanced surface area and absorptive activity to omnivore diets (El-Bakary and El-Gammal, 2010). As a result, grey mullet and grass carp digested and utilized the FW A were more efficiently than other food waste feeds, according to results of PER, FCR, RWG and SGR.

The differences in fish growth may be related to the protein quality e.g. amino acid profile and digestibility of dietary protein. The amino acid profiles of the control, FW A and FW B were similar (Table 2.4), possessing similar levels of amino acid e.g. lysine, glycine, proline, phenylalanine and tyrosine which are important for fish growth (Garg, 2007; Aksnes *et al.* 2008; Li *et al.*, 2008). The availability and digestibility of proteins are critical for fish growth in this study. The differences of protein solubility in KOH in feed were closely related to the growth performance of fish. KOH protein solubility is a convenient, cheap and fast method for monitoring the protein quality of soy bean meal (Araba and Dale, 1990). A study showed that the growth of chicken was correlated to the protein solubility and protein dispersibility index of feeds (soybean meal with different heat treatments) (Batal *et al.*, 2000). However, KOH solubility is seldom use in evaluating aquaculture feed and growth performance of fish, the present study attempted to use this index to estimate the growth of grass carp and grey mullet. The present results showed similar correlations,

with significantly higher growth rates in fish in the control and FW A, with the protein solubility 60.57% and 51.91% respectively, which were significantly higher than FW B and FW C ($p<0.05$) (Table 2.3).

Both FW B and FW C contained meat product resulted in poor growth in both grass carp and grey mullet, implying that meat product may not be suitable for these two species. Poultry by-products are good alternative protein sources for manufacturing fish feed (Higgs *et al.*, 1979; Rawles *et al.*, 2009), but it may be more suitable for carnivorous fish. Erturk and Sevgili (2003), and Rawles *et al.* (2006; 2009) showed that the poultry by-products could replace the fish meal by 35% and 40% or even entirely in hybrid striped bass (carnivore) diet. On the contrary, poor growth in common carp (*Cyprinus carpio*) fingerlings was observed in all tested feeds with 33, 67 and 100% replacement of fish meal by poultry by-product meal (Emre *et al.*, 2003).

2.4.2 Utilizations of different food waste feeds by grass carp and grey mullet

Grass carp fed with the control feed showed the most efficient use of protein and lipid, reflected by ANLU, ANPU, and apparent protein and lipid digestibility. A similar trend was also observed in grey mullet, except that FW B showed the poorest performance according to different parameters (no digestibility of nutrients was investigated as different fish culture setup was used and fish feces were not collected due to the land-based setup of fish tank in AFCD Center, siphoning out the feces was not applicable). High protein digestibility and ANPU in grass carp demonstrated that the control feed protein was better utilized than food waste feed proteins, implying

that the sources of protein may be responsible for the efficiency on feed utilization. The major protein source in the control feed and FW A was derived from plant based substances, e.g. soy bean, rice bran, wheat. These plant based proteins are more efficiently digested by grass carp than animal based proteins and resulted in higher retention of protein in grass carp body (Javed and Watanabe, 2000). Besides, varied protein sources may result in the deficiency of some essential amino acids contained in feed, which may lead to poor utilization of dietary protein (Halver and Hardy, 2002), as demonstrated by the lower protein digestibility of food waste groups (~73-83%). Therefore, the digestive systems of both grass carp and grey mullet preferred plant based than animal based protein.

As the experimental feeds were isonitrogenous (same protein level), only significant differences in carbohydrates and lipid contents were noted between the 4 experimental feeds. The protein sources contained in FW B and FW C were partially derived from animal products, e.g. fish visceral, pork, beef and chicken which may possess a higher amount of lipid leading to a poor growth observed in both grey mullet and grass carp. The non-protein energy sources i.e. lipids and carbohydrates could affect the efficiency of protein utilization in fish (Wilson and Halver, 1986). However, fish are capable to generate carbohydrate metabolites e.g. glucose and glycogen from excessive lipid and protein as energy, while carnivorous and omnivorous fish are more capable to utilize lipid as energy than herbivorous fish (Du *et al.*, 2009). Sufficient lipids in the diet promoted growth and feed utilization in various fish species, as protein is spared in fish body and converting carbohydrates or

lipids into energy (De Silva *et al.*, 2001; Skalli *et al.*, 2004). As a result, the growth performance and body composition are affected by the lipid and carbohydrate contents (carbohydrate to lipid (CHO: L) ratio) which served as major energy sources in feeds.

The quantity of energy from carbohydrates and lipids are critical to fish growth, but excessive amounts should be avoided. The poor growth performance of grey mullet and grass carp fed with FW B and FW C may be due to the high lipid and low carbohydrates contained in feed. FW C contained the highest amount of lipid (~19%), followed by FW B (~13%), while FW A and the control feed showed similar lipid contents (5-6%). The majority of energy contained in FW B and FW C was mainly from lipids, rather than from carbohydrates, as shown by low CHO: L ratio (1.83 and 1.04 respectively). Poor utilization of lipid (lower ANLUs) was found in both grey mullet and grass carp, but it was surprised that ANLU in grass carp fed with the low lipid content FW A was the lowest. The lower value of ANLU also indicated less retention of lipid in body, with more lipid converted to energy. Du *et al.* (2005) also observed that dietary lipid contents varied from 0 to 12%, which were adjusted by fish and corn oil, in their study involving grass carp. Gao *et al.* (2010) also reported that grass carp showed the best growth at 7.5 of dietary CHO: L ratio, the tested ratios ranged from 1.74 – 202.5, with dietary energy and protein levels maintained at 16.2 kJ/g and 39%, respectively.

In general, dietary lipids are mainly converted to visceral fat around different organs e.g. livers and kidneys, rather than other parts of body (Sheridan, 1994). The

high dietary lipid (>10%) inhibited the growth of green grouper (*Epinephelus coioides*) with a reduced feed intake quantity (Luo *et al.*, 2004; Luo *et al.*, 2005). A possible reason for lowered feed intake may be due to adequate energy supply from feed lipid and finally resulted in stunted growth with less protein intake (Luo *et al.*, 2005). Based on the observation after 20 minutes of feeding, some feeds were not consumed in all food wastes feed groups for grass carp and grey mullet but the amount was not significant. This lowered feed intake may be due to the high lipid content in FW B and FW C, or the lower palatability of fish feeds due to the food waste components, which also implied that the food waste may reduce fish appetite.

High mortality (10-46%) and low utilization of lipid (~20-60% of ANLU) and protein (~8-20% of ANPU) were found in the experiment dealing with grey mullet fry (<1 g). The digestive system of fry is less developed and much simpler than that in adult (Govoni *et al.*, 1986); the proteolytic digestion in fry is dependent on exogenous sources e.g. live prey (Lauff and Hofer, 1984). The feed utilization in grey mullet fry was the best when energy in feed was mainly from carbohydrate than lipid (El-Dahhar, 2000a). The present results also showed that control and FW A (more carbohydrates) had better growth indices (SGR, FCR and PER) and survival rate. Hence, the fry would be more susceptible to feed nutrients leading to a high mortality when feed protein and lipid utilizations were especially low in FW B and FW C.

Apart from growth performance, the fish feed could also affect the body composition of fish. Both grass carp and grey mullet fed with high lipid diets (FW C) showed the highest carcass lipid content and stunted growth. Positive correlation

between carcass lipid content and dietary lipid level was observed in the present study, but the body lipid in FW A was significantly lower than the control ($p<0.05$). Similar correlation between carcass lipid content and dietary lipid level was also found in grass carp (Gao *et al.*, 2010). Extensive studies indicated that excessive fat deposition in fish visceral and tissues resulted from high lipid feeding (Lanari *et al.*, 1999; López *et al.*, 2006). Ali and Al-Asgah (2001) showed that dietary lipid content over 14% (CHO: L = ≤ 2.06) would hinder growth and increase FCR in Nile tilapia, indicating poor utilization of feed, with higher carcass lipid contents. The lipid requirement varied among different fish species. The present result illustrated a lower lipid content (~6%) in feed favored the growth of grass carp and grey mullet than a high level of lipid (>13%). Köprücü (2012) also noted that protein utilization and growth performance of juvenile grass carp were the best in 6% lipid at 33% feed protein level, but not higher protein (37%) or lipid (8%). In general, the growth of grass carp is more liable to lipid content in feed as poor utilization of excessive protein and lipids was found in grass carp, but carnivorous or omnivorous fish showed better utilization (Du *et al.*, 2009).

For the above mentioned reasons, the lipid content in food waste would impose adverse effects on fish growth, which should be removed prior making fish feeds. In general, the growth rates of fish fed with food waste feeds were slower than the control feed, further studies focusing on promoting feed utilization by upgrading FW A should be investigated. Supplementation of exogenous enzymes in feed may enhance digestion and absorption of nutrients by fish, leading to a better weight gain

and a higher retention of protein in the body. It also reduced water pollution as fewer nutrients were excreted to the environment (Kolkovski *et al.*, 1997). Enzymes e.g. papain have shown positive effects on growth rate, FCR, protein digestibility and nitrogen retention efficiency, compared to diet without enzyme (Singh *et al.*, 2011).

Moreover, nutrients deficiency in feeds, especially protein and lipid would impair fish metabolic functions, e.g. proteins and glycogen synthesis and breakdown, and hence reduce fish growth, immunity and disease resistance. It has been noted that the deficiency of essential fatty acids damaged the antibody production and macrophage function in rainbow trout (Kiron *et al.*, 1995) and serum complement activity in Nile tilapia (Lim *et al.*, 2009). To our knowledge, there is a severe lack of information on the effects of food waste treated with enzymes on fish immunity. Fish immunity is a crucial factor for fish farmers to adopt food waste for culturing fish.

2.5 Conclusion

The hypothesis of this experiment was rejected due to the poor growth performance of fish fed with food waste feeds, further upgrading of food wastes should be investigated in order to utilize them as alternative protein sources. In general, both grass carp and grey mullet performed the best growth (in terms of SGR, RWG, FCR and PER) fed with cereal dominant food waste fish feed (FW A), among all food waste feeds, but the growth was lower than the control. Meat product contained feeds, FW B and FW C showed inferior growth on both species, indicating grass carp and grey mullet utilized plant proteins better than animal proteins. Being a

herbivorous fish, grass carp would utilize carbohydrate as a major energy source than lipid. The high lipid content in feed was also a possible reason for hindering growth, which could also be accumulated in body.

Moreover, a higher level of lipid was observed in fish feeds containing meat products. It is suggested that lipid should be removed in the preparation of food waste feed for grass carp. The growth performance (reflected by RWG, SGR, FCR and PER) of fish fed with food waste was not satisfactory, and further investigations on upgrading FW A by adding enzymes are needed.

Chapter 3

Upgrading food wastes by means of bromelain and papain to enhance growth and immunity of grass carp (*Ctenopharyngodon idellus*) and grey mullet (*Mugil cephalus*)

3.1 Introduction

Protein is the most expensive component in feed which is majorly from fish meal. Energy in feed is critical to the utilization of dietary protein in fish (Wilson and Halver, 1986), energy is obtained from the catabolism of proteins if the dietary energy is inadequate. The protein is also catabolized into energy if dietary protein is excessive (Alatise *et al.*, 2006). Therefore, the feed is more cost effective if dietary protein is used for growth e.g. repairing and building up tissue as much as possible and with the least amount broken down to energy (Gauquelin *et al.*, 2007). Protein utilization in fish could be enhanced by adjusting feed protein to energy (P/E) ratio (McGoogan and Gatlin, 2000) or applying feed supplements such as medicinal herbs (Li *et al.*, 2009) and baker's yeast (Osman *et al.*, 2010).

Increasing the utilization of feed in aquaculture is important and beneficial to aquaculture industry as well as the environment, as better feed conversion could reduce nutrient loss and lower the feed cost in aquaculture production. In previous experiments (Chapter 2), the fish feeds (FWB & C) contained meat product showed poor growth in both grass carp and grey mullet while cereals dominant feed (FW A) is most suitable to both species. This showed the meat product may be more suitable for carnivores, but not omnivores and herbivores. Although both grass carp and grey

mullet fed with the cereal dominant food waste feed (FW A) had the best growth performance (in terms of SGR, RWG and PER) among the food waste feeds, the growth performances were poor when compared to the control, though not significantly.

Supplementation of enzymes was adopted for the upgrading food waste feeds in this study. Nowadays, exogenous enzymes are extensively applied as animal feed additives to improve the nutritional value and reduce water pollution (Kolkovski *et al.*, 1997). Addition of enzymes in feed could enhance the availability and utilization of plant protein by fish could be due to elimination of anti-nutritional factors in plant, e.g. phytates (Liebert and Portz, 2005; Singh *et al.*, 2011). Enzyme mixtures (Farmazyme®, including fungal xylanase, hemicellulase, pectinase and cellulose) also showed a significant improvement on the growth of African catfish (*Clarias gariepinus*) (Yildirim and Turan, 2010).

Bromelain and papain could be considered as possible feed supplements used in aquaculture, with fruit waste is also a possible source for these two enzymes. Bromelain could be extracted from the stem and fruit of pineapple and papain obtained from papaya leaf, unripe fruit and papaya latex. Bromelain increased the digestibility of animal and vegetable proteins by partially hydrolyzing the molecules into smaller peptides (Fennema, 1996). Papain can hydrolyze proteins, lipids and carbohydrates and function at wide range of pH and temperature (Miyamoto *et al.*, 2004). Soy protein peptides were shortened by papain treatment and hence increased its solubility (Wu *et al.*, 1998). It has been demonstrated that pre-digestion of soybean

residues by papain could enhance the feed conversion in common carp (*Cyprinus carpio*) (Wong *et al.*, 1996).

A remarkable mortality of grey mullet fries (20% more than commercial feed) was observed when fed with food waste feed (FW B and C) in the previous experiment (Chapter 2). It raised a concern on the effects of food waste on fish health, probably due to poor nutrient conversion from food waste feeds. Protein is constructed from various amino acids, which also forms different biochemical components e.g. hormone, enzyme and immunoglobulin for metabolic functions. Hence, poor nutrition from diet could impair the fish immunity and disease resistance (Chandra, 1992). There is also a lack of information on the effects of food waste on the immunity of fish and also on the feasibility of upgrading food waste by adding enzymes.

In the present study, more corn starch and lipid (peanut cooking oil) were added into FW A to replace part of the food wastes. The new food waste formulation (FW D), contained 60% food wastes, whereas corn starch and lipid contents were increased from 15% to 25% and 6% to 12% respectively. The use of peanut cooking oil was to mimic the worst scenario of high lipid content in food wastes, which was also observed in FW B and FW C formulations. It has been shown that the growth of grey mullet fed with lower dietary protein could be enhanced by increase the energy content in the form of lipids (El-Dahhar, 2000b). Therefore, the present experiment was intended to investigate whether lower protein of feed could support the growth of grass carp and grey mullet by increasing the lipid content.

It is hypothesized that the enzymes mixture of bromelain and papain could enhance the utilization of food waste feeds by fish. As a result, the objectives of present study were to investigate 1) the growth performance of grass carp and grey mullet; 2) the fish immunity when fed with food waste and enzyme supplemented food waste; and 3) the feasibility of improving the protein efficiency using lower protein and higher lipid contents in feeds.

3.2 Materials and Methods

3.2.1 Effects of food wastes formulation upgraded by papain and bromelain on grass carp growth performance

The procedures of food waste feed production were described in details in Section 2.2.1. In the present experiment, more corn starch and lipid (peanut cooking oil) were added into FW A to replace part of the food wastes as the new food waste formulation (FW D), contained 60% food wastes. Corn starch and lipid contents contained in FW D were increased from 15% to 25% and 6% to 12 % respectively. Papain and bromelain were mixed with the food waste diets (FW A and FW D containing 75% and 60 % food waste respectively, Table 3.1). The composition of FW D was similar to FW A with dominant cereal proportion, but a higher portion of corn starch and lipid was replaced part of the food waste. Papain (Sigma 76220, papain from *Carica papaya*) and bromelain (Sigma B-4882, EC 3.4.22.32, bromelain from pineapple stem) were mixed with the food waste diets (FW A and FW D containing 75% and 60% food waste respectively) with (50% by mass of fish diet) 0.025 mol/L sodium

dihydrogen phosphate and adjusted to pH 8 with NaOH, at 37ºC for 5 h. The commercial fish feed, Jinfeng®, 613 formulated feed (~30 % protein) and two food waste diets (FW A and D) without enzyme treatment were used as the control. Then the substrates were heated to 95°C for 5 min to inactivate the enzyme (Wu *et al.*, 1998). The fish feed dough was pelletized (3 mm diameter) with a meat grinder and dried at 50 ˚C for 24 h. The proximate composition (ash, moisture, protein, fibre, lipid and nitrogen free extracts) and the feed protein solubility in KOH were determined as mentioned in Section 2.2.5 and shown in Table 3.2

Five hundred fingerlings of grass carp *Ctenopharyngodon idella* (herbivore) were used for testing different fish feeds, with 18 individuals (~9-12 g) stocked in each tank (~60 L water) in triplicates. The water temperature, pH and dissolved oxygen were measured three times a week using a portable Hanna pH meter and a YSI digital Dissolved Oxygen (DO) meter and the values ranged from 20.2-22.1°C, 6.1-6.7 and 6.3-7.2 mg/mL respectively.

Feeding with the control feed was carried out at the rate of 2% body weight daily in each experimental feeding group and the fish were acclimated for 3 weeks before the onset of experiment. The experiment was conducted for a period of 56 days, with the fish blood samples collected at Day 1, 14, 28 and 56, by caudal venous puncture at vertebral column of the fish (fish were anasthzied by MS-222). The oxidative radical production (Nitroblue tetrazolium (NBT) assay) in blood, total protein and total immunoglobulin of plasma were determined (refer to Section 3.2.4 for details).

Table 3.1 Food waste feed formulations (FW A & D, containing 75 & 60% food waste respectively)

Formulation	Food waste products					Non-food waste products		peanut oil	Total (%)
	fruit/ vegetables	meat products	cereals	bone meal	other	fish meal	corn starch		
Food waste A	10	0	53	8	4	10	15	0	100
Food waste D	10	0	38	8	4	10	25	5	100

Table 3.2 Composite analysis of experimental feeds with 1% or 2% mixture of bromelain and papain in food waste A and D (FW A & D)

Formulation	Control	FW A 0%	FW A 1%	FW A 2%	FW D 0%	FW D 1%	FW D 2%
Dry matter (%)	93.68±0.20a	95.69±0.02a	94.79±0.23a	94.24±0.33a	93.58±0.35a	93.64±0.31a	93.54±0.27a
Ash (%)	8.24±0.09a	9.18±0.46b	8.81±0.29ab	8.68±0.25ab	10.48±0.36c	10.41±0.33c	10.27±0.39c
Protein (%)	30.16±1.55a	31.44±0.31ab	31.17±0.08ab	32.07±1.68b	19.41 ±0.35c	20.00±0.55 c	20.39±0.39 c
Lipid (%)	5.17±0.94a	6.12±1.66a	6.02±0.81a	6.08±0.56a	12.88±1.66b	12.76±0.98b	12.71±1.05b
Fibre (%)	9.57±0.21a	9.62±0.63a	9.60±0.60a	9.54±0.76a	8.25±0.88a	8.10±0.84a	8.08±0.85a
Carbohydrates (%)[1]	40.53	39.64	39.19	37.86	42.55	42.37	42.09
Energy (kJ/g diet)[2]	16.17	16.64	16.51	16.52	17.03	17.09	17.11
CHO/L ratio [3]	7.84	6.43	6.51	6.23	3.30	3.32	3.31
P/E (mg/kJ)[4]	1865.0	1889.1	1887.5	1941.2	1139.9	1170.3	1191.5
Protein solubility (%)[5]	60.57±2.64a	51.81±1.43b	72.89±4.18c	73.48±3.63c	56.21±2.51ab	76.58±3.39cd	78.95±3.69d

*Different superscripts (a, b, c) among feeding groups are significantly different ($p<0.05$)

[1] Carbohydrates (%) = 100 − (crude protein % + crude lipid % + moisture % + ash % + fibre %) (Castell and Tiews, 1980)

[2] Energy (kJ/g diet) = (% crude protein × 23.6) + (% crude lipids × 39.5) + (% carbohydrates × 17.3) (Chatzifotis *et al.*, 2010)

[3] Carbohydrates to Lipid (CHO: L) ratio = % wt. in CHO / % wt. in lipid

[4] Protein to energy (P/E) **(**mg/kJ) = crude protein (%) / Energy

[5] Protein solubility (%) = Protein in KOH / Protein in Sample x 100% (Araba and Dale, 1990)

The fish were starved for 24 h before collecting the carcass for chemical analyses, with 5 fish collected at the beginning of the experiment. After 56 days of feeding experiment, 3 fish from each tank were collected and killed by MS-222, weighed, freeze dried, ground into a homogeneous sample and kept at -20ºC until analysis. The lipid, ash, moisture and protein contents of the carcass were analysed as described in Section 2.2.5.

Different parameters: specific growth rate, relative weight gain, feed conversion ratio, protein efficiency ratio, apparent net protein and lipid utilization (Section 3.2.3 for details) were calculated for monitoring the growth performance, based on different treatments.

3.2.2 Effects of food wastes upgraded by papain and bromelain on grey mullet growth performance

This experimental setup was almost the same as stated in Section 3.2.1, except the fish species was grey mullet (~15-20g), instead of grass carp. The water temperature, pH and dissolved oxygen were measured three times a week using a portable Hanna pH meter and a YSI digital Dissolved Oxygen (DO) meter and the values ranged from 20.5-22.3°C, 6.2-6.9 and 6.5-7.0 mg/mL respectively.

3.2.3 Fish growth performance parameters

Growth rates were calculated for each aquarium as a specific growth coefficient resulting from the following expression:

a) Daily feeding rate (% of body weight/day) = Daily feed intake (g/fish) / initial average body weight (g)

b) Specific growth rate, SGR (%/day) = 100 (Ln Wf- Ln Wi) / t

c) Relative Weight Gain, RWG (%) = (Wf – Wi) x 100 / Wi

where:

Wf is the mean final body weight (g) for the fish in each aquarium, Wi is the mean initial body weight of the fish in the same aquarium, and t is time in days.

d) Feed conversion ratio, FCR = feed intake (g) / (Final biomass – Initial biomass (g))

e) Protein Efficiency Ratio, PER = weight gain (g) / protein intake (g).

f) Apparent Net Protein Utilization, ANPU (%) = 100 × (final fish body protein (g) - initial fish body protein (g)) / total crude protein intake (g)

g) Apparent Net Lipid Utilization, ANLU (%) = 100 × (final fish body lipid (g) - initial fish body lipid (g)) / total lipid intake (g)

3.2.4 Fish immunological parameters

Plasma total protein and total immunoglobulin assays

The total immunoglobulin (IgI) in plasma was determined using the method described in Siwicki *et al.* (1994) with slight modifications. The protein concentration of the plasma was determined according to the modified colorimetric method based on Bradford protein assay (Bradford, 1976). Briefly, 5 μL of plasma and 250 μL of Bradford solution were added to 96-well microtiter plates. After 20 min of incubation at 22±1ºC, the absorbance was measured with a microplate reader (Infinite 200, Tecan,

Austria) at 595 nm. The protein concentration (g/L) was calculated from the standard curve.

For total immunoglobulin, 50 μL plasma and 50 μL polyethylene glycol (10%) (PEG) were incubated at 22±1ºC for 2 h and the mixtures were centrifuged at 1000 G for 15 min. The protein content of the supernatant was determined by the assay mentioned above. The total immunoglobulin concentration (Total IgI) of the plasma (g/L) was the difference between the total protein (TP) level in plasma and PEG treated plasma.

Nitroblue tetrazolium (NBT) assay

The Nitroblue tetrazolium (NBT) assay was carried out based on the method described in Anderson and Siwicki (1995). Heparinized fish blood (100 μL) was added to an equal volume of 0.2% NBT (Sigma-Aldrich, St. Louis, Missouri, USA) solution. The mixture was then incubated at 22±1ºC for 30 min. The resultant suspension (50 μL) was added into a glass tube 1.0 ml N, N-dimethyl formamide (Sigma-Aldrich, St. Louis, Missouri, USA) and centrifuged at 3000 G for 5 min. The optical density (OD) of the supernatant was measured by a spectrophotometer (UV-1601, Shimadzu, Tokyo, Japan) at 540 nm.

3.2.5 Statistical analyses

The effects of different feed types on the feed compositions, fish growth and fish carcass compositions and in two fish species were analyzed by one-way ANOVA,

comparing the mean value with Duncan's multiple range tests ($p<0.05$) (SPSS Statistics 17.0, Chicago, Illinosis, USA). The correlation between fish carcass lipid and feed types was tested by Spearman's test ($p<0.01$).

3.3 Results:

3.3.1. Results of grass carp feeding trial with different food waste feeds upgraded by papain and bromelain

3.3.1.1 Grass carp growth performance fed with different food waste feeds upgraded by papain and bromelain

For the growth performance, the grass carp fed with FW A and 1% mixed enzymes (FW A 1%) showed the highest specific growth rate (SGR),which was significantly higher than the control ($p<0.05$) (Table 3.3). The fish fed with FW D 2% showed the lowest SGR which is significantly lower than FW D 0% and FW D 1% ($p<0.05$). The SGR in FW D with or without enzymes (all FW D feed groups) were significantly lower than the control ($p<0.05$). The relative weight gain (RWG) in FW A 1% was the highest, and RWG in FW A 1% and FW A 2% were significantly higher than all other groups ($p<0.05$), including the control. Besides, RWG of all FW D feed groups were significantly lower than the control ($p<0.05$), while FW D 2% showed the lowest RWG. The feed conversion ratio (FCR) of grass carp in all FW D feed groups was significantly higher than the control and all FW A feed groups ($p<0.05$).

The apparent net protein utilizations (ANPUs) of FW A groups and the control were comparable with FW D 1% had the highest ANPU, but not significantly different

from the control ($p>0.05$), but significantly higher than FW A 2% ($p<0.05$) which was the lowest. The apparent net lipid utilizations (ANLUs) was the highest in FW A 1%, which was significantly higher than the control, while the ANLU in all FW D feed groups were significantly lower than the control ($p<0.05$). The ANLU of the FW A 2% was the lowest among FW A feed groups, with same observation was found in FW D feed groups. The protein efficiency ratio (PER) of FW A 1% was the highest among all feeding groups, which was significantly higher than the control group and FW A 0% ($p<0.05$). It was also noted that PER of FW D 2% was significantly lower than the control and FW D 0% ($p<0.05$).

3.3.1.2 Grass carp carcass composition fed with different food waste feeds upgraded by papain and bromelain

The carcass moisture contents of grass carp fed with FW A 1% and FW A 2% were significantly higher than the control (Table 3.4). On the contrary, carcass moisture contents in FW D 0% and FW D 1% were significantly lower than the control ($p<0.05$). No significant difference in terms of ash contents were observed between all feeding groups ($p>0.05$). The carcass protein contents in FW D 0% and FW D 2% were significantly lower than control ($p<0.05$). The carcass lipid contents of FW A 1% and FW A 2% were significantly lower than control ($p<0.05$) with FW D 0% had higher carcass contents than control ($p<0.05$). The decreasing trends of lipid contents were significantly correlated with the increase of enzyme supplements (0%, 1%and 2%) in FW A (r=0.630) and FW D (r=0.669) groups (Spearman's test, $p<0.01$).

Table 3.3 Growth performance of grass carp fed with different food waste feeds upgraded by papain and bromelain

Measurement	Control	FW A 0%	FW A 1%	FW A 2%	FW D 0%	FW D 1%	FW D 2%
Initial weight (g)	10.76±0.07a	11.05±0.14b	10.55±0.13d	10.89±0.05c	11.10±0.09b	10.96±0.08c	10.64±0.1a
Final weight (g)	15.02±0.14a	14.96±0.26a	15.77±0.41b	15.66±0.17b	14.23±0.06c	13.68±0.33d	12.63±0.16e
Feeding rate (%b.w./day)	2.08±0.05ab	2.07±0.02ab	2.08±0.05ab	2.05±0.06a	2.14±0.05b	2.11±0.05ab	2.12±0.01ab
SGR (%b.w./day)	0.60±0.02ab	0.54±0.05a	0.72±0.07c	0.65±0.02b	0.44±0.01d	0.40±0.04d	0.31±0.02e
RWG (%)	39.56±1.66ab	35.43±3.65a	49.51±5.55c	43.80±1.40b	28.24±0.70d	24.86±2.48d	18.78±1.38e
FCR	3.14±0.04ab	3.46±0.29b	2.64±0.28ab	2.85±0.09a	4.3±0.14c	4.80±0.46c	6.19±0.46d
ANPU (%)	30.66±3.64ab	30.04±1.77ab	33.66±3.56ab	28.05±0.98a	30.95±4.93ab	37.54±6.55b	32.11±4.01ab
ANLU (%)	93.96±15.64a	107.91±9.19a	132.98±19.53b	47.11±6.50c	53.95±10.67c	41.71±5.88cd	25.31±8.39d
PER	1.05±0.01ab	0.92±0.08bc	1.22±0.14d	1.09±0.04ad	1.19±0.04ad	1.05±0.10ab	0.80±0.06c

*Different superscripts (a, b) among feeding groups are significantly different ($p<0.05$)

Table 3.4 Proximate compositions (%, wet weight basis) of grass carp carcass fed with different food waste feeds upgraded by papain and bromelain

	Control	**FW A 0%**	**FW A 1%**	**FW A 2%**	**FW D 0%**	**FW D 1%**	**FW D 2%**
Moisture (%)	72.50±1.26a	72.76±1.46a	75.28±1.37cd	76.05±0.83d	69.64±2.60b	69.93±1.71d	73.33±2.33ac
Ash (%)	2.97±0.06a	2.81±0.20a	2.84±0.32a	2.80±0.24a	2.90±0.25a	3.08±0.17a	2.95±0.20a
Protein (%)	10.04±1.31a	9.77±0.86a	9.83±1.17a	9.35±0.86ab	7.28±0.81c	8.92±1.74ab	8.01±0.85bc
Lipid (%)	7.96±0.81ab	7.95±1.11ab	6.08±0.68c	5.84±0.86d	10.47±1.47d	8.74±1.07b	6.85±1.03ac

*Different superscripts (a, b) among feeding groups are significantly different ($p<0.05$)

3.3.1.3 Grass carp immunological parameters

The NBT activities of FW D 0% and FW D 1% were significantly lower than the control at Day 14 ($p<0.05$) (Fig. 3.1a). At Day 28, the activities of FW A 0% and all FW D feed groups were significantly lower than the control, while FW A 1% showed a significantly higher activity than the control at Day 56 ($p<0.05$).

In general, the plasma protein and total IgI in grey mullet fed with food waste without enzymes were lower than the control (Fig. 3.1b & c). The plasma protein level of FW A 0% and FW D 0% were significantly lower than the control at Day 14 and 28 respectively ($p<0.05$). After 56 days, FW A 0%, FW A 2% and FW D 2% showed lower plasma protein than the control ($p<0.05$). The total IgI of FW A 0% was significantly lower than the control at Day 14 and 28 ($p<0.05$), but all the treatments were not significantly different at Day 56 ($p>0.05$).

3.3.2. Results of grey mullet feeding trial with different food waste feeds upgraded by papain and bromelain

3.3.2.1 Grey mullet growth performance fed with different food waste feeds upgraded by papain and bromelain

The SGR and RWG of FW A 1% were the highest, which were significantly higher ($p<0.05$), while those parameters of FW A 0%, FW D 0% and FW D 2% were significantly lower than the control ($p<0.05$) (Table 3.5). The FCR of FW A 1% was the lowest among all treatments but not significantly different from the control ($p>0.05$), while FCR of FW A 0%, FW D 0% and FW D 2% were significantly higher

than the control ($p<0.05$). The ANPU of FW A 0% and FW D 1% was significantly lower than control ($p<0.05$), and FW A 1% and FW A 2% were significantly higher than FW A 0% ($p<0.05$). The ANLU of FW A 2% was the highest and was significantly higher than all other treatments ($p<0.05$). It was also noted that all FW D feed groups showed significantly lower ANLU than the control ($p<0.05$). The PER of FW A 1% and FW A 2% were significantly higher than FW A 0% ($p<0.05$), but not significantly different from the control ($p>0.05$), while PER of FW D feed groups were significantly higher than the control and all other groups ($p<0.05$). It was also noted that FER of FW D 0% and FW D 1% were significantly higher than those of FW D 2%.

3.3.2.2 Grey mullet carcass composition fed with different food waste feeds upgraded by papain and bromelain

No significant difference in carcass moisture between all treatments ($p>0.05$) was observed. All treatments, except FW A 0% and FW A 2% showed higher carcass ash contents than the control ($p<0.05$) (Table 3.6). The carcass protein contents in FW D 1% and FW D 2% were significantly lower than the control ($p<0.05$). The carcass lipid content of FW D 2% was the highest which was significantly higher than the control ($p<0.05$).

3.3.2.3 Grey mullet immunological parameters

There was no significant difference for the NBT activity between all

treatments at Day 14 and 28 ($p>0.05$) (Fig. 3.2a). At Day 56, the NBT activity of FW D 2% was significantly lower than the control. The activity of FW A 1% was the highest at Day 56 which was significantly higher than FW A 0% and all FW D groups ($p<0.05$). The plasma protein level of FW A 2% was significantly higher than the control at Day 14 ($p<0.05$) (Fig. 3.2b). The plasma protein levels of FW A 1%, FW A 2% and FW D 2% were significantly higher than the control at Day 28 ($p<0.05$). However, the plasma protein levels of FW A 0% and FW D 1% were significantly lower than the control, while FW A 2% was higher than the control significantly at Day 56 ($p<0.05$).

The total IgI level of FW A 2% was significantly higher than the control at Day 14 ($p<0.05$) (Fig. 3.2c). At Day 28 and 56, the IgI levels of FW A 1% and FW A 2% were not significantly different from the control ($p>0.05$) but significantly higher than FW A 0% ($p<0.05$). FW A 2% showed the highest IgI level at Day 56 which significantly higher than the control ($p<0.05$). The same trend of IgI level was also observed in FW D groups, with the total IgI levels of FW D 1% and FW D 2% significantly higher than FW D 0% ($p<0.05$).

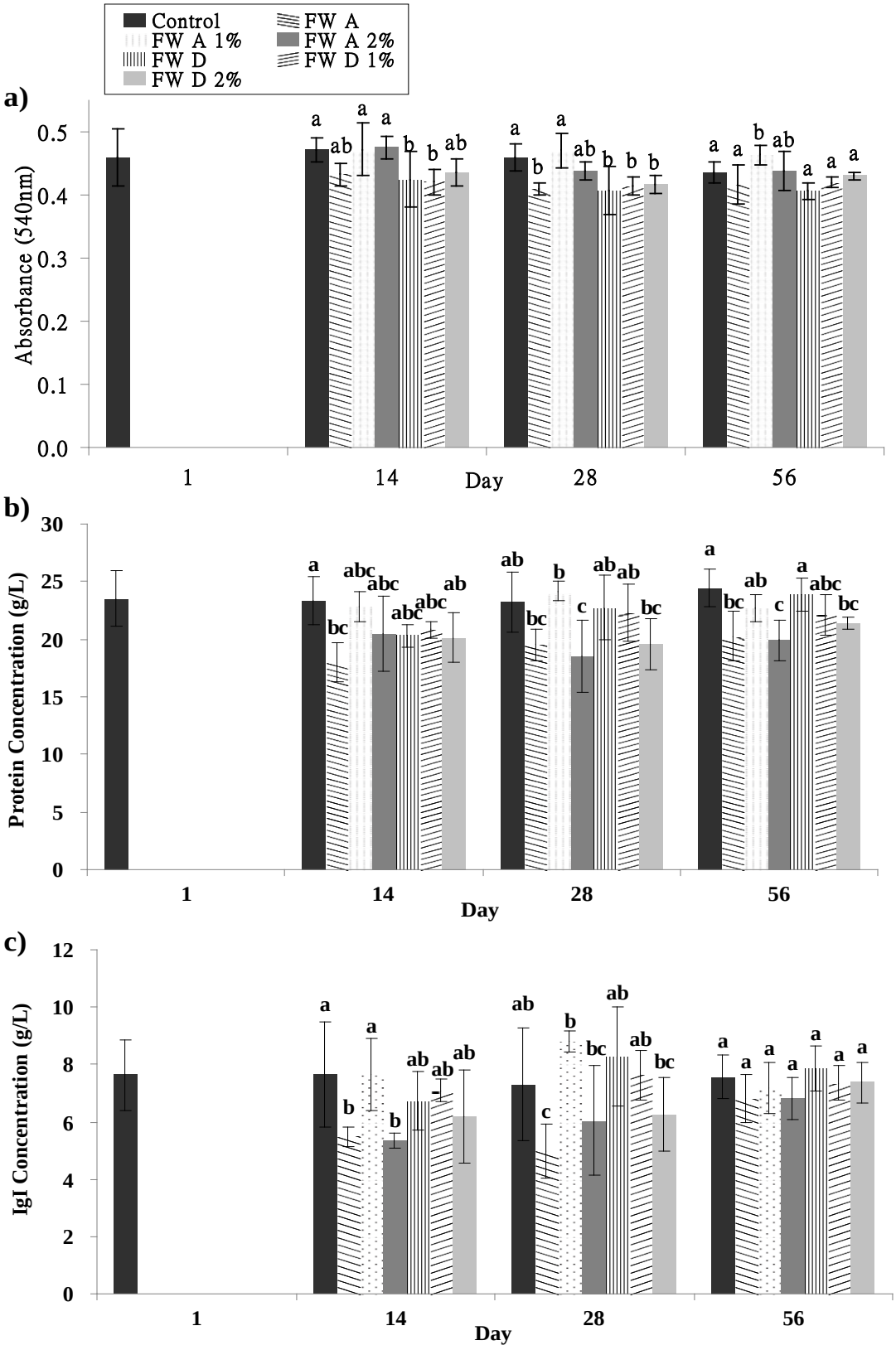

Fig. 3.1 a) Nitroblue tetrazolium (NBT) activity in blood, b) plasma total protein (g/L) and c) total immunoglobulin (g/L) of grass carp in the control and food waste feeding groups, different superscripts (a, b) within in same sampling day are significantly different (*p<0.05*).

Table 3.5 Growth performance of grey mullet fed with different food waste fish feeds upgraded by papain and bromelain

Measurement	Control	FW A 0%	FW A 1%	FW A 2%	FW D 0%	FW D 1%	FW D 2%
Initial weight (g)	14.78±0.14ab	14.76±0.25ab	14.60±0.82ab	15.44±1.30b	15.17±0.80ab	13.86±0.33a	14.82±0.62ab
Final weight (g)	21.10±0.47abc	19.20±0.23d	21.53±1.42bc	21.90±1.60c	20.33±1.01abcd	19.50±0.78ab	19.70±0.43abd
Feeding rate (%b.w./day)	2.10±0.07ab	2.30±0.03b	2.15±0.09ab	2.04±0.26a	2.06±0.08ab	2.24±0.08ab	2.18±0.11ab
SGR (%b.w./day)	1.27±0.06a	0.94±0.03b	1.39±0.09c	1.25±0.05a	1.05±0.02b	1.22±0.06a	1.02±0.08b
RWG (%)	42.73±2.22a	30.08±1.04b	47.41±3.74c	41.89±1.89a	34.04±0.80b	40.61±2.42a	33.00±3.01b
FCR	2.32±0.12ab	3.43±0.08e	2.18±0.13a	2.28±0.23a	2.76±0.13cd	2.58±0.20b	3.00±0.15d
ANPU (%)	21.15±1.88ab	14.16±0.47c	20.60±3.40ab	22.07±2.65a	19.12±1.07ab	14.10±4.15c	16.29±2.86bc
ANLU (%)	71.65±2.61a	69.97±1.12a	72.04±9.95a	83.51±7.38b	35.19±3.95c	32.38±2.66c	52.44±4.44d
PER	1.43±0.07a	0.93±0.02b	1.46±0.09a	1.40±0.14a	1.87±0.09d	1.94±0.15d	1.64±0.08c

*Different superscripts (a, b) among feeding groups are significantly different ($p<0.05$)

Table 3.6 Proximate composition (%, wet weight basis) of grey mullet carcass fed with different food waste fish feeds upgraded by papain and bromelain

	Control	**FW A 0%**	**FW A 1%**	**FW A 2%**	**FW D 0%**	**FW D 1%**	**FW D 2%**
Moisture (%)	65.79±1.79ab	64.65±1.73ab	67.03±1.84a	65.76±2.26ab	65.02±1.02ab	63.72±1.31b	64.00±1.61b
Ash (%)	5.21±0.18ab	5.15±0.14a	5.61±0.21c	5.51±0.08bc	5.78±0.37c	5.57±0.04c	5.80±0.24c
Protein (%)	16.67±0.59a	16.45±0.56a	16.79±1.48a	16.60±1.13a	15.63±0.75ab	14.87±0.98b	14.82±0.58b
Lipid (%)	10.05±1.59a	11.20±0.88ab	11.14±3.19ab	11.90±1.80ab	11.04±1.46ab	11.64±1.75ab	13.29±1.95b

*Different superscripts (a, b) among feeding groups are significantly different ($p<0.05$)

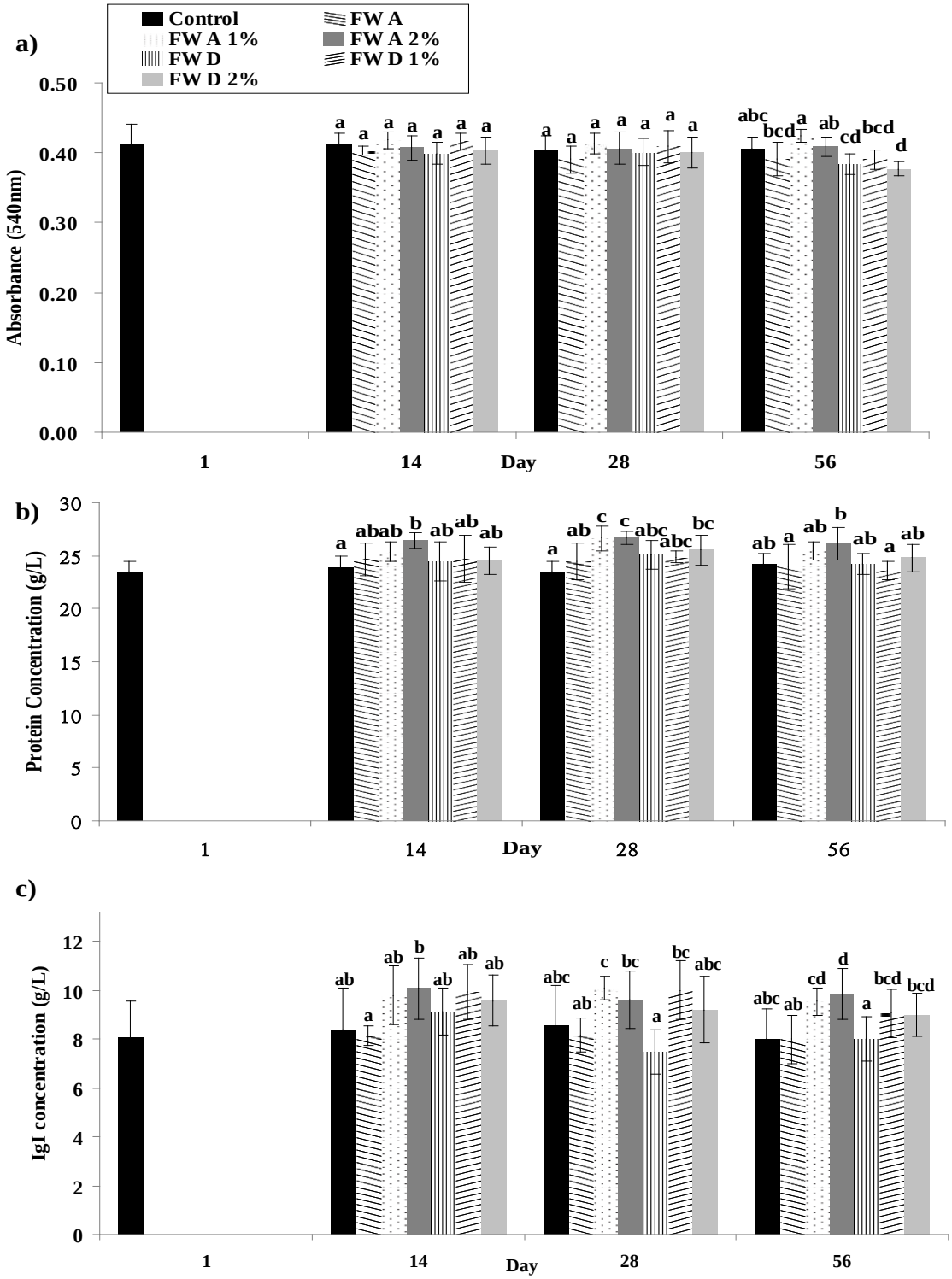

Fig. 3.2 a) Nitroblue tetrazolium (NBT) activity in blood, b) plasma total protein (g/L) and c) total immunoglobulin (g/L) of grey mullet in control and food waste feeding groups, different superscripts (a, b, c) within in same sampling day are significantly different (*p<0.05*)

3.4. Discussion

3.4.1 Growth of grass carp and grey mullet fed with food waste supplemented with bromelain and papain mixture

In general, the growth performance of grass carp and grey mullet was the best when feeding with food waste A supplemented with 1% of mixed bromelain and papain (FW A 1%). The growth performance was evaluated by SGR, RWG, FCR and PER, and grass carp fed with FW A 1% had the best performance, while grey mullet achieved the best SGR and RWG in the same treatment. The results were in line with the previous experiment (Chapter 2) on feeding with different food waste formulations, with FW A 0% showed comparable growth performance in both grey mullet and grass carp when compared with the control feed. The FW D formulation was similar to FW A but contained lower protein level and lower CHO: lipid ratio. The growth performance of both fish species in most feeding groups of FW D were significantly lower than the control ($p<0.05$).

The poor growth of fish could be due to the difference in carbohydrate and lipid (CHO/L) ratios in feed, which were about 3.3 in FW D and 6.3 in FW A. A study showed that an inclusion of 10-20% of corn starch could promote growth and PER in yellowfin seabream (*Sparus latus*), but adverse effect was observed when percentage reached 26% (Wu *et al.*, 2007). There is no specific information on the optimum carbohydrates in feeds (NRC, 1993), although warmwater fish like grass carp (Lin, 1991) are believed to have better utilization of carbohydrates than coldwater fish like Atlantic salmon (*Salmo salar*) (Helland *et al.*, 1991). In general, 28-32% and 36.5-

42.5% dietary proteins and carbohydrates accordingly are optimal for grass carp juvenile (Hasan *et al.*, 2007), similar to the control feed and FW A in the present study.

Low P/E value in FW D indicated a large portion of energy was derived from non-protein source and low CHO/L ratio indicated that energy is mainly from feed lipids. The non-protein energy sources could affect the efficiency of protein utilization in fish (Wilson and Halver, 1986). However, it has been noted that carbohydrates are more suitable energy source than lipid for grey mullet (El-Dahhar, 2000a), while a high lipid content could hinder the growth in various fish, including green grouper (*Epinephelus coioides*), Nile tilapia (*Oreochromis niloticus*) and grass carp (Ali and Al-Asgah, 2001; Luo, 2004; Luo, 2005; Du *et al.*, 2009). The high lipid content in feed may also affect the lipid content in carcass and the feed utilization. The grass carp fed with FW D 0% retained higher lipid content in body and showed poor growth. Ali and Al-Asgah study (2001) also showed that Nile tilapia fed with high dietary lipid (>14%) resulted in poor growth and higher carcass lipid.

However, in the present study, both fish showed impaired growth responses when fed with feeds containing high dietary lipid and low protein contents, and the lipid retentions in carcass were different. High lipid retention in the grey mullet was not observed when fed with FW D without enzymes supplement (FW D 0%), while high retention was observed in grass carp. Lower carcass protein retentions were observed in both fish fed with FW D and protein sparing effects were not shown. Protein sparing effect occurred when fish are more capable to metabolize feed protein

into body tissue from dietary energy of non-protein sources. This was observed in many fish species e.g. Jundia (*Rhamdia quelen*) and hybrid clarias catfish (*Clarias macrocephalus* x *Clarias gariepinus*) (Jantrarotai *et al.*, 1998; Meyer and Fracalossi, 2004). The high dietary lipid did not compensate the effect of low dietary protein based on the retarded growth and lower carcass protein contents, a higher dietary energy level may be required in both fish to spare protein.

On the contrary, higher PER and similar RWG were observed in grey mullet of all FW D groups compared to the control, but low retentions of protein and lipids and higher ash contents were found in carcass. This may also indicate the inadequate dietary energy in FW D and protein was partially converted into energy to support the growth of grey mullet (NRC, 1983). However, further study on the effects of higher level of dietary energy with low protein diet is needed to prove this. The present study also suggested that grey mullets possess different utilization efficiency of lipid in the body, and are more capable to utilize dietary lipid as energy source especially with low protein and high lipid diet.

The present results showed that grey mullet required less dietary protein than grass carp, the PER in grey mullet was improved when feeding with low protein diet (20%). Du *et al.* (2009) suggested that dietary protein and lipid at 35% and 3% respectively are optimal for grass carp (body weight was about 3 g), while grey mullet fry (1.6 g) had the best growth performance at 28% feed protein (El-Dahhar, 2011). El-Dahhar (2000a) also revealed that grey mullet utilized energy with a low CHO/L ratio more efficiently.

3.4.2 Utilizations of food waste supplemented with bromelain and papain mixture

The addition of bromelain and papain may enhance the feed utilization or stimulate fish immunity. The present results showed the enhanced growth and reduced lipid retention in grass carp when fed with enzyme supplemented feed. The decreased carcass lipid was significantly correlated with the increase of bromelain and papain in FW A (r=0.630) and FW D (r=0.669) (Spearman's test, $p<0.01$). Addition of 1% and 2% of bromelain and papain mixture could enhance ANLU in both grass carp and grey mullet. The exogenous enzymes in feed may enhance metabolism and utilization of lipid in grass carp, with more energy converted from lipid, leading to more protein reserved in carcass as observed. However, there is no specific study on the effects of exogenous enzymes on the lipid utilization in fish.

Exogenous enzyme supplementation could assist feed digestion and utilization in fish. The ANPU of grass carp and grey mullet was also improved when adding 1% of mixed enzymes in FW A. Bromelain and papain could hydrolyze proteins and release shorter peptides in feed, the key factor to increase protein digestibility (Fennema, 1996; Singh *et al.* 2011). A few studies also showed that an addition of exogenous papain in feed could enhance the growth of common carp and improve water quality. Wong *et al.* (1996) showed common carp could digest the soybean residues treated with 1% papain more efficiently, the turbidity of tank water and hence fish mortality were reduced in the papain feed groups. A recent study also showed the growth of common carp with 2% papain was promoted, although the water parameters were not

affected (Singh *et al.*, 2011). Another study on palm kernel meal also indicated that addition of enzyme (Ronozyme VP) could enhance the growth and net protein utilization in tilapia (Boonyaratpalin *et al.* 2000). It was also revealed that exogenous enzymes (commercial products) could improve the protein digestibility and feed conversion in rainbow trout (*Oncorhynchus mykiss*) (Farhangi and Carter, 2007).

The cost of enzyme supplemented fish feed could be further reduced if fruit processing wastes were utilized instead of pure enzymes. The use of pineapple wastes could also promote the growth of *Labeo rohita* fingerling, with the SGR, PER were significantly higher in feed containing 25% pineapple wastes, than the control and other fruit waste feeds such as orange and lime (Deka *et al.*, 2003). In Hawaii, the growth of *Litopenaeus vannamei* was comparable to commercial feed when fed with 50% inclusion of fermented papaya waste in shrimp feed. The production cost could be further reduced under a large scale production (Kang *et al.*, 2010).

3.4.3 Hematological parameters of fish fed with food waste supplemented with enzyme mixture

The hematological parameters were affected by the two food waste feeds and supplemented enzymes. The total protein, total IgI and NBT activity of fish feeding with food waste feeds were depressed to some extent during the experiment, e.g. significantly reduced NBT activities were found in grass carp fed with FW A 0% and all FW D groups at Day 28 ($p<0.05$). The production of oxidative radical by neutrophil was quantified by the NBT assay (Siwicki *et al.* 1994), and noted that the

oxidative radical is an important defense system in fish (Anderson *et al.* 1992).

The total IgI and total serum protein levels of grass carp in FW A 0% were also significantly lower than the control during several samplings ($p<0.05$). Serum protein level indicated a balance between anabolic and catabolic activity in fish. When anabolic processes exceeded catabolic ones, the reserved protein is released into blood stream more to meet higher metabolic requirements (Helmy *et al.*, 1974). Serum proteins are responsible for innate immune response of fish and a higher level of serum protein provided stronger response (Wiegertjes *et al.* 1996; Sahu *et al.* 2007a). The rise of plasma proteins could be resulted from the higher digestion of dietary protein (Lundstedt *et al.,* 2002), while the lowered serum protein levels may show nutritional deficiencies in fish (Siwicki *et al.*, 1994). The nutrients contained in food waste in this study may not be utilized and absorbed by fish efficiently, and therefore the growth and immunity were also affected. Moreover, it is noted that the impaired immunity was less obvious in grey mullet than that of grass carp. This further suggested different fish species possess different requirements on feed nutrients and different feed utilization mechanisms for their growth.

Although the food waste feeds have negative impacts on the tested hematological parameters, enzyme supplementation could minimize the impacts and even stimulate the NBT activity, total protein and total IgI levels when fed with 1% or 2% mixed enzyme. Mixed exogenous enzymes of pepsin, papain and α-amylase could increase the total serum protein and globulin level and enhance the growth of Nile tilapia in terms of better SGR, PER and FCR (Goda *et al.,* 2012). A similar study adding lipase,

pepsin, trypsin in feed for common carp fry also showed a better growth performance and elevated total serum protein, compared to feed without enzymes (Yousefian *et al.*, 2013). However, the effects of papain and bromelain on fish immunity are not known, and current study provided an induction on their effects on grey mullet and grass carp. The use of enzyme as feed supplement may be an applicable and viable approach in enhancing feed utilization and fish immunity, but further investigations on specific fish species are needed.

To sum up, the present results indicated on growth performance and haematological parameters of grass carp and grey mullet could be improved by adding enzymes to the food wastes. Other feed supplements such as baker's yeast could be added into food waste for further enhancement on feed utilizations. Baker's yeast is also one of the popular supplements in aquaculture industry, serving as immuno-stimulant and growth promoters.

3.5 Conclusion

The hypothesis of this experiment was accepted, the addition of bromelain and papain could enhance the utilization of food waste feeds by fish. In general, the growth and immunity of grass carp and grey mullet fed with food waste feeds without enzymes were impaired compared to the control, however, improvement superior to the control was observed with the addition of bromelain and papain supplement. Addition of 1% and 2% mixture of bromelain and papain could significantly enhance the ANLU in grass carp and ANPU in grey mullet. The results also showed that grey

mullet was more capable to utilize protein and lipid in the low protein diet than grass carp, but the high dietary lipid did not compensate the effect from low dietary protein based on the retarded growth. Further experiments on the effects of baker's yeast (*Saccharomyces cerevisiae*) on grass carp immunity and disease resistance were conducted in Chapter 4.

Chapter 4

Adding baker's yeast to food waste to enhance growth performance and immunity of grass carp (*Ctenopharyngodon idellus*)

4.1 Introduction

The use of probiotics in aquaculture industry has been extensively investigated (Aly *et al.*, 2008; Wang and Xu, 2004; Wang e*t al*., 2005a). "Probiotics" is defined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance" (Fuller, 1989). The probiotics can be applied in the form of feed supplements or directly introduced to the aquaculture system, for enhancing feed value, enzymatic digestion, pathogens inhibition, growth and immune response of hosts (Ziaei-Nejad *et al.*, 2006; Wang and Xu, 2004; Wang *et al.*, 2005a).

Probiotics can enhance the establishment of favorable microbial communities e.g. *Bacillus* sp. and other lactic acid bacteria in the gastrointestinal system. These beneficial communities could alter gut morphology and generate enzymes for digestion and absorption of nutrients and also stimulate the host immunity (Verschuere *et al.*, 2000). It was observed that the food waste feed was not efficiently utilized by grass carp and grey mullet, leading to impaired growth (Chapter 2). Although supplementation of bromelain and papin could enhance the utilization of food waste, the immunity of grass carp was not affected significantly (Chapter 3). Therefore, attempts will be made to apply baker's yeast as a feed supplement to enhance growth and host immunity.

The yeast, *Saccharomyces cerevisiae* has been used in baking and fermenting alcoholic beverages for a thousand years. More recently, it has been used to improve growth and feed utilization in aquaculture, in various carp species (Mohanty *et al.*, 1996; Swain *et al.*, 1996), hybrid striped bass (*Morone chrysops* × *M. saxatilis*) (Li and Gatlin, 2005) and Nile tilapia (*Oreochromis niloticus*) (Abdel-Tawwab *et al*, 2008). *S. cerevisiae* also alleviated the transportation stress of tilapia and strengthened their disease resistance against *Aeromonas hydrophila* (Zaki, 2004).

The positive effects of probiotics on fish growth may be due to the adhesion of yeast cells to the intestinal wall, with their amylase enzymes increasing the digestibility of the diet (Scholz *et al.* 1999). *S. cerevisiae* also significantly enhanced amylase activity in Nile tilapia (Essa *et al.*, 2010). Furthermore, the cell wall constituents of yeast could stimulate innate immune responses and protect fish against infections (Esteban *et al.*, 2001). Baker's yeast consisted of β-glucans, mannan oligosaccharides and nucleic acid which are regarded as immune-stimulants (White *et al.*, 2002). The β-glucan isolated from yeast has shown immune-stimulating properties on various fish species, such as Asian catfish (*Clarias batrachus*) and common carp (*Cyprinus carpio*), enhancing their disease resistances to *A. hyrdophila* (Kumari and Sahoo, 2006a & b; Selvaraj *et al.*, 2005). The addition of whole yeast in diet stimulated the immune response of channel catfish (*Ictahrus punctatus*), seabream (*Sparus aurata*) and tilapia (*O. niloticus*) (Chen and Anisworth, 1992; Rodriguez *et al.*, 2003; He *et al.*, 2009). Applying whole yeast would be superior to extracted and purified constituents, as the cost and technique are lessened.

In addition, the whole baker's yeast is a potential alternative for fish meal protein (Oliva-Teles and Goncalves, 2001). Yeast is one of the major by-products in brewing industry followed by brewer spent grain and could be collected easily and economically. It is hypothesized that the baker's yeast could enhance the growth and immunity of grass carp through better feed utilization. In this Chapter, the baker's yeast was used as a feed supplement (2.5% and 5% w/w) by incorporating into 3 different types of feeds: commercial feed (Jinfeng®, 613 formulation) (as the control) and food waste feeds with (FW A-E) and without (FW A) addition of bromelain and papain mixtures. The objectives of this study were to investigate 1) the effects of adding baker's yeast to food waste feed on growth and feed utilization of grass carp; 2) changes of fish immunity and 3) disease resistances of fish to *Aeromonas hydrophila*.

4.2 Materials and Methods

4.2.1 Experimental setup and fish feed preparation

The food waste feed formulation A (FW A) containing 75% food wastes with mainly plant materials such as cereals, fruit and vegetables (Chapter 2, Table 2.2), was prepared according to the procedures mentioned in Section 2.2.1. Papain and bromelain in the proportion of 0.5+0.5% (weight by weight) were added into FW A by mixing with (50% by mass of fish diet) 0.025 mol/L sodium dihydrogen phosphate and adjusted to pH 8 with sodium hydroxide (NaOH), at 25ºC for 3 h. The commercial fish feed (Jinfeng®, 613 formulation) and FW A without enzyme treatment were used as the controls. The resulted substrates were heated to 95°C for 5

min to inactivate the enzyme (Wu *et al.*, 1998).

The three types of feeds, i.e. the control feed (C-0%Y), enzyme treated FW A (A-0%Y) and non-enzyme treated FW A (A-E-0%Y) were supplemented with 2.5% and 5% w/w of baker's yeast, *Saccharomyces cerevisiae* (Type II, YSC2, Sigma-Aldrich) (nine groups in total). Deionized water was added and the fish feed dough was pelletized with a meat grinder and dried at 50˚C for 24 h. The proximate compositions (ash, moisture, protein, fibre, lipid and nitrogen free extracts) were determined in Section 2.2.5 with the results shown in Table 4.1

4.2.2 Feeding experiment design

Seven hundred fingerlings of grass carp *Ctenopharyngodon idella* (herbivore) were purchased from a local fish farm. Eighteen individuals (~20-25g) were placed into each tank (~60L). The water temperature, pH and dissolved oxygen were measured three times a week using a portable Hanna pH meter and a YSI digital Dissolved Oxygen (DO) meter and the values ranged from 21.8-23.7°C, 6.3-6.9 and 6.6-7.5 mg/mL respectively.

The control feed was fed to fish at the rate of 2% body weight daily for 3 weeks before starting the experiment. The experiment was conducted for 56 days and there were triplicates for each treatment (9 in total).

Table 4.1 Composite analysis of three types of feeds: control (C) and food waste formulation A (with (A-E) and without (A) enzyme) feed supplement with or without baker's yeast (Y)

Formulation	C-0%Y	C-2.5%Y	C-5%Y	A-0%Y	A-2.5%Y	A-5%Y	A-E-0%Y	A-E-2.5%Y	A-E-5%Y
Dry matter (%)	93.68±0.20a	94.78±0.32a	94.53±0.64a	95.69±0.02a	94.89±0.28a	94.67±0.23a	94.79±0.23a	94.26±0.29a	93.86±0.16a
Ash (%)	8.24±0.09a	8.22±0.21a	8.13±0.34a	9.18±0.46b	8.68±0.34ab	8.48±0.25ab	8.81±0.25ab	8.25±0.33ab	8.10±0.20ab
Protein (%)	30.16±1.55a	29.38±1.72a	30.90±1.68abc	31.44±0.31ab	30.14±0.35ab	31.65±0.81bc	31.17±0.08ab	30.74±0.35ab	32.85±0.82c
Lipid (%)	5.17±0.94a	5.43±0.86a	5.33±0.58a	6.12±1.66a	6.18±0.95a	6.22±0.82a	6.02±0.81a	6.08±0.97a	6.16±0.67a
Fibre (%)	9.57±0.21a	10.01±0.25a	10.45±0.31a	9.62±0.63a	9.84±0.41a	9.95±0.68a	9.60±0.60a	10.26±0.25a	10.59±0.49a
Carbohydrate (%)[1]	40.53	41.74	39.72	39.34	40.05	38.37	39.19	38.94	36.16
Energy (kJ/g diet)[2]	16.17	16.30	16.27	16.64	16.48	16.56	16.51	16.39	16.44
CHO/L ratio [3]	7.84	7.69	7.45	6.43	6.48	6.17	6.51	6.40	5.87
P/E (mg/kJ)[4]	1865	1802	1899	1889	1829	1911	1888	1875	1998

*Different superscripts (a, b, c) between feeding groups are significantly different, $p<0.05$

[1] Carbohydrates (%) = 100 − (crude protein % + crude lipid % + moisture % + ash %+ fibre %) (Castell and Tiews, 1980)

[2] Energy (kJ/g diet) = (% crude protein × 23.6) + (% crude lipids × 39.5) + (% carbohydrates × 17.3) (Chatzifotis *et al.*, 2010)

[3] Carbohydrates to Lipid (CHO: L) ratio = % wt. in CHO / % wt. in lipid

[4] Protein to energy (P/E) (mg/kJ) = crude protein (%) / energy

4.2.3 Fish immunological and growth parameters

Fish blood samples were collected by caudal venous puncture at Day 1, 14, 28 and 56, and the bactericidal activity (described as follows), neutrophil activity (Nitroblue tetrazolium (NBT) assay) in blood, total protein and total immunoglobulin of plasma were determined (refer to Section 3.2.4 for details).

Various parameters: specific growth rate, relative weight gain, feed conversion ratio, protein efficiency ratio, apparent net protein utilization and apparent net lipid utilization were monitored as parameters to indicate fish growth performance of different groups (Section 2.2.4).

Bactericidal activity of plasma

The bactericidal activity of blood plasma was conducted based on the method of Abidov and Mirismailov (1979). *A. hydrophila* (same bacteria used for the infection, Section 4.2.4) was inoculated in LB broth at 28ºC for 18 h. The cultures were centrifuged at 850 G for 15 min. The supernatant was removed and the bacteria pellet was washed by sterile 0.9% saline twice. The concentration of bacteria was adjusted to 1×10^8 cfu/mL, based on the optical density of suspension and diluted to 10^{-4} by sterile saline. Equal volumes (80 µL) of diluted bacterial suspension and plasma (0.9% sterile saline for growth control) were mixed and incubated at 28ºC for 30 min. After incubation, 50 µL of mixed solution was poured onto the LB agar plate and the dishes were incubated for 18 h at 28ºC. The bactericidal activity was represented by the percentage decreased of colony counts in the sample compared to the control.

4.2.4 *A. hydrophila* injection to grass carp

Aeromonas hydrophila (isolated from diseased fish in the Pearl River fishery Research Institute, China fishery Science China) was inoculated in LB broth overnight at 28 ºC**.** The cultures were centrifuged at 850 G for 15 min. The supernatant was removed and the bacteria pellet was washed twice in sterile 0.9% saline. The suspension was adjusted to 1 x 10^8 cfu/mL, based on the optical density of suspension with sterile saline (~0.13 absorbance at 625 nm). Suspended bacteria (0.1 ml) was injected into the peritoneal cavity of fish (12 fish for each tank) at Day 56 of the feeding experiment. The mortality rate was recorded in the following 10 days after infection.

4.2.5 Statistical analysis

The results obtained at each sampling day (Day 1, 14, 28, 56) were compared using one-way ANOVA and Duncan's multiple range tests (SPSS Statistics 17.0, Chicago, Illinosis, USA). Significant differences between experimental groups were expressed at the significance level of $p<0.05$.

4.3 Results

4.3.1 Feeding trial with yeast supplement incorporated in different feeds

4.3.1.1 Growth performance of grass carp

The highest values of SGR and RWG were shown in A-E-5%Y and SGR and RWG of A-E-2.5%Y, A-E-5%Y and C-5%Y were significantly higher than those of C-

0%Y ($p<0.05$) (Table 4.2). The lowest values of SGR and RWG were observed in A-0%Y which were significantly lower than C-0%Y ($p<0.05$).

The yeast supplemented groups, C-5%Y, A-5%Y, A-E-2.5%Y and A-E-5%Y also showed significantly higher growth rate (in terms of SGR and RWG) than its base feed without yeast supplement i.e., C-0%Y, A-0%Y and A-E-0%Y correspondingly.

Similar observations were also found in FCR, with FCR of A-5%Y significantly lower than A-0%Y, while FCR of A-E-2.5%Y and A-E-5%Y significantly lower than A-E-0%Y ($p<0.05$). However, FCR of A-0%Y and A-2.5%Y were significantly higher than C-0%Y ($p<0.05$). The PER of A-0%Y was significantly lower than C-0%Y ($p<0.05$), while A-E-2.5%Y was significantly higher than C-0%Y ($p<0.05$).

The ANPU of C-0%Y was significantly lower than all other groups ($p<0.05$) and ANPU of A-E-2.5%Y was higher than A-E-0%Y ($p<0.05$). A significantly lower ANLU was only observed in A-2.5%Y ($p<0.05$).

4.3.1.2 Carcass composition of grass carp

The moisture content of A-0%Y was significantly lower than the control ($p<0.05$). The ash content of C-2.5%Y was significantly higher than C-0%Y ($p<0.05$) (Table 4.3). Carcass protein contents of most of the groups (except C-5%Y, A-2.5%Y and A-E-5%Y) were significantly higher than C-0%Y ($p<0.05$). The yeast supplemented groups C-2.5%Y and A-E-2.5%Y showed significantly higher carcass protein contents than C-0%Y and A-E-0%Y (feed groups without yeast) ($p<0.05$). A significantly higher carcass lipid content was observed in A-0%Y than C-0%Y, while A-2.5%Y was significantly lower than A-0%Y ($p<0.05$).

Table 4.2 Growth performance of grass carp fed with the control (C) and food waste (with (A-E) and without (A) enzyme) feeding groups with or without baker's yeast (Y)

	C-0%Y	C-2.5%Y	C-5%Y	A-0%Y	A-2.5%Y	A-5%Y	A-E-0%Y	A-E-2.5%Y	A-E-5%Y
Initial weight (g)	14.10±0.21a	13.92±0.31a	13.92±0.34a	14.26±0.27a	14.25±0.33a	13.89±0.32a	13.94±0.32a	14.17±0.21a	13.80±0.31a
Final weight (g)	21.18±0.32bc	21.14±0.59bc	22.89±0.67cd	19.26±1.03a	20.05±1.04ab	22.40±1.20cd	20.23±1.06ab	23.19±1.24d	22.87±0.97cd
Feeding rate (%/day)	2.01±0.04b	2.03±0.08b	2.04±0.07b	2.05±0.04b	2.17±0.12c	2.04±0.02b	2.05±0.06b	1.88±0.04a	1.94±0.06ab
SGR (%/day)	0.73±0.03bc	0.75±0.07bcd	0.89±0.08de	0.53±0.09a	0.61±0.12ab	0.85±0.08cde	0.66±0.08ab	0.88±0.08de	0.90±0.04e
RWG (%)	50.2±2.6bc	51.9±5.7bc	64.5±7.0d	35.0±6.6a	40.9±9.4ab	61.3±7.4cd	45.1±6.5ab	63.6±6.8d	65.6±3.5d
FCR	2.81±0.11ab	2.78±0.35ab	2.36±0.23ab	3.94±0.77c	3.73±1.01c	2.45±0.27ab	3.16±0.38bc	2.20±0.24a	2.21±0.16a
PER	1.18±0.05bcd	1.24±0.15cde	1.38±0.14de	0.83±0.15a	0.93±0.23ab	1.30±0.13de	1.03±0.12abc	1.49±0.16e	1.38±0.09de
ANPU (%)	28.17±9.64a	43.17±2.66b	36.30±3.06b	36.79±2.39b	35.97±3.91b	38.50±2.93b	37.87±2.05b	52.57±4.55c	38.96±1.21b
ANLU (%)	102.6±41.4ab	117.7±10.5ab	117.7±4.2ab	128.3±6.6b	97.6±7.5a	121.6±1.8ab	102.5±11.1ab	105.4±1.3ab	112.9±7.5ab

*Different superscripts (a, b) among feeding groups are significantly different ($p<0.05$)

4.3.1.3 Hematological parameters of grass carp fed with different feeds

The plasma protein in each group did not vary significantly during the experimental period (Fig. 4.1a). Significantly higher plasma protein levels were only observed in A-2.5%Y and A-5%Y at Day 14 and 28, compared to A-0%Y ($p<0.05$), while the plasma protein level of A-0%Y was significantly lower than all other groups at Day 56 ($p<0.05$).

There was no significant difference between all feed groups and the control in total IgI levels at Day 14 ($p>0.05$) (Fig. 4.1b). The IgI levels of A-5%Y and A-E-5%Y were significantly higher than A-0%Y at Day 28 and 56 respectively ($p<0.05$).

There was no significant difference between all feed groups and C-0%Y (the control) in NBT activity at Day 14 ($p>0.05$) (Fig. 4.2a). The NBT activity of A-E-5%Y was significantly higher than C-0%Y, A-E-0%Y and A-E-2.5%Y at Day 28 ($p<0.05$). The NBT activities of C-2.5%Y, C-5%Y, A-E-2.5%Y and A-E-5%Y were significantly higher than C-0%Y at day 56 ($p<0.05$). It was also noted that the activity of A-5%Y was significantly higher than A-0%Y (without yeast) at Day 56 ($p<0.05$).

The serum bactericidal activities of A-5%Y, A-E-2.5%Y and A-E-5%Y were significantly higher than C-0%Y at Day 14 ($p<0.05$) (Fig. 4.2b). The activities of A-E-2.5%Y and A-E-5%Y were significantly higher than A-E-0%Y (without yeast) and C-0%Y at Day 14 and 28 ($p<0.05$). The activities of C-5%Y and A-5%Y were also significantly higher than C-0%Y at day 28 ($p<0.05$). All groups except A-0%Y and A-E-0%Y, i.e. all yeast supplemented groups showed significantly higher bactericidal activities than C-0%Y ($p<0.05$).

Table 4.3 Proximate composition of grass carp carcass (%, wet weight basis) fed with the control (C) and food waste (with (A-E) and without (A) enzyme) feeding groups with or without baker's yeast (Y)

	C-0%Y	C-2.5%Y	C-5%Y	A-0%Y	A-2.5%Y	A-5%Y	A-E-0%Y	A-E-2.5%Y	A-E-5%Y
Moisture (%)	75.8±1.3a	73.0±5.0ab	75.2±1.6a	71.1±2.0b	74.0±0.8ab	73.0±2.1ab	74.9±3.4ab	74.8±1.2ab	74.3±1.6ab
Ash (%)	2.59±0.12a	3.24±0.62b	2.68±0.42ab	3.15±0.42ab	2.58±0.24a	2.60±0.32a	2.69±0.11ab	2.77±0.49ab	3.00±0.26ab
Protein (%)	9.48±0.50a	11.63±2.55b	10.30±0.60ab	11.53±0.60b	11.22±0.58ab	11.38±1.06b	11.45±0.95b	13.72±1.06c	11.16±0.21ab
Lipid (%)	6.34±0.65ab	6.00±0.35a	5.77±0.21a	7.83±0.21c	6.26±0.68ab	6.99±0.20bc	5.99±1.16a	5.45±0.27a	6.07±0.73ab

*Different superscripts (a, b) among feeding groups are significantly different ($p<0.05$)

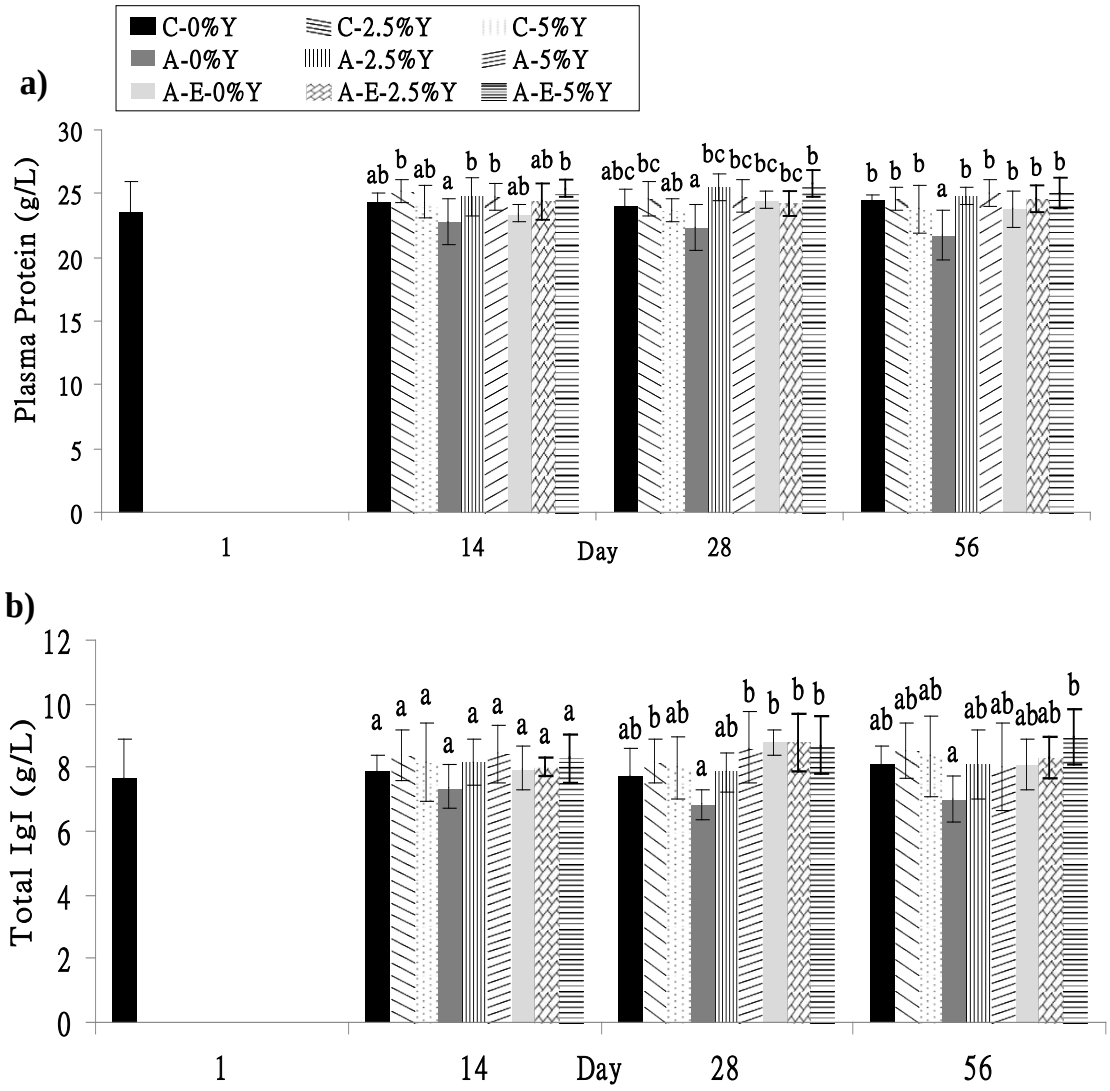

Fig. 4.1 a) Total plasma protein (g/L) and b) total immunoglobulin (g/L) of grass carp feeding with the control (C) and food waste (with (A-E) and without (A) enzyme) feeds groups with or without baker's yeast (Y), different superscripts (a, b) within one sampling day are significantly different (*p<0.05*)

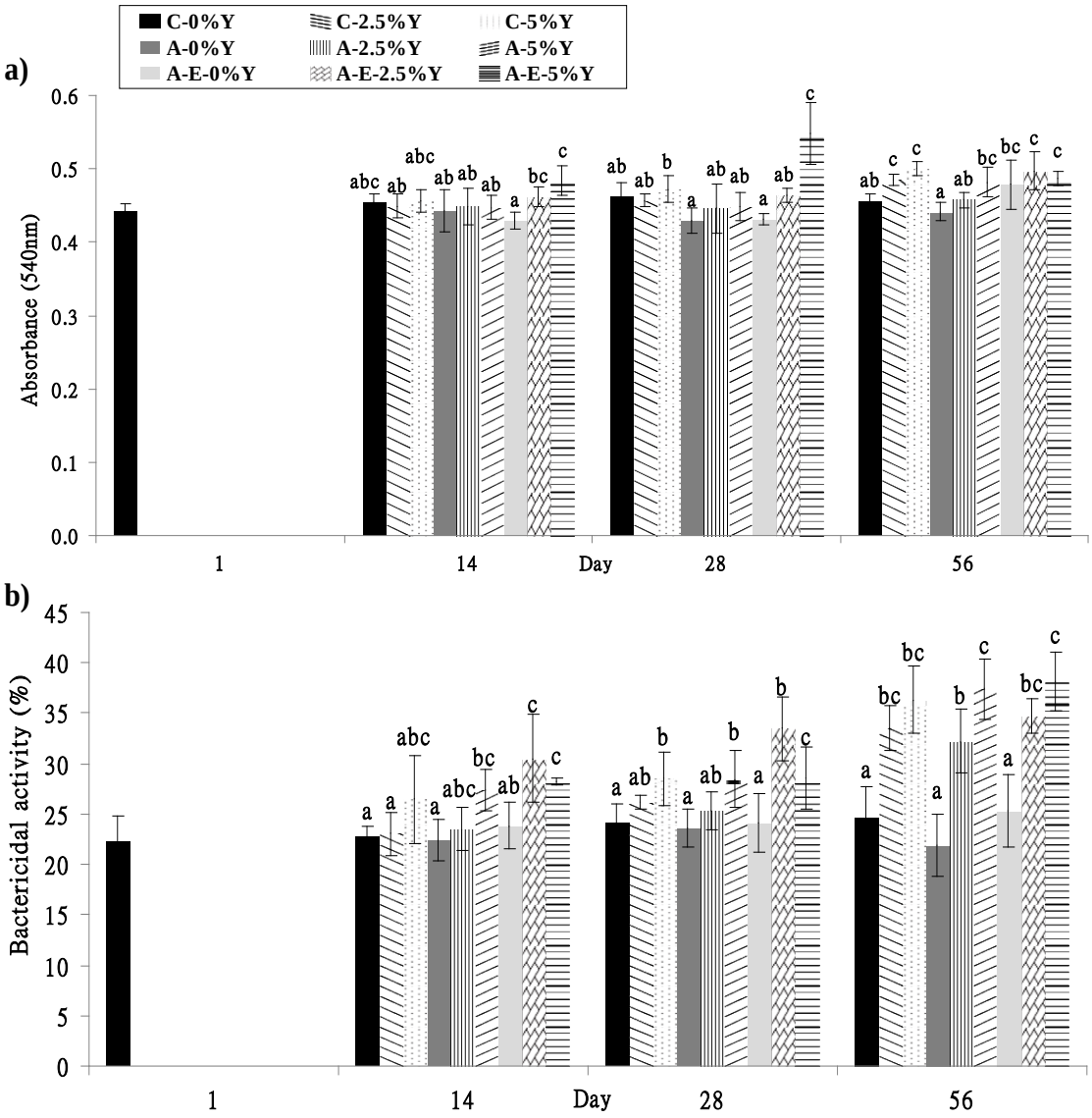

Fig. 4.2 a) Nitroblue tetrazolium (NBT) activity and b) plasma bactericidal activity (%) of grass carp feeding with the control (C) and food waste (with (A-E) and without (A) enzyme) feeds groups with or without baker's yeast (Y), different superscripts (a, b) within one sampling day are significantly different (*p<0.05*).

4.3.1.4 Disease resistance to *Aeromonas hydrophila* of grass carp

The diseased grass carps showed lethargy, expanded abdomen with red blotches and redden and swollen anus, with yellow mucus released from the anus when slight pressure was applied to the abdomen. They lost their appetite after infection. These signs matched with enteritis infection observed in grass carp (NACA, 1989; Zheng *et al.*, 2012).

Mortalities of yeast supplemented groups, C-5%Y, A-2.5%Y, A-E-2.5%Y and A-E-5%Y were significantly lower than C-0%Y after 10 days of injections ($p<0.05$). The highest mortality was 69.4% noted in A-0%Y, while lowest was 33.3% in A-E-2.5%Y (Fig. 4.3).

4.4 Discussion

4.4.1 Growth and feed utilizations of grass carp fed with baker's yeast supplemented feed

In general, the yeast supplemented feed groups showed better growth rates than the groups without yeast, in terms of FCR, PER, SGR and RWG. The highest growth rate was observed in the corresponding groups with 5% *S. cerevisiae* (baker's yeast) with different types of feeds, i.e. FW A with and without bromelain and papain and the control feed. This indicated the yeast could facilitate fish growth in different feeds. Lara-Flores *et al.* (2003) showed a higher protein digestibility of feed in Nile tilapia by adding *S. cerevisiae*, and similar results were also observed in sea bass and tilapia (Tovar *et al.*, 2002; Ozório *et al.*, 2012). Better feed utilization hence lower FCR suggested that the feed quantity and cost could be substantially reduced.

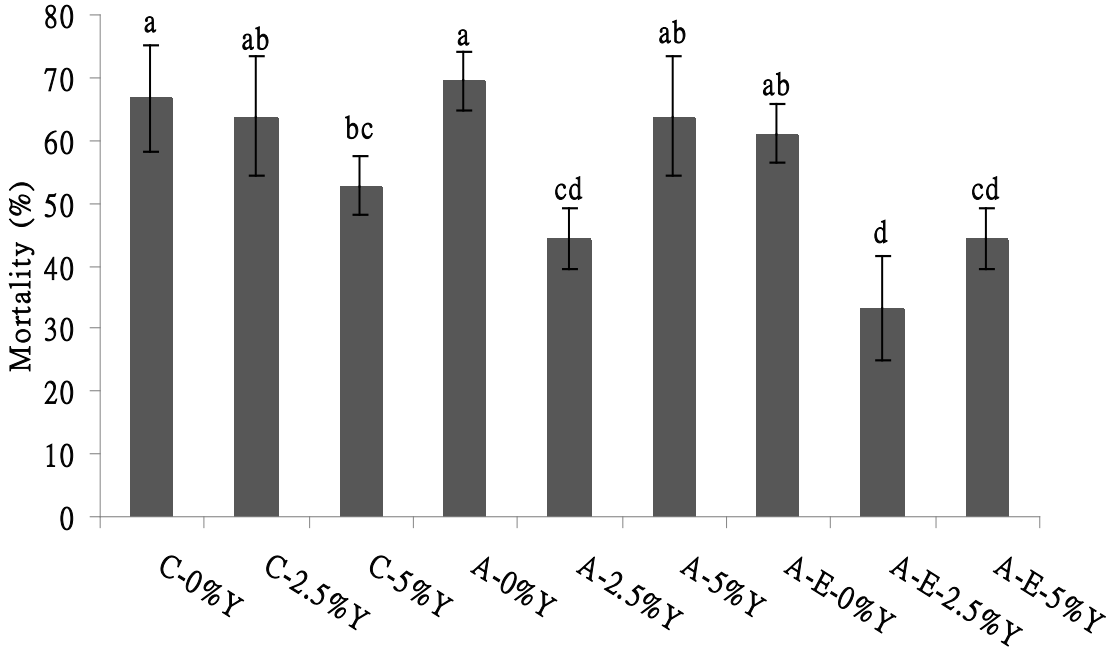

Fig. 4.3 Mortality of grass carp against *Aeromonas hydrophila* injection after feeding with the control (C) and food wastes (with (A-E) and without (A) enzyme) feeds groups with or without baker's yeast (Y), different superscripts (a, b) within one sampling day are significantly different (*p<0.05*).

Baker's yeast itself is rich in protein and is a good feed alternative for *Cyprinus carpio* fingerlings, replacing 30% of fish meal without impairing fish growth (Korkmaz and Cakirogullari, 2011). The baker's yeast mainly comprised of mannan, glucans and small amounts of chitin and nucleic acid (Anderson *et al.*, 1995; Nguyen *et al.*, 1998), which would be beneficial to fish (Li and Gatlin, 2003). The administration of β-glucan also stimulated the immunity and enhanced the survival of *L. rohita* fingerlings, apart from promoting growth (Misra *et al.*, 2006).

Protein utilization as well as fish growth was also promoted by the yeast supplements in the present study. All yeast supplemented groups showed significantly higher ANPU than the control feed without yeast. The yeast may also help the establishment of beneficial intestinal microbial community, which could also enhance enzyme activities for digestion (Tovar *et al.*, 2002; Waché *et al.*, 2006). For example, the amylase activity in tilapia was increased after feeding with *S. cerevisiae* (Essa *et al.*, 2010). The gastrointestinal bacteria are also involved in the breakdown of nutrients and provided physiologically active nutrients to hosts (Bairagi *et al.*, 2004; Ramirez and Dixon, 2003). As a consequence of better nutrient digestion and absorption, higher protein was retained in fish body fed with 2.5% or 5% yeast supplemented feeds in the current study. The growth rate and protein utilization were also higher in both enzyme and yeast (2.5%) supplemented group than the groups without enzymes. This suggested that the yeast could further enhance the nutrient utilization in feeds, in addition to the initial improvement after adding bromelain and papain.

Lower lipid retentions in grass carp carcass could be due to more lipids being converted to energy and hence protein was reserved (Meyer and Fracalossi, 2004). Significant reductions of lipid retentions were found in enzyme and yeast supplemented groups. On the contrary, Quentel *et al.* (2005) showed that *S. cerevisiae* increased muscle lipids in rainbow trout muscle. The contradictory results may be due to the addition of papain and bromealin which reduced the lipid retention in grass carp (Chapter 3).

4.4.2 Immunity of grass carp fed with baker's yeast supplemented feed

The immunity of grass carp could be stimulated by implementing yeast in feeds, reflected by the higher oxidative radical production activity in blood (NBT activity) and bactericidal activity of plasma. The NBT assay quantified the production of intracellular superoxide radicals by leukocytes (Sahu *et al.*, 2007b; Ardó *et al.*, 2008). In general, higher NBT activities were found in the grass carp fed with yeast supplemented feeds than feeds without yeast, e.g. C-2.5%Y and C-5%Y showed significantly higher activities than C-0%Y at Day 56 ($p<0.05$) (Fig. 4.2a). The NBT activity reached highest level at Day 28 in A-E-5%Y which was significantly higher than all other groups. Li and Gatlin (2003) also obtained similar results with 2% of brewer's yeast improved the extracellular superoxide anion and blood neutrophil oxidative radical productions of head kidney macrophages in hybrid striped bass (*M. chrysops* × *M. saxatilis*). The reactive oxygen radicals in fish immune system are harmful for bacterial pathogens and are important for killing bacteria in blood (Hardie *et al.*, 1996; Itou *et al.*, 1996)

A more evident response on the immuno-stimulating effect of yeast was also observed in the plasma bactericidal activity. All yeast supplemented groups showed remarkable increases in bactericidal activities, compared to the corresponding types of feeds without yeast (C-0%Y, A-0%Y and A-E-0%Y). Compared to whole yeast *S. cerevisiae*, β-glucan (extracted from *S. cerevisiae*) and laminaran (glucan from brown algae), β-glucan is more effective to stimulate serum bactericidal activity and lysozyme activity in Nile tilapia (El-Boshy *et al.*, 2010). Similar stimulations on lysozyme, serum bactericidal and complement activities by β-glucan were found in *Labeo rohita* (Misra *et al.*, 2006). As a result, the immune-stimulating effect of *S. cerevisiae* should be mainly due to the presence of β-glucan.

On the other hand, the yeast did not affect the total plasma protein and IgI levels to a great extent. Higher IgI levels in yeast supplemented groups and lower levels in non-yeast supplemented groups were generally observed, though not significantly different, when compared to the control feed without yeast. Moreover, depressed total protein level was found in FW A without any yeast and enzymes, this was in line with the previous study (Chapter 3). This could be due to the poor utilization of feed, as nutritional deficiencies may cause lower a plasma protein in fish (Siwicki *et al.*, 1994) and the level of immunoglobulin may also be lowered.

4.4.3 Fish disease resistance to *A. hydrophila*

The disease resistance to *A. hydrophila* of grass carp was also greatly enhanced

when supplemented with 2.5% yeast in enzyme supplemented FW A. Similar result was obtained in Nile tilapia against *A. hydrophila* with 0.1% w/w of live baker's yeast (Abdel-Tawwab *et al.*, 2008). The mortality of Nile tilapia was closely related to the doses added in feed and it was significantly reduced as the yeast supplementation increased from 0.1 to 0.6% (Osman *et al.*, 2010). The well-built resistance to *A. hydrophila* in grass carp may be due to the enhanced NBT activity and bactericidal activity. The present study about grass carp fed with Traditional Chinese Medicine (TCM) also showed higher resistance to *A. hydrophila* accompanied with a higher bactericidal activity (Chapter 6).

In addition, the modifications of bacteria flora in guts by yeast could also affect the disease resistance to pathogens and fish immunity. The dietary yeast products containing *S. cerevisiae* could alter the intestinal microbial community into a more beneficial one, and provide stronger protection for gibel carp (*Carassius auratus*) against Gram-negative pathogens such as *A. hydrophila* (He *et al.*, 2011)

It is envisaged that combination of bromelain, papain and baker's yeast may further promote fish growth and immunity. More prominent resistance to *A. hydrophila* was observed on the group combining bromelain (0.5%), papain (0.5%) and also yeast (2.5%). Only 33.3% mortality was recorded, compared with nearly 70% in the food waste groups without any supplements. Positive results related to growth and bactericidal activity were apparent after feeding fish with these supplements. The enzymes may further facilitate the immuno-stimulating effects of yeast, but no study has been conducted on the

combined effects of enzymes and baker's yeast.

Baker's yeast is a natural substance and has no adverse effects on animals and the environment, it is also a by-product readily available from industries such as brewery with low-cost (Tewary and Patra, 2011). The application of baker's yeast could be beneficial to both aquaculture industry and the environment, serving as an immuno-stimulant and as an alternative to antibiotics. The present study indicated that the whole yeast feed supplement yielded positive results on fish growth and immunity. The whole yeast is more economical in terms of the net profit of fish farmers as it promotes higher growth rates and better feed conversion.

4.5 Conclusion

The hypothesis was accepted as the baker's yeast enhanced the growth and immunity of grass carp through better feed utilization. The yeast supplemented feed groups generally showed better growth rates than feeds without yeast, in terms of FCR, PER, SGR and RGW. The optimal dose was 2.5% yeast (*S. cerevisiae*) with bromelain and papain (FW A) added. Protein utilization was enhanced by the yeast supplements. Grass carp immunity was stimulated by implementing yeast in feeds, reflected by the higher NBT activity in blood and bactericidal activity of plasma. This indicated that baker's yeast could facilitate fish growth as supplements in different feeds. It was concluded that there may be additive effects on growth and immunity by mixing enzymes and yeast. This inexpensive industrial product could reduce feed quantity and enhance

fish immunity in aquaculture, which subsequently reduces feed cost accompanied with higher yields.

Chapter 5

The antimicrobial activity of Traditional Chinese Medicines (TCMs), a potential drug alternative on dealing with fish pathogens

5.1 Introduction

Aquaculture provides important food sources in the worldwide; its importance is increasing as the capture fishery production has been saturated during the past 20 years (FAO, 2012). The aquaculture industry has expanded rapidly in the past few decades, but the high stocking density in fish farms easily triggered off the disease outbreak such as parasitic and bacterial diseases. *Aeromonas hydrophila*, *Streptococcus* spp. and *Vibrio* spp. are the common fish pathogens and could cause relentless economical loss in fish farms. In fact, bacterial infection is one of the major factors causing mortality in aquaculture (Grisez and Ollevier, 1995). In general, drugs and antibiotics such as Malachite green, formalin, terramycin, potassium permanganate, sulfonamides and tetracyclines are commonly used for controlling diseases (Rukyani, 1994; Rawn *et al.*, 2009).

The application of drugs and antibiotics may not be beneficial and sustainable approaches dealing with diseases, especially antibiotics. The effectiveness of antibiotics decreased as a result of improper use and abuse. The potential hazard of antibiotics to public health has also been raised since the development and spread of drug resistant bacteria were found. The frequent occurrences of antibiotic resistant bacteria have been

observed in recent years and are probably due to the abuse of veterinary antibiotics and sub-therapeutic doses used in aquaculture (Smith *et al.*, 2002). As a consequence, more and more antibiotics resistant fish pathogens have been detected around aquaculture sites (Furushita *et al.*, 2005; Cabello, 2006; Sørum, 2006). Terrestrial veterinary pathogens and even human pathogens may gain antibiotic resistance genes through horizontal gene transmissions from bacteria in the aquaculture environment (Angulo and Griffin, 2000; Heuer *et al.*, 2009).

The presence of antibiotics residues in aquaculture products and the environment is also associated with human health risks (Cabello, 2006). The drugs and bacteria in the contaminated products may be transferred with resistant genes developed in the human intestine (Salyers *et al.*, 2004) leading to bacterial infections. Senderovich *et al.* (2010) revealed that fish digestive systems are reservoirs of *Vibrio cholerae* and therefore improper management of aquaculture diseases and drug applications would endanger human health. The Marine Product Export Development Authority of India (MPEDA) has recommended the hatchery operators and fish farmers not to use various antibiotics (Sanandakumar, 2002), the European Union has also banned the use of antibiotics as feed additives in food-producing animal unless authorized after January 2006. The Food and Drug Administration (FDA) also restricted the use of various aquaculture drugs, e.g. oxytetracycline and chloramphenicol (FDA, 2011). World Health Organization (WHO), Food and Agriculture Organization of the United Nations (FAO) and the World Organisation for Animal Health (OIE) jointly issued the guidelines for the responsible

use of antimicrobial agents for veterinary uses (FAO/OIE/WHO, 2006). Unfortunately, the uses of antibiotics e.g. quinolones in fish farming in developing countries such as China and Chile are still common (Cabello, 2004; Jacoby, 2005). Therefore, there is an urgent need to seek alternatives for antibiotics to deal with bacterial infections in aquaculture systems, otherwise more resistant strain bacteria would be spread in the environment.

Traditional Chinese Medicines (TCMs) is one of the novel alternatives to antibiotics, which can be served as antimicrobial agent and immuno-stimulant. TCMs have been traditionally used in China aquaculture industry but only being explored scientifically in recent years. Herbal medicines showed distinctive antimicrobial activities on fish pathogens (Bakkali *et al.*, 2008; Van Vuuren and Viljoen, 2008), which have been suggested as an alternative to antibiotics (Galina *et al.*, 2009). Using herbal medicines for treating human pathogens with multi-drug resistance (MDR) has become another popular topic, antimicrobial activity on various pathogenic organisms, such as *Staphylococcus aureus, Pseudomonas aeruginosa, E. coli, Bacillus subtilis, Salmonella typhimurium* and *Enterococcus faecalis* have been studied extensively (Singh *et al.*, 2010; Chan *et al.*, 2011; Ibrahim *et al.*, 2011). However, there is a lack of information on drug resistance development in TCMs on whether drug resistant bacteria would be developed when exposed to TCM. Therefore, there is a need to study the longer term effects of using TCMs in aquaculture.

TCM have been used for human therapeutic purposes with thousands year of history,

details of usage and formulations of TCM have been recorded in substantial national literatures, e.g. Shen Nong's Materia Medica (The Complier Group, 2009), Compendium of Materia Medica (Li, 2009) and Dictionary of Chinese Materia Medica (Jiangsu New Medical College, 1977). Drug resistance of bacteria to TCMs and natural herbs is seldomly recorded in literature, even for its long history in applications. To the best of our knowledge, there is limited information focusing on the development of drug resistance in bacteria to TCM. The present study will focus on the antimicrobial activities of selected TCMs and drug resistance development in fish pathogens to these TCMs. Seventeen types of TCMs used for treating disease in human digestion systems were selected for the investigations on their antimicrobial activities related to some common fish pathogens.

Three common fish pathogens, *V. cholerae*, *A. hydrophila* and *Lactococcus garvieae* were selected for this study. *V. cholerae* is the causative agent of cholera in human and vibrosis in fish. It is commonly found in natural aquatic environments and the intestine of freshwater fish e.g. grass carp, grey mullet, common carp and marine fish e.g. soldierfish, and fish serving as reservoirs of these pathogens (Senderovich *et al.*, 2010). *A. hydrophila* could cause the infections on skins, blood and intestine in both human and fish (Castro *et al.*, 2008). *L. garvieae* is a gram-positive pathogen causing lactococcosis in both marine and freshwater species such as yellowtail, rainbow trout and grey mullet (Akhlaghi and Keshavarzi, 2002; Chen *et al.*, 2002; Vendrell *et al.*, 2006). It is also infectious on human urinary tract, blood and skin (Elliot *et al.*, 1991).

It has been recognized that total contents of single herb could show more prominent effect than a single active ingredient or constituent in herb (Nahrstedt and Butterweck, 2010). It was hypothesized that the TCM possessed good antimicrobial activity which would defer the development of drug resistance in fish pathogens, and is therefore an ideal alternative to antibiotics. The major objectives of this experiment were to 1) screen TCMs to identify which one(s) possess strong antimicrobial activity; 2) investigate the antimicrobial activities of TCM extracts in different solvents; 3) reveal the combined effects of selected aqueous TCM extracts; and 4) investigate the development of drug resistant pathogens exposed to TCM extracts, compared with that of antibiotics.

5.2 Methods and Materials

5.2.1 Antimicrobial activity of boiled aqueous extracts of 17 TCMs

Seventeen Traditional Chinese Medicines (TCMs) *Rhizoma coptidis, Radix astragali, Herba andrographis, Herba houttuyniae, Radix scutellariae, Radicis angelicae sinensis, Astemisia capillaries, Cnidium monnieri, Radix isatidis, Folium isatidis, Radix glycyrrhizae, Rhizoma rhei, Cortex phellodendri, Semen sinapis, Fructus forsythiae, Fructus gardeniae jasminoidis* and *Radix sophorae flavescentis* were obtained from and identified by the School of Chinese Medicine, Hong Kong Baptist University were tested for their antimicrobial activity of TCM extracts on various bacteria as stated below. The herbs were dried at 40ºC for 24 h and then pulverized to powder using a mechanical blender.

In the present experiment, 3 species of gram-positive (*Lactococcus garvieae*, *Staphylococcus aureus* and *Enterococcus faecalis*) and 4 species of gram-negative bacteria (*Escherichia coli*, *Aeromonas hydrophila, Vibrio cholerae* and *Serratia marcescens*) were involved. The minimum inhibitory concentrations (MICs) of TCM extracts on 7 species of bacteria, ATCC 25922 *E. coli*, ATCC 35548 *S. aureus*, ATCC 29212 *E. faecalis, L. garvieae, V. cholera,* ATCC 43861 *S. marcescens* and *A. hydrophila* were determined by micro-dilution method (NCCLS, 2001). *L. garvieae* and *V. cholerae* were isolated from liver of diseased grey mullet, from the fish farm in Yuen Long, Hong Kong and *A. hydrophila* from the intestine of diseased grass carp in Institute of Hydrobiology, Jinan University, Guangzhou. The *A. hydrophila* and *L. garvieae* was identified by API 20NE kit and API 32 Strep kit, the *V. cholerae* was identified by 16SrDNA analysis. Three laboratory strains of bacteria, ATCC 35548 *S. aureus*, ATCC 29212 *E. faecalis*and ATCC 43861 *S. marcescens* are also common pathogens for aquatic diseases, while ATCC 25922 *E. coli* was used as the control for the bacteria testings.

The bacteria were cultured from -80 ºC bacteria stock in Mueller-Hinton Broth at 37ºC for 18h (except *S. marcescens* and *A. hydrophila* at 28 ºC). The overnight culture was spread on Mueller-Hinton Agar for several generations and then single colony was re-transferred from agar plate into Mueller-Hinton broth and cultured at optimum temperature (37 or 28 ºC) for 18 h. The cultures were centrifuged at 850G for 15 min and the supernatant removed. The bacteria pellets were washed with sterile saline with the turbidity of suspension adjusted with saline to ~0.13 absorbance at 625nm

spectrophotometrically. The bacteria were diluted by MHB and the final concentration of bacteria in the well was about 5×10^5 cfu/ml.

The powdered TCM was extracted by boiling in deionized water. The powder was then soaked for 30 min in deionized water (1:10 w/v), and boiled for 30 min. The extract was removed and another portion of deionized water was added and boiled for another 30 min. The extracts were pooled then filtered with Whatman No. 1 filter paper by suction filtration and were dried at 50°C. The dried extracts were then kept at desiccators for 12 h before recording the weight. The extracts yield (%) was calculated as follows:

(Mass of the dried extract / mass of the ground plant sample) x 100%

The dried extracts were then redissolved in 50% ethanol to final concentration (328 mg/mL) and were stored at -20 ºC.

Two antibiotics chloramphenicol (0.0625 to 64 μg/mL) and streptomycin (0.125 to 256 μg/mL) were used as positive controls. The tested concentrations of TCM extracts were fixed at 0.04 to 40.96 mg/mL, as MIC values within 1 to 8 mg/mL were generally considered as possessing antimicrobial activity (Fabry *et al.*, 1998; Gibbons, 2004; Rios and Recio, 2005). The powder of chloramphenicol (Sigma, Aldrich) and streptomycin (Sigma, Aldrich) was weighed and dissolved in ethanol and sterile deionized water at the appropriate concentration (10 fold of highest tested concentration) respectively, and kept at -20ºC as stock solution.

100 μL of each TCM extract (81.92 mg/mL) was serially diluted two-fold with

sterile deionized water in the well. 100 µL of bacteria culture (~5 x 10^5 cfu/ml) in MHB was added. 2, 3, 5-Triphenyltetrazolium chloride was used for indicating the bacteria growth (Lee *et al.*, 2007), pink solution indicated the growth of bacteria and the well at the lowest concentration with no sign of growth was recorded as minimum inhibition concentration (MIC). 10% Ethanol (concentration of ethanol in diluted TCM extracts) and sterile deionsied were included as solvent control to test whether any inhibitory effect on the growth of tested bacteria. Growth control without TCM extracts or antibiotics was included to confirm that the growth of bacteria. The negative controls with TCM extracts or antibiotics with sterile broth were included to confirm the prepared solutions were not contaminated.

5.2.2 Antimicrobial activity of selected aqueous and organic TCMs extracts

TCMs were extracted by sonication bath in various solvents: hexane, dichloromethane (DCM), 90% ethanol (EtOH) and deionized water (20ml/g) using the procedure described by Ncube *et al.*, (2012) with modifications. The extraction was repeated 3 times and each sonciation lasted for 45 min. The crude extracts were pooled and then filtered with Whatman No. 1 filter paper by suction filtration. The organic extracts were concentrated by a rotary evaporator below 50ºC and then dried at room temperature (~23ºC) under a stream of air until compete dryness. Water extracts were freeze-dried and kept in airtight containers. The TCM was also extracted by boiling in deionized water for comparing antimicrobial activity of water extracts. The extraction

procedures and the calculation of extract yield of each solvent (%) were the same as stated in Section 5.2.1. The dried extracts were then redissolved in 50% ethanol to final concentration (328 mg/mL) and stored at -20ºC. The minimum inhibitory concentrations (MICs) of each extraction on 7 species of bacteria were determined by microdilution method as mentioned in Section 5.2.1. The ratio of MICs between non-boiled and boiled water extracts (results from this section and Section 5.2.1) were calculated.

5.2.3 Checkerboard method for the combined effect of *C. phellodendri, R. scutellaria, R. coptidis* and *F. forsythiae*

The four herbs (*C. phellodendri, R. scutellaria, R. coptidis* and *F. forsythiae*)that showed strongest bacterial inhibition in Section 5.2.1 were selected for investigating the combined effects of the boiled aqueous extracts at equal ratio. MIC values were determined for each of these combinations to establish any interaction effects following Section 5.2.1. The fractional inhibitory concentration (FIC) indices were calculated to evaluate the interaction of two extracts, but not the effects of mixtures with more than two extracts. The concentration of each tested extracts in combination is expressed as a fraction of the concentration that produced the same effect when used independently (Berenbaum, 1978). The FIC was calculated as the MIC of the combination divided by the MIC of each individual component extract. The FIC index was the sum of each extract FIC in a combination. Synergistic (≤0.5), additive (>0.5–1.0), indifferent (1–4.0) or antagonistic (≥4.0) were interpreted based on the value of FIC index (Schelz *et al.*,

2006).

5.2.4 Development of drug resistant fish pathogens exposed to selected aqueous TCM extracts

The aqueous extract was used to investigate the development of resistant fish pathogens as it is the traditional practice and the most common form prepared in homesteads. Three fish pathogens, *L. garvieae, V. cholerae* and *A. hydrophila* were cultured in three of the most inhibiting TCM extracts (i.e. with lowest MIC values) respectively at sub MIC (half of MIC), with *E. coli* included as control (Section 5.2.1). The boiled aqueous extracts used were *R. coptidis, R. Scutellariae, F. forsythiae* and *C. Phellodendri.*

The changes of MIC in 21 consecutive days of experiment were investigated by the modified method of Clark *et al.* (2011). The development of resistant fish pathogens on two antibiotics, Chloramphenicol and Streptomycin were also investigated. In brief, the concentrations of TCM extracts applied on the bacteria species were based on the results obtained in Section 5.2.1. The bacteria were cultured in 200 mL of Mueller-Hinton broth with half of the MICs (Table 5.1) as the initial dose. The MIC of TCM extracts on the bacteria was tested using the same micro dilution method (Section 5.2.1) at Passage 1, 4, 7, 14 and 21. The subculture doses of antibiotics or TCM extracts were adjusted until a significant increase of MIC (>4-fold) was observed, compared to the initial or previous subculture doses. The MIC of TCM extracts of each bacteria species was tested again

after 7 further passages (Passage 28), without antibiotics or TCM extracts.

5.3 Results

5.3.1 Antimicrobial activity of boiled aqueous extracts of 17 TCMs

The extract yields of TCMs in boiled extracts ranged from 9.52 (*S. sinapis*) to 50.26% (*R. scutellaria*). Fourteen out of seventeen water boiled TCM extracts showed inhibition on 7 species of bacteria (Table 5.1), no MICs were determined in the tested concentrations (0.04-40.96 mg/mL) for the 3 speices: *S. sinapis, F. isatidis* and *R. isatidis*. In this study, MICs values equal or below 1.28 mg/mL were considered as possessing strong antimicrobial activity (highlighted in bolded in Table 5.1) and MICs values equal or below 1.28 mg/mL. *C. phellodendri* and *R. coptidis* demonstrated the strongest antimicrobial activities, with a broad range of inhibition on both gram-positive and -negative bacteria. Both extracts also showed strong antimicrobial activity on the two fish pathogens, *L. garvieae* and *V. cholreae.*

S. aureus was the most susceptible pathogen to TCM extracts, inhibited (MICs ≤10.24 mg/mL) by 64.7%, 11 out of the 17 tested TCMs, while *S. marcescens* was the most tolerant bacteria species (0%), the only recorded MIC was 20.48 mg/mL of *F. forsythiae*. Based on the results, four TCMs, *C. phellodendri*, *R. scutellaria*, *R. coptidis* and *F. forsythiae* possessed strong antimicrobial activities and were selected for further studies on antimicrobial activity.

Table 5.1 Antimicrobial activity screening (mg/mL) using boiled aqueous extracts of 17 Traditional Chinese Medicines (TCMs)

Herbs	Extract yield (%)	Bacteria strains						
		Vc	Lg	Ah	Sa	Ef	Sm	Ec
R. scutellaria	50.26	5.12	10.24	2.56	**1.28**	*	*	40.96
F. forsythiae	12.21	5.12	10.24	10.24	10.24	5.12	20.48	10.24
F. gardeniae jasminoidis	23.11	20.48	*	*	5.12	40.96	*	40.96
C. phellodendri	12.47	**1.28**	**0.64**	20.48	10.24	**1.28**	*	*
R. coptidis	17.55	**0.08**	**0.08**	**0.32**	**1.28**	2.56	*	**1.28**
H. andrographis	16.99	10.24	20.48	10.24	10.24	20.48	*	*
H. houttuyniae	10.53	20.48	*	*	5.12	*	*	*
F. lonicerae japonicae	27.83	20.48	*	*	10.24	*	*	*
R. astragali	40.17	*	40.96	*	*	*	*	*
F. cnidii	12.89	10.24	*	*	5.12	*	*	*
R. glycyrrhizae	19.48	20.48	10.24	*	2.56	*	*	*
R. angelicae sinensis	60.71	40.96	*	*	40.96	*	*	*
S. sinapis	9.52	*	*	*	*	*	*	*
R. sophorae flavescentis	22.07	20.48	*	*	5.12	*	*	*
F. isatidis	14.37	*	*	*	*	*	*	*
H. astemisia capillaris	17.97	20.48	*	*	*	*	*	*
R. isatidis	12.04	*	*	*	*	*	*	*
Chloramphenicol (μg/mL)	/	0.5	4	1	8	8	16	4
Streptomycin (μg/mL)	/	8	16	2	4	16	4-8	2

Ah= *Aeromonas hydrophila*, **Vc**= *Vibrio cholerae*, **Lg**= *Lactococcus garvieae*, **Ec**= ATCC 25922 *Escherichia coli*, **Sa**= ATCC 35548 *Staphylococcus aureus*, **Ef**= ATCC 29212 *Enterococcus faecalis*, **Sm**= ATCC 43861 *Serratia marcescens*

* No minimum inhibition concentration (MIC) was found in tested concentrations (0.04-40.96 mg/mL)

5.3.2 Antimicrobial activity of aqueous (boiled and non-boiled) and organic extracts of 4 selected TCMs

Higher extract yields in aqueous extracts (6.51-50.76%) than organic solvents (0.30-8.00%) were generally observed, especially *R. coptidis* and *R. scutellaria* (Table 5.2). The yields in non-boiled extracts were also higher than boiled aqueous extracts, except *C. phellodendri* which possessed more non-polar content and strongly inhibited *S. marcescens* (0.16 mg/mL). The aqueous, ethanol and DCM extracts of *R. coptidis* and *R. scutellaria* possessed similar antimicrobial abilities to the three field isolated pathogens: *V. cholerae, L. garvieae* and *A. hydrophila*, e.g. the aqueous, ethanol and DCM extracts of *R. coptidis* on *L. garvieae* were 0.08 to 0.16 mg/mL. However, these ethanol and DCM extracts generally possessed higher antimicrobial activities to the other four bacteria species: *S. marcescens, E. faecalis, S. aureus* and *E. coli*, compared to aqueous extract. The DCM and ethanol extracts of *R. coptidis* on *L. garvieae* were 0.16 and 0.32 mg/mL respectively, compared to 2.56 mg/mL of aqueous extracts. Among the four selected extracts, *F. forsythiae* and *R. coptidis* possessed the weakest and strongest antimicrobial ability, respectively.

Table 5.2 Antimicrobial activities (mg/mL) of different solvent extracts of *Radix scutellaria, Rhizoma coptidis, Cortex phellodendri and Fructus forsythiae*

Herbs	Solvent #	Yield (%)	MIC^ on different bacteria strains						
			Vc	Lg	Ah	Sa	Ef	Sm	Ec
R. scutellaria	**H**	0.73	**0.32**	**0.64**	**0.64**	**0.16**	**1.28**	2.56	2.56
	D	0.78	**0.16**	**0.32**	**0.32**	**0.16**	**0.16**	2.56	2.56
	E	6.24	**0.64**	**0.64**	**0.64**	**0.64**	**0.64**	2.56	2.56
	Wn	50.73	**1.28**	**1.28**	**1.28**	2.56	10.24	20.48	20.48
	Wb	50.26	5.12	10.24	2.56	**1.28**	*	*	*
F. forsythiae	**H**	1.64	2.56	**1.28**	2.56	2.56	**1.28**	**1.28**	5.12
	D	7.08	5.12	5.12	2.56	2.56	5.12	10.24	5.12
	E	8.00	2.56	2.56	5.12	2.56	5.12	5.12	10.24
	Wn	9.70	5.12	2.56	2.56	5.12	2.56	5.12	5.12
	Wb	6.51	2.56	5.12	5.12	5.12	2.56	5.12	5.12
C. phellodendri	**H**	4.96	5.12	2.56	5.12	**0.16**	**1.28**	5.12	5.12
	D	7.08	2.56	2.56	5.12	**0.64**	2.56	**1.28**	5.12
	E	6.94	2.56	2.56	2.56	**0.32**	**1.28**	2.56	**1.28**
	Wn	9.69	2.56	**0.64**	2.56	5.12	2.56	10.24	5.12
	Wb	12.47	**1.28**	**0.64**	20.48	10.24	**1.28**	*	*
R. coptidis	**H**	0.30	**1.28**	**0.64**	**1.28**	2.56	**0.64**	2.56	2.56
	D	0.93	**0.64**	**0.08**	**0.64**	**0.16**	**0.08**	**1.28**	**0.64**
	E	1.89	**0.32**	**0.08**	**0.32**	**0.32**	**0.64**	**0.64**	**0.32**
	Wn	24.56	**0.32**	**0.16**	**1.28**	2.56	**0.64**	10.24	2.56
	Wb	17.55	**0.08**	**0.08**	**0.32**	2.56	5.12	*	2.56
Chloramphenicol			1	2	0.5	8	4	16	4
Streptomycin			8	4	4	2	8	2	4

Ah= *Aeromonas hydrophila*, **Vc**= *Vibrio cholerae*, **Lg**= *Lactococcus garvieae*, **Ec**= ATCC 25922 *Escherichia coli*, **Sa**= ATCC 35548 *Staphylococcus aureus*, **Ef**= ATCC 29212 *Enterococcus faecalis*, **Sm**= ATCC 43861 *Serratia marcescens*

^: MIC unit of TCM extracts (mg/mL) and antibiotics (μg/mL)

#: Wn= non boiled water extract, Wb= boiled water extracts, D=dichloromethane, E=ethanol, H=hexane

Solvent polarity index: Water 9.0, ethanol 5.2, dichloromethane 3.1, hexane 0.0

In comparison, antimicrobial abilities varied in non-boiled and boiled aqueous extracts among 7 bacteria, only 3 out of 21 extracts showed a lower ratio (ratio of MIC between Non-boiled and boiled aqueous extracts below 0.5) and 2 extracts showed a higher ratio (>2) (Table 5.3). Lower ratios were observed in *R. scutellaria* on *V. cholerae* and *L. garvieae* and *R. coptidis* on *E. faecalis* while higher ratios were found in *R. coptidis* on *V. cholerae* and *A. hydrophila*.

5.3.3 Antimicrobial activities of boiled aqueous mixtures of *C. phellodendri, R. scutellaria, R. coptidis* and *F. forsythiae*

The FIC indices were generally lager than 1, indicated the mixed TCM extracts possessed weaker antimicrobial activities than that involved only single herbs, and therefore antagonistic effects on antimicrobial activities were found in the combinations of these four TCMs (Table 5.4). For example, the MIC of *R. scutellaria* and *R. coptidis* mixture was 0.64 mg/mL (FIC index=2.25) to *A. hydrophila*, compared to 2.56 (*R. scutellaria*) and 0.32 (*R. coptidis*) mg/mL. Additive interactions (FIC index >0.5-1.0) were found in combinations of *R. scutellaria* and *F. forsythiae* on *E. coli* and *A. hydrophila* (FIC index =0.63) and *R. scutellaria* and *C. phellodendri* on *A. hydrophila* (FIC index =0.56). Combination of *C. phellodendri* and *F. forsythiae* showed synergistic effect (FIC index ≤0.5) on the inhibition on *A. hydrophila* (FIC index =0.38), further

enhancement on inhibition observed when added with *R. scutellaria* (FIC index =0.34). Various antagonistic effects on antimicrobial activities (FIC index ≥4.0) were observed in the combinations which contained *R. coptidis.*

5.3.4 Development of drug resistant fish pathogens exposed to selected TCM extracts

In general, development of drug resistant bacteria to TCM extracts was not found, with the exception of *L. garvieae* exposed to *C. phellodendri* and *E. coli* exposed to *R. coptidis*, with 4 folds and 8-16 folds increases of MICs were found after 21 passages respectively, but all of the MICs fell back to original values after 7 extracts free passages (Table 5.5). Remarkable increases of MICs were observed in bacteria species treated with streptomycin, ranged from 16-128 folds, the increases were generally obvious after 14 passages. The MIC of *E. coli* maintained at 128 μg/mL to streptomycin after 7 passages without antibiotics. Less drug resistance was found in bacteria species exposed to chloramphenicol, only 2-8 folds increases were observed and MICs of each bacteria species returned near to the initial values after 7 passages without chloramphenicol.

Table 5.3 Ratio of MIC between non-boiled and boiled aqueous extracts

Herbs	Ratio of MIC on different bacteria strains*						
	Vc	**Lg**	**Ah**	**Sa**	**Ef**	**Sm**	**Ec**
R. scutellaria	**0.25**	**0.125**	0.5	2	**/**	/	/
F. forsythiae	2	0.5	0.5	1	1	1	1
C. phellodendri	2	1	**/**	0.5	2	/	/
R. coptidis	4	2	4	1	**0.125**	/	1

Ah= *Aeromonas hydrophila*, **Vc**= *Vibrio cholerae*, **Lg**= *Lactococcus garvieae*, **Ec**= ATCC 25922 *Escherichia coli*, **Sa**= ATCC 35548 *Staphylococcus aureus*, **Ef**= ATCC 29212 *Enterococcus faecalis*, **Sm**= ATCC 43861 *Serratia marcescens*

*: The values below 1 indicated lower MIC for non-boiled water extracts, the values below 0.5 were highlighted in bolded.

/: No numerical value was shown if MIC greater 10.24 mg/mL.

Table 5.4 The minimum inhibition concentration (MICs) fractional inhibitory concentration (FIC) indices of boiled aqueous mixtures of four Traditional Chinese Medicines (TCMs)

	MIC (mg/mL) and FIC indices of bacteria			
TCM combinations	***A. hydrophila***	***L. garvieae***	***V. cholerae***	***E. coli***
Rc Rs	0.64 (2.25)	1.28 (16.13)	1.28 (16.25)	2.56 (2.06)
Rc Cp	0.64 (2.03)	0.32 (4.50)	0.32 (4.25)	1.28 (2.00)
Rc Ff	0.64 (2.06)	0.32 (4.03)	0.64 (8.13)	1.28 (1.13)
Rs Cp	1.28 *(0.56)*	1.28 (2.13)	1.28 (1.25)	5.12 (4.13)
Rs Ff	1.28 *(0.63)*	5.12 (1.00)	1.28 (**0.50**)	5.12 *(0.63)*
Cp Ff	2.56 **(0.38)**	2.56 (4.25)	--	--
Rc Rs Ff	0.32	1.28	1.28	5.12
Rc Rs Cp	0.32	1.28	0.64	5.12
Rc Cp Ff	0.64	0.32	1.28	2.56
Rs Cp Ff	0.64	1.28	1.28	--
Rc Rs Cp Ff	0.64	0.64	0.64	--

Rs: *Radix scutellaria,* Rc: *Rhizoma coptidis,* Cp: *Cortex phellodendri,* Ff: *Fructus forsythiae*
a: Synergistic interactions (≤0.5) are printed in bold
b: Additive interactions(>0.5-1.0) are printed in italics
--. :Not tested as the MIC value of mixture >40.96 mg/mL, no FIC index was determined.

Table 5.5 Development of drug resistant fish pathogens after serial passages of *Radix scutellaria, Rhizoma coptidis, Cortex phellodendri and Fructus forsythiae*

Herbs	No. of passage	MIC [a] on different bacteria strains			
		Vc	Lg	Ah	Ec
R. scutellaria	**Control** [b]	5.12	10.24	2.56	40.96
	4	10.24	10.24	2.56	40.96
	7	5.12	10.24	5.12	40.96
	14	5.12	10.24	5.12	40.96
	21	5.12 (1)[d]	10.24 (1)	10.24 (4)	40.96 (1)
	28[c]	5.12 (1)	10.24 (1)	2.56 (1)	40.96 (1)
F. forsythiae	**Control**	--	--	10.24	10.24
	4	--	--	10.24	10.24
	7	--	--	10.24	20.48
	14	--	--	10.24	20.48
	21	--	--	20.48 (2)	20.48 (2)
	28	--	--	10.24 (1)	10.24 (1)
C. phellodendri	**Control**	1.28	0.64	--	--
	4	1.28	0.64	--	--
	7	1.28	1.28	--	--
	14	1.28	2.56	--	--
	21	1.28 (1)	2.56 (4)	--	--
	28	1.28 (1)	0.64 (1)	--	--
R. coptidis	**Control**	0.08	0.08-0.16	0.16-0.32	0.64
	4	0.08	0.16	0.16	0.64
	7	0.08	0.16	0.64	0.64
	14	0.08	0.16	0.64	10.24
	21	0.08 (1)	0.16 (1-2)	0.64 (2-4)	10.24 (16)
	28	0.08 (1)	0.16 (1-2)	0.16 (1)	0.64 (1)
Chloramphenicol	**Control**	0.5	2-4	1-2	4
	4	2	4	4	16
	7	1	4	2	16
	14	2	8	2	64
	21	2 (4)	16 (4-8)	4 (2-4)	64 (8)
	28	0.5 (1)	8 (2-4)	2 (2)	16 (4)
Streptomycin	**Control**	4-8	16	2-4	2-4
	4	8	32	16	2
	7	8	64	32	4
	14	128-256	128-256	64	128
	21	512 (64-128)	512 (32)	64 (16-32)	128 (32-64)
	28	32 (4-8)	64 (4)	16 (4-8)	128 (31-64)

a MIC unit for TCM extracts (mg/mL) and antibiotics (μg/mL) (-- Not Tested)

b Control: passages without antibiotics or TCM extracts

c Passages from No. 22-28 were without antibiotics or TCM extracts

d Fold of increase of MIC at 21 and 28 passages compared to control are shown in bracket

5.4 Discussion

5.4.1 Antimicrobial activities of boiled aqueous extracts of 17 TCMs

The present study results showed strong inhibiting effects on the growth of some fish pathogens which demonstrated potential uses of TCMs as antimicrobial agent for pathogens in aquaculture. MIC values below 8 mg/mL suggested that the TCM extracts possessed antimicrobial activity (Fabry *et al.*, 1998) and MIC values below 1 mg/mL are considered as possessing remarkable antimicrobial activity (Gibbons, 2004; Rios and Recio, 2005). In this study, due to the difference in tested concentrations, MICs equal to or below 10.24 mg/mL were regarded as possessing antimicrobial activities, and equal to or below 1.28 mg/mL as possessing strong activities.

Only 2 TCM extracts (*R. coptidis* and *C. phellodendri*) tested in this study showed strong inhibitions on the tested bacteria, *R. coptidis* exhibited strong antimicrobial activity on two common fish pathogens *V. cholerae* and *L. garvieae* (0.08 mg/mL) and also effective (MIC:0.32-1.28 mg/mL) on other bacteria e.g. *A. hydrophila*, *S. aureus* and *E. coli*. Berberine hydrochloride is the active component of *R. coptidis* and *C. phellodendri*, which showed strong antimicrobial activity to various fish pathogens: *A.hydrophila*, *Pseudomonas fluorescens*, *Vibrio harveyi*, *Edwardsiella ictaluri* and *Streptococcus dysgalactiae*, with MIC ranged from 0.1-0.5 mg/mL (Zhang *et al.*, 2010). Li *et al.* (2006) also found similar results, with *R. coptidis*, *R. scutellaria* and *C. phellodendri* exhibited stronger antimicrobial activity among 10 tested TCMs. *R. coptidis* extracts also inhibited *S. aureus* (Yu *et al.*, 2005) and *A. hydrophila* (Zhang and Yang

2006).

However, Rattanachaikunsopon and Phumkhachorn (2009) found that *Andrographis paniculata* possessed strong antimicrobial activity on *Streptococcus agalactiae* (31.25 µg/mL), while the present result did not showed weak antimicrobial activities on *H. andrographis* (leaf of *Andrographitis*) (MIC ≥10.24 mg/mL). A study showed that *Andrographitis* extracts had an inhibitory effect on *Bacillus cereus*, *E. coli* and *Pseudomonas aeruginosa* (Singha *et al.,* 2003), but no inhibition was revealed on *E. coli* in the present study. The inhibitory effect of *Andrographitis* extract is possibly due to the presence of both arabinogalactan proteins and andrographolides (Singha *et al.,* 2003). The difference in inhibitory effects on bacteria varied according to the types of solvent such as methanol, chloroform and water for extractions and bacteria strains. Similar variations of bacterial inhibitions exposed to TCM extracts were also found in a recent study (Philip *et al.*, 2009), with different active compounds dissolved in different solvents based on different extraction methods.

5.4.2. Antimicrobial activities of organic and aqueous extracts of *R. coptidis* and *R. scutellaria, C. phellodendri* and *F. forsythiae*

In general, the present results showed that the organic extracts such as ethanol and DCM possessed stronger antimicrobial activities on bacteria than aqueous extracts, especially in *R. scutellaria*. Voravuthikunchai *et al.* (2006) and Mulaudzi *et al.* (2011) also reported the organic extracts (DCM, methanol and ethanol) of medicinal plants

possessed higher antimicrobial activity than aqueous extracts.

In terms of the solvent polarity, stronger antimicrobial activities were found in DCM and ethanol extracts (polarity index =3.1 and 5.2 respectively) of *R. coptidis* and *R. scutellaria* than hexane extracts, except a prominent antimicrobial activity was found in hexane extracts (polarity index =0) (0.16 mg/mL) of *C. phellodendri* on *S. aureus*. This result was contradictory to other findings that non-polar extracts (petroleum ether and DCM) usually demonstrated stronger activity than polar extracts (e.g. ethanol and water) (McGaw *et al.*, 2001; Ncube *et al.*, 2012). Gram-positive bacteria i.e. *S. aureus, L. gariveae* and *E. faecalis* did not possess outer membrane as gram-negative bacteria. The outer membrane could regulate the entry of hydrophobic substance into the cell; therefore gram positive bacteria are more susceptible to antimicrobial substance especially non-polar contents, resulted in the distortions of the electron flow, proton motive force, cytoplasmic membrane, and coagulation of cell contents (Burt, 2004). Most relevant studies found that gram-positive bacteria are more susceptible to TCM extracts than gram-negative bacteria (Lopez *et al.*, 2005, Shan *et al.*, 2007), but the present experiment did not show such observations. Although *S. marcescens* (gram-negative) and *S. aureus* (gram-positive) were the most tolerant and sensitive species which were inhibited by 0% and 64.7% of 17 types of TCMs respectively, other gram-positive bacteria, *L. garvieae* (29.4%) and *E. faecalis* (17.6%) showed similar susceptibilities as *V. cholerae* (35.3%) and *A. hydrophila* (23.5%). Further antimicrobial studies of these 17 types of TCMs on more bacteria species should be investigated for concluding the susceptibility of gram-

positive and -negative bacteria.

The heating during extractions may affect the antimicrobial activities of TCMs, but the effects could be varied and would be depended on the bacteria and herb species. Non-boiled aqueous extracts showed stronger inhibition compared to boiled ones, e.g., lower MICs were found in the non-boiled extracts of *R. coptidis* on *E. faecalis* and *R. scutellaria* on *V. cholerae* and *L. gariveae*. This indicated heating during extractions may impair the antimicrobial actions of TCMs. Herbal extracts contained various groups of chemicals (such as tannins, saponins, flavonoids, alkaloids, phenols, glycosides) which can be photo-degraded when exposed to light (Oyi *et al.*, 2007). Heating could reduce the bioavailability and hence antimicrobial activity of active components such as saponins and tannins in aqueous extracts (Bolaji *et al.*, 1997). On the contrary, stronger antimicrobial activities were observed in heated aqueous extracts found in *R. coptidis* on *V. cholerae* and *A. hydrophila*. The extraction methods could affect the antimicrobial ability of TCM extracts, but the present results suggested the antimicrobial activities of heated and non-heated extracts could be related to the specific interactions between chemicals and bacteria. Further studies should be performed on the mechanisms of inhibition of various pathogens by TCM extracts.

5.4.3 Development of resistant bacteria to single TCMs and antimicrobial activities of mixed TCM extracts

In the study on the development of resistant bacteria to TCMs, MICs of *V. cholerae*,

L. gariveae and *A. hydrophila* against TCM extracts did not increase dramatically (<4 folds). Although 8-16 folds increase of MICs were found in *E. coli* against *R. coptidis*, the MICs returned to the original level (0.64 mg/mL) after 7 passages without TCM extract. Similar trends were also observed in the treatment of *R. scutellaria* with *A. hydrophila* and *C. phellodendri* with *L. garvieae*, which was completely different from pathogens against streptomycin. Pathogens treated with streptomycin developed tough drug resistance and high degree of resistances (≥4 folds MIC of original values) persisted after drug free passages. The results indicated drug resistance was less easily developed and retained in bacteria treating with TCM extracts than antibiotics. The results were in line with findings of Meng *et al.* (2003) who studied *R. coptidis* and claimed that it was probably due to the multiple actions of TCM extracts to inhibit bacteria.

Synergistic effects between selected TCMs were only found in 2 out of 22 combinations in 4 bacteria species in this study. *F. forsythiae* mixed with *C. phellodendri* exerted a stronger inhibition on *A. hydrophila* (MIC= 2.56mg/mL, FIC index = 0.38) than single herbs. The combination of *F. forsythiae* and *R. scutellaria* also showed a stronger inhibition on *V. cholerae* (MIC= 1.28mg/mL, FIC index = 0.50). It is surprising that those three TCMs showed weak or no inhibition on *A. hydrophila* when on their own (MICs ranged from 2.56-20.48 mg/mL). Synergistic antimicrobial action of TCM mixture may occur in the presence of some substances with low antimicrobial activity. Stermitz *et al.* (2000) showed that antimicrobial activity of berberine was greatly enhanced in the presence of 5'-methoxyhydnocarpin (5'-MHC), a compound isolated from chaulmoogra

oil and an inhibitor of multidrug resistance (MDR) pumps of *S. aureus*. MDR pump is the common way of drug resistance in both gram-positive and gram-negative bacteria which could block the entry of antimicrobial agent into the bacteria (Li and Nikaido, 2004; Poole, 2007). Similar effects were also noted in berberine bounded with 5-nitro-2-phenylindole (INF-55), an inhibitor of MDR pump on inhibiting *S. aureus* and *E. faecalis* (Ball *et al.*, 2006; Tomkiewicz *et al.*, 2010). In South Africa, van Vuuren and Viljoen (2008) also reported synergistic effects of the combined of traditional herb extracts on inhibiting *Bacillus* cereus, *Candida albicans* and *Cryptococcus neoformans*. These studies indicated the presence of multiple ingredients in herbs which may be responsible for the defense against the drug resistance development. However, the synergistic effects in combined TCM extracts should not be always assumed, combinations with antagonistic effects are commonly found in different studies, including the present one (Ndhlala *et al.*, 2009; Ncube *et al.*, 2012). Investigations on antimicrobial agents from mixed herbal extracts could be ineffectual if only focused on the combinations of herbs with strong antimicrobial activities.

Some TCMs that have low antimicrobial ability might enhance the inhibition on bacteria when combined, e.g. combinations of *C. phellodendri*, *F. forsythiae* and *R. scutellaria*. It is possible to investigate new antimicrobial agents by combining different TCMs, and not only focusing on herbs possessing strong antimicrobial activities. However, it is time consuming to study in depth the synergistic effect between copious types of herbs and the result is not guaranteed. A more efficacious step would be on

synergistic effects between different solvent extracts from single herb which is beneficial to combination chemotherapy to treat infectious diseases (Ncube *et al.*, 2012).

TCMs can serve as antimicrobial agents, it also can act as immunostimulants, as well as growth promoters and potential alternative to vaccines to fish (Anderson, 1992; Secombes, 1994). The herbal medicine can be effective on enhancing fish disease resistance through oral administration (Sharma *et al.* 2010) or injection (Wu *et al.*, 2010), and this environmental friendly drug should be further investigated for actual application in aquaculture, on a large scale.

5.5 Conclusion

The hypothesis was accepted that the TCMs (*R. coptidis R. scutellaria* and *C. phellodendri*) generally deffered the development of drug resistance pathogens, compared to the tested antibiotics. To conclude, the results of this study provided significant information on using TCMs as alternatives to antibiotics in aquaculture. *R. coptidis* seems to be superior with strong inhibitions to fish pathogens e.g. *V. cholerae* and *L. garvieae* but with less drug resistance developed. The antimicrobial activities of TCM extracts varied in different solvents with ethanol and DCM extracts (especially *R. scutellaria*) possessed stronger antimicrobial activities on bacteria than aqueous extracts. Besides, *R. coptidis* did not show any synergetic effect on antimicrobial activity when combined with other TCMs, only two combinations of TCM extracts: *F. forsythiae and C. phellodendri* on A. hydrophila, and *F. forsythiae and R. scutellaria* on *V. cholerae* showed synergetic

effects (FIC index ≤0.5). Application of TCMs may ease the problem of the abuse of antibiotics and other undesirable chemicals used in aquaculture.

Chapter 6

Effects of Traditional Chinese Medicines (TCM) on the Immune Response of Grass Carp (*Ctenopharyngodon idellus*)

6.1 Introduction

Grass carp is the second largest production (after silver carp) in freshwater aquaculture worldwide, which is more than 3 million tonnes, and China contributed 95.7% of the global production in 2002 (FAO, 2009). Enteritis is one of the most common diseases in grass carp, especially for young fish, with mortality around 50% - 90% (Yang, 2008). *Aeromonas* spp in the intestine proliferated rapidly and caused dysfunction of capillaries on the intestine and released endotoxin leading to septicemia, the major symptom of enteritis (Xu *et al.,* 1988). Due to the high stocking density, fish diseases have a crucial effect on aquaculture yield. Antibiotics are commonly used to control and treat diseases in aquaculture, with sulphaguanidine and furazolidone commonly used for treating enteritis in grass carp (FAO, 2009).

The frequent occurrence of antibiotic resistant bacteria near aquaculture site is probably due to the abuse of veterinary antibiotics and sub-therapeutic doses applied in aquaculture (Smith *et al.,* 2002). As a consequence, more and more antibiotics resistant fish pathogens have been detected around aquaculture sites (Furushita *et al.,* 2005; Cabello, 2006; Sørum, 2006). Terrestrial veterinary pathogens and even human pathogens may gain antibiotic resistance determinants through horizontal gene

transmission from bacteria in the aquaculture environment (Angulo and Griffin, 2000; Heuer *et al.*, 2009). Due to the various threatening effects of antibiotics, the European Union has banned its use as feed additives in food-producing animals after January 2006. Other feed supplements which are environmentally safe and cost-effective should be used, with herbal medicines one of the popular options.

The possible use of Traditional Chinese Medicine (TCM) for aquaculture has been explored in recent years, Direkbusarakom (2004) confirmed that the usage plays an important role in the Asian aquaculture industry. Herbs are known to exert positive effects on growth, combat viral infection, stimulate appetite and relief stress (Francis *et al.*, 2005; Citarasu *et al.*, 2006; Venketramalingam *et al.*, 2007). Therefore the use of TCMs may greatly reduce the disease outbreak in aquaculture production, and replace the use of antibiotics as an effective measure for disease control.

The principle of TCM is quite different from the western pharmacological and therapeutic principles which target on diseases or pathogens directly. The rationale of TCM is mainly based on the theories, such as the five elements theory and the Yin-Yang balance, which are considering the overall balance in the human body (Cheng, 2000). In other words, TCM is aimed at maintaining and restoring the balance or enhancing the host immunity to defend diseases.

The medicinal values of TCMs in fish have been studied, but they mainly focused on the non-specific immune responses and disease resistances of fish to bacterial infections. Rohu (*Labeo rohita*) fed with *Achyranthus* showed enhanced superoxide

anion production level, lysozyme and serum bactericidal activity (Rao *et al.*, 2006). Mortalities in TCM fed fish due to bacterial infections e.g. *Aeromonas hydrophila* in Nile tilapia (*Oreochromis niloticus*) and common carp (*Cyprinus carpio*) (Ardó *et al.*, 2008; Yin *et al.*, 2009) and *Vibrio harveyi* in grouper (*Epinephelus tauvina*) (Punitha *et al.*, 2008) were significantly reduced. Better disease resistance was shown in different studies using herbal mixture rather than single herb. Abasali and Mohamad (2010) showed the formulation containing *Ocimum basilicum*, *Cinnamomum zeylanicum*, *Jugpans regia* and *Mentha piperita* enhanced the non-specific immunity and disease resistance to *A. hydrophila* in common carp. In general, herbal medicine seems able to exert antimicrobial and immuno-stimulatory effects in the treated fish.

Based on TCM theories, TCM compound formulations involved complex interactions between drugs and may exert better effects on immune-stimulation or improvement on disease resistance of fish. However, there is a lack of information on the use of compound formulation containing more than three types of TCM as feed supplements. Besides, there is limited research investigating the feasibility of its application in real aquaculture practice, especially on its cost effectiveness. It was hypothesized that the TCM formulation possess stimulatory effects on grass carp immunity and would be cost effective in preventing *Aeromonas* infection in grass carp. The TCM extract mixture was also expected to be more effective on activating plasma bactericidal activity than single herb.

In this study, a compound formulation of TCMs, in a ratio of 1:1:2:3 with *Rhizoma coptidis*: *Radix scutellaria*: *Herba andrographis: Radix sophorae flavescentis* was concocted. Two herbs (*R. coptidis* and *R. scutellaria*) which showed strong antibacterial activities on *A. hydriphila* (Chapter 5) were combined with another two herbs (*Herba andrographis: Radix sophorae flavescentis*) which showed immuno-stimulating properties or enhanced disease resistance (Li *et al.*, 2005; Rattanachaikunsopon and Phumkhachorn, 2009). The effects of this formulation (in a form of TCM supplemented fish feed) on immune parameters of grass carp i.e. oxidative radical production in blood (Nitroblue Tetrazolium (NBT) assay), total protein and total immunoglobulin and bactericidal activity in plasma were investigated. Finally, the cost effectiveness of applying the TCM feed in aquaculture was evaluated. The major objectives of this study were to 1) investigate the effects of the TCM formulation on grass carp immunity and disease resistance; 2) evaluate its effectiveness in aquaculture both biologically and economically via laboratory experiments and field trial; and 3) investigate the *in vitro* effects of single and combined TCM extracts on plasma bactericidal activity.

6.2 Materials and Methods

6.2.1 Experimental fish feed preparation

Traditional Chinese Medicines *Rhizoma coptidis, Radix scutellaria, Herba andrographis* and *Radix sophorae flavescentis* were obtained from the School of Chinese

Medicine, Hong Kong Baptist University for all the experiments on TCMs. The herbs were dried at 30ºC for 24 h and then pulverized to powder using a mechanical blender. The powdered herbal medicines were mixed according to the following ratio: *Rhizoma coptidis*: *Radix scutellaria*: *Herba andrographis*: *Radix Sophorae flavescentis* = 1:1:2:3. 0.5, 1 and 2% w/w of TCM powder (1:10 w/v, TCM powder: water) was boiled for 30 min with 200 mL of deionized water and the aqueous extracts were filtered through Whatmen No.1 filter paper. The TCM residues were boiled with another 200 mL of deionized water. The extracts and the TCM residues were pooled and mixed with powdered commercial grass carp feed thoroughly. The commercial fish feed used was Jinfeng®, 601 Grass carp formulated feed, with 33.7 % protein, 4.2% crude fat and 7.7% ash. Deionized water was added and the fish feed dough was pelletized with a meat grinder and dried at 50 ˚C for 24 h. The fish feed containing no TCM was used as the control.

6.2.2 Identifications of TCMs

The four selected TCMs were verified based on morphological, microscopic and thin-layer chromatography (TLC) indentifications by the School of Chinese Medicine of Hong Kong Baptist University. The morphological and microscopic identifications validated the plants species of TCMs, according to the procedure listed in the Hong Kong Chinese Materia Medica Standards (HKCMMS) Volume III (Department of Health, HKSAR). The TLC identification was conducted using standard solutions of

corresponding herbs, the TLC plates were observed under UV light (254nm) and visible light (The State Parmacopoeia Commission of PR China, 2005). The corresponding components in TCMs are identified and lised in Table 6.1.

6.2.3 Fish feeding experiment and blood sampling

Healthy grass carp fingerlings (27.1± 4.2 g) were bought from a fish farm in Hong Kong, and placed in 12 tanks (65 L) with 20 fish per tank, and the remaining fish were cultured in 3 stock tanks (~200L). All the fish tanks were continuously aerated with the water temperature maintained at 22±2^{o}C. The water temperature, values of pH and dissolved oxygen were measured three times a week using a portable Hanna pH meter and a YSI digital Dissolved Oxygen (DO) meter and the values were 21.9±1.7°C, 6.5±0.28 and 6.7±0.48 mg/mL.The fish were fed with experimental control feed, by 1% of body weight (g) per meal and two meals per day.

The fish were acclimatized for 2 weeks and adapted to the experimental control feed, the experimental fish were evaluated by a careful examination of physical appearance and behavior (e.g. rapid responses to light, and active feeding behavior) and showing no sign of infection (body lesion, scratching, lethargy). After 2 weeks acclimation in laboratory condition, the fish were redistributed into the 12 tanks (20 fish per tank, 4 treatments in triplicates) randomly. Dead fish were discarded and replaced by qualified fish from the stock tanks, which were acclimated for further 2 weeks (without any mortality during this period) before start of experiment.

Table 6.1 The major chemical components and their medical values of *Rhizoma coptidis, Radix scutellaria, Herba andrographis* and *Radix sophorae flavescentis* identified by Thin-layer Chromatography (TLC)

Herbs	The chemical components (Rf value)*	Medical values of herbs
Rhizoma coptidis	Berberine chloride (0.41) and Palmatine chloride (0.27)	Berberine reduced the mRNA expression level of inflammation factors (Choi *et al*., 2006); this herb showed effects on treating diarrhea, deintoxication, anti-inflammatory (Liu & Ng, 2000)
Radix scutellaria	Baicalin (0.06), Baicalein (0.30) and Wogonin (0.48)	Baicalin enhanced the phagocytosis of macrophages (Cai *et al.*, 1994); this herb is responsible for the treatments of fever, ulcer, cancer, and inflammation in humans (Horvath *et al.*, 2005)
Herba andrographis	Dehydroandrophilide (0.49) and Andrpgrapholide (0.27)	Andrpgrapholide enhanced reactive oxygen species production by neutrophil (Shen *et al.*, 2000)
Radix sophorae flavescentis	Oxymatrine (0.32)	Oxymatrine markedly inhibit the growth of tumors (H22 murine hepatoma and S180 murine sarcoma) (Li *et al*., 2006; Shen *et al.*, 2005)

*The Rf values of the TCMs were matched with corresponding standard chemicals.

Three different dosages of the mentioned TCM formulation were used in the experiment: 0.5%, 1%, and 2% (w/w). A treatment without TCM added in fish feed was used as the control. There were triplicates for each treatment i.e., 12 tanks in total. The fish blood samples (two fish per tank) were collected by caudal venous puncture at Day 1, 7, 14 and 21 after euthanized by 100 mg/L of Anesthetic Tricaine, MS-222 (Sigma-Aldrich, St. Louis, Missouri, USA). The Nitroblue Tetrazolium (NBT) assay, bactericidal activity and protein and immunoglobulin assays were conducted. No mortality was observed during the 21 days of experiment.

6.2.4 *A. hydrophila* injection to grass carp

Aeromonas hydrophila was inoculated in LB broth overnight at 28ºC**.** The cultures were centrifuged at 850 G for 15 min. The supernatant was removed and the bacteria pellet was washed twice in sterile 0.9% saline. The suspension was adjusted to 1 x 10^8 cfu/mL, based on the optical density of suspension with sterile saline (~0.13 absorbance at 625 nm). Suspended bacteria (0.1 ml) were injected into the peritoneal cavity of fish (12 fish for each tank) at Day 21 of the feeding experiment. The mortality rate was recorded in the following seven days after infection.

6.2.5 Immunological parameters analysis

The total immunoglobulin in plasma was determined using the method described in Siwicki *et al.* (1994) with modifications, the protein concentration of the plasma was

determined according to the modified colorimetric method based on Bradford protein assay (Bradford, 1976). The Nitroblue Tetrazolium (NBT) assay was carried out based on the method described in Anderson and Siwicki (1995). The above procedures were described in details in Section 3.2.4. The bactericidal activity of blood plasma was conducted, based on the method of Abidov and Mirismailov (1979) (Section 4.2.3 for details).

6.2.6 Field trial in Yuen Long and cost evaluation on the application of TCM feed

The production cost of TCM feed for field trial experiment was estimated, based on the cost of control feed same as the laboratory experiment, and compared with the cost of the TCM added feed. Both the control fish feed and the two types of TCM feeds (1% and 2%) were processed by a commercial fish feed manufacturer in Lau Fau Shan, Hong Kong. The feeding costs using the 2% TCM feed in grass carp culture were estimated with the following assumptions: the feeding period of 2% TCM feed was 60 days at 2% daily feeding rate on young grass carp (~0.028 kg, mean weight with 1% daily weight gain) at two stocking densities (9000 and 18000 fish/ha) with the marketable grass carp size of 1.5 kg (FAO, 2009). The wholesale sale price of grass carp was converted from the average wholesale prices ($15.62 HKD/kg) in Hong Kong during 2006-2008 (CSD 2009), which was $2.0 USD/kg (1 USD = 7.8 HKD). The profit of improved yield when using the TCM feed was also evaluated based on the reduced mortality observed in field trial.

Calculation on applying 2% TCM feed

(i) Applying 2% TCM feed cost (USD/ha)

= DFR (% of b.w.) x MBW x SD (fish/ha) x FD x TCM feed cost (USD/kg)

(ii) Profit of improved yield when using the TCM feed (USD/ha)

= SD (fish/ha) x RD (%) x HFW (kg/fish) x WP (USD/kg)

where SD: Stocking density = 9000 or 18000 fish/ha (FAO, 2009)

HFW: Harvested fish weight = 1.5 kg/fish (FAO, 2009)

DFR: Daily feeding rate = 2% body weight per day

MBW: Mean body weight in the 60 feeding days= 0.028 kg (calculated from 1% specific growth rate)

FD: Feeding Day = 60 days

2% TCM feed cost = $ 1.02 USD/kg

RD: Reduced mortality due to feeding TCM feed = 20% (refer to field trial results)

WP: Wholesale price = 2.0 USD/kg (CSD, 2009)

A field trial on the effects of TCM formulation on the resistance of grass carp to *A. hydrophila* was conducted at the fish pond in Yuen Long, Au Tau Fisheries Centre of Agriculture, Fisheries and Conservation Department (AFCD), Hong Kong SAR Government. Two types of TCM feeds (only 1% and 2% were tested, based on laboratory

results) and the feed without adding TCM serving as the control were used in this field trial. Fifty individuals of grass carp (~20 g) were cultured in a fish cage (~2 m x 2 m x 1.5 m) placed in a fish pond and acclimatized for two weeks before experiment.

The fish were fed about 1% of body weight (g) per meal and twice per day (average weight of fish was about 20g). The pond water temperature, pH and dissolved oxygen levels were recorded 5 times a week by a handheld Multiparameter Instrument YSI 556MPS which were 19.5±3.3ºC, 6.7±0.31 and 6.2±0.35 mg/mL respectively. The same screening procedures for fish followed those of the laboratory study and the clinical signs in diseased fish were also examined.

6.2.7 Effects of TCM extracts on plasma bactericidal activity of grass carp against *A. hydrophila*

The experiment was performed based on Ji *et al.* (2012) with modifications. Blood of grass carp was collected from 5 grass carp with an average weight of 500 g. The blood samples were taken from the caudal vein to serum tubes with Lithium heparin as anticoagulant. The plasma were separated by centrifugation at 4000 g for 15 min and pooled and stored at -80ºC. The complement activity in grass carp plasma was inactivated at 52 ºC for 30 min (Sakai, 1981). Single herbs and combinations of 2 to 4 herbs of *Rhizoma coptidis* (Rc), *Radix scutellaria* (Rs), *Herba andrographis* (Ha) and *Radix sophorae flavescentis* (Rsf) were tested. The aqueous herbal extracts were prepared as described in Section 5.2.1. The bactericidal activity of plasma was tested based on the

modified methods of Abidov and Mirismailov (1979) and Ji *et al.* (2012). Same strain of pathogens, *A. hydrophila* in the bacterial challenge of Chapter 4 and 6 (Section 6.2.3 and 6.2.6) was inoculated in TSB at 28 ºC for 18 h. The cultures were centrifuged at 850 G for 15 min. The supernatant was removed and the bacteria pellets were washed by sterile 0.9% saline twice. The concentration of bacteria was adjusted to about 1×10^{8} cfu/mL, based on the optical density of suspension and diluted to 10^{-4} by sterile saline.

A volume of 60 µl grass carp plasma or heat-inactivated grass carp plasma was mixed thoroughly with 30 µl of diluted *A. hydrophila*, and then 30 µl of TCM extracts (24, 80 or 240 mg/L) was added. The final concentrations of TCM extracts were 6, 20 or 60 mg/L, respectively. After being incubated at 28 ºC for 30 mins, 50 µL of mixed solution was spread onto the LB agar plate and the dishes were incubated for 18 h at 28ºC. The plasma and the TCM extracts were replaced by sterile phosphate buffered saline (PBS, pH 7) as growth control. The TCM extracts were replaced by PBS to demonstrate the bactericidal activity of grass carp without TCMs. The bactericidal activity was represented by the percentage decreased of colony counts in the sample compared to growth control.

6.2.8 Statistical analysis

The results were compared at each sampling days (Day 1, 7, 14, 21) using one-way ANOVA and Duncan's multiple range tests (SPSS Statistics 17.0, Chicago, Illinosis,

USA). Significant differences between experimental groups were expressed at the significance level of $p<0.05$.

6.3 Results

6.3.1 Immune parameters in Grass carp blood feeding with TCM formulation

No significant difference in total protein was found among all TCM feed groups compared to the control feed group at all sampling days ($p>0.05$) (Fig. 6.1a). A significantly ($p<0.05$) higher total immunoglobulin level (IgI) was observed in 1 and 2% TCM feed groups than the control feed group at Day 21 ($p<0.05$), and the total IgI of 1% TCM group at Day 14 was also significantly ($p<0.05$) higher than the control group (Fig. 6.1b). All groups feeding the TCM formulation showed a higher bactericidal activity of plasma at Day 21 as compared to the control group. However, only significantly higher ($p<0.05$) bactericidal activity of plasma was shown in 2 % TCM group, compared to the control feed group at Day 21; while the bactericidal activity of 2% feed group was gradually increased since Day 1, and significantly improved at Day 21 compared to the value at Day 1 (Fig. 6.2a). A higher NBT activity was found in 2% TCM group at Day 14 significantly, but no significant difference was found among all treatments for the neutrophil oxidative activity in the NBT assay ($p>0.05$) at Day 21 (Fig. 6.2b). In general, 2% TCM feed enhanced the total immunoglobulin and bactericidal activity of plasma.

6.3.2 Fish growth and disease resistance to *A. hydrophila* in laboratory experiment

The mortality of fish was monitored for seven days after bacterial infection. The diseased grass carps in laboratory and field studies showed lethargy, expanded abdomen with red blotches and loss of appetite after infection; redden and swollen anus was observed, with yellow mucus released from the anus when slight pressure was applied to the abdomen. Hemorrhages in the intestinal wall and liver enlargement were observed in diseased fish. These signs matched with enteritis infection observed in grass carp (NACA, 1989; Zheng *et al.*, 2012).

Generally, all groups fed with TCM showed lower mortalities after feeding for 21 days (Fig. 6.3a). A significant reduced mortalities ($p<0.05$) were observed in 1% and 2% TCM groups, with mortalities of 43.3% and 26.7% respectively, compared to the control group (60.0%). The TCM feeding groups i.e. 0.5, 1 and 2% also showed a higher relative weight gain and specific growth rate (Table 6.2) compared to the control group, but the difference was not significant ($p>0.05$).

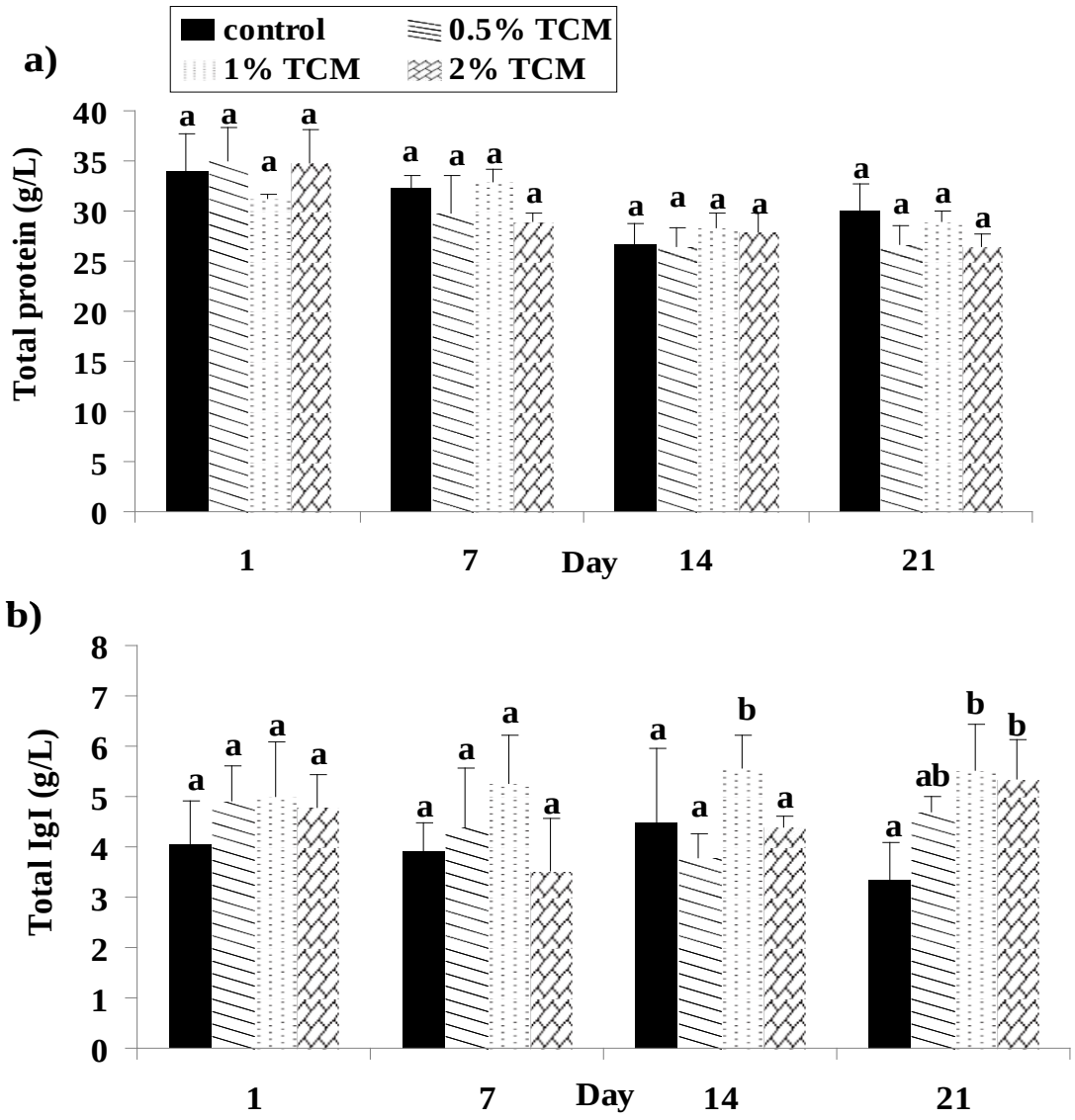

Fig. 6.1a) Total protein (g/L) (Mean ± SD) and b) Total immunoglobulin (IgI) (g/L) (Mean ± SD) of grass carp plasma in the control feed group and feeding various doses of formulated TCM feed groups. Mean in same sampling day with different superscripts are significantly different at *p<0.05*.

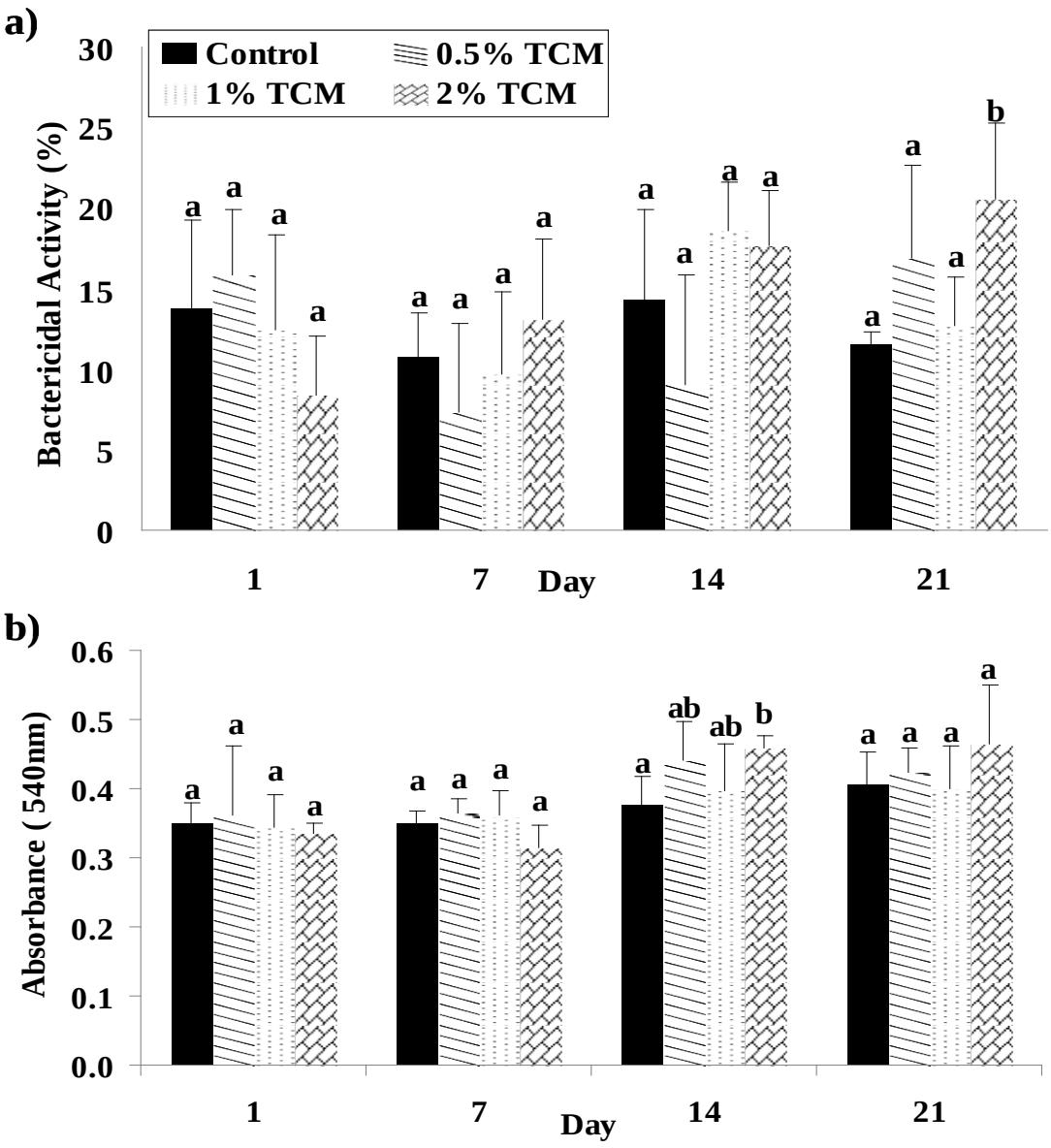

Fig. 6.2a) Bactericidal activity in plasma (%) (Mean ± SD) and b) Optical density of NBT assay in blood (Mean ± SD) of grass carp in the control feed group and feeding various doses of formulated TCM groups. Mean in same sampling day with different superscripts are significantly different at *p<0.05.*

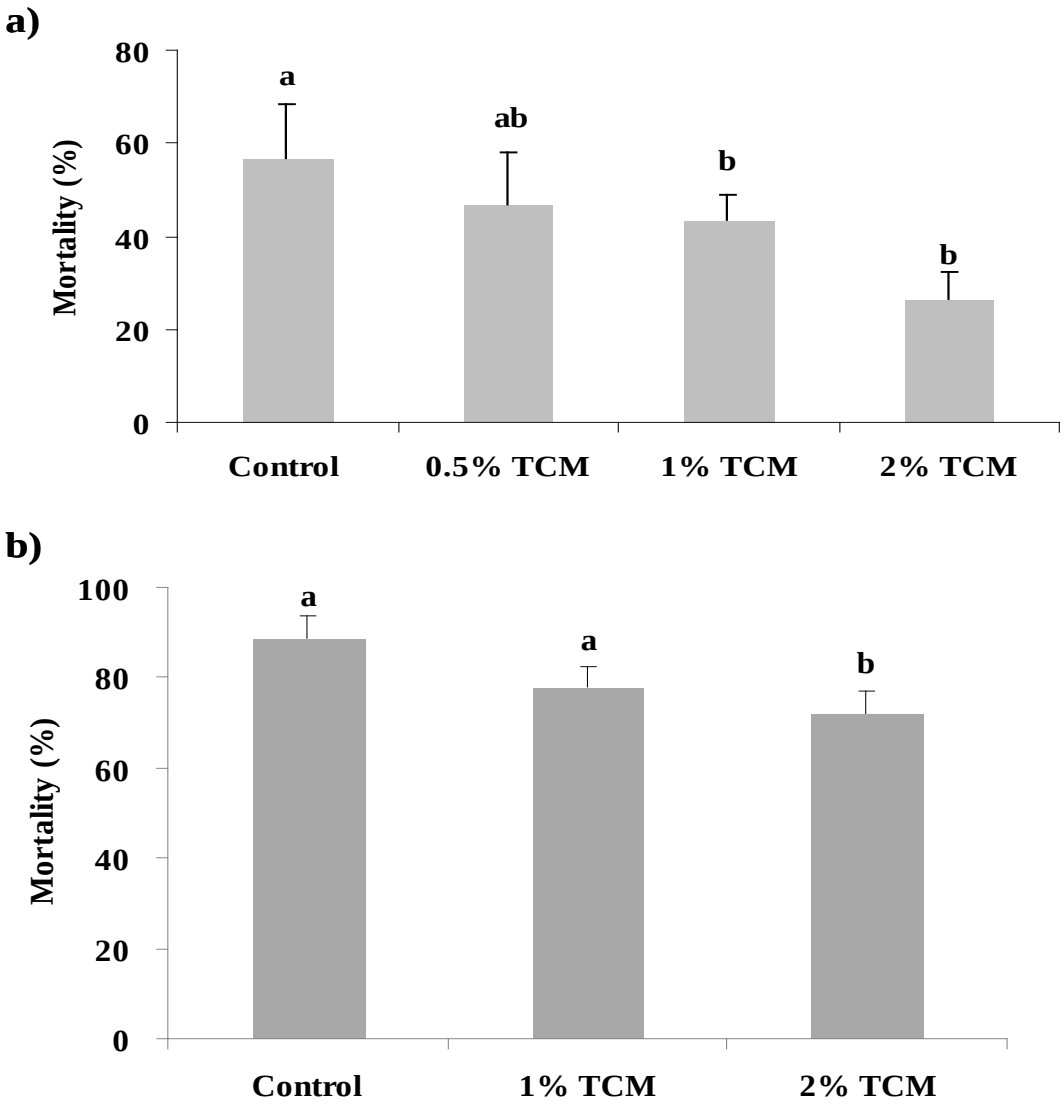

Fig. 6.3 Mortality (%) (Mean ± SD) of grass carp of different feeding groups after intra-peritoneal injection of *A. hydrophila*, a) in laboratory experiment (Control, 0.5%, 1% and 2% formulated TCM) and b) field trial (Control, 1% and 2% formulated TCM). Mean with different superscripts are significantly different at *p<0.05*

Table 6.2 Weight gain rate (%) and specific growth rate (%/day)

Treatment	Tank experiment (n=20)		Pond Trial (n=30)	
	^WGR, %	#SGR, %/day	^RWG, %	#SGR, %/day
Control	12.66±2.58 a	0.57 ±0.11 a	17.20 ±0.71 a	0.55 ±0.08 a
0.5% TCM	15.00±1.14 a	0.66 ±0.17 a	N.T*	N.T*
1% TCM	13.42±1.60 a	0.60 ±0.07 a	18.69 ±3.56 a	0.60 ±0.11 a
2% TCM	13.58±2.09 a	0.61 ±0.09 a	18.47 ±2.08 a	0.59 ±0.12 a

Values in the same column with different superscripts are significantly different ($p<0.05$)
*N.T.: Not tested
^Relative Weight Gain, RWG = [(Final body weight - Initial body weight)/Initial body weight] X 100%
#Specific Growth Rate, SGR= (Ln Weight final-Ln Weight Initial) x 100/day

6.3.3 Disease resistance to *A. hydrophila* in field trial at Yuen Long and cost evaluation on TCM feed application

The diseased grass carp showed same clinical signs of enteritis as observed in laboratory study. There were lower fish mortalities in both 1% and 2% TCM feeding groups (77.8% and 69.4%) respectively, compared to the control group (88.9%), significant ($p<0.05$) mortality reduction was only observed in 2% TCM (Fig. 6.3b). The TCM feeding groups i.e. 1% and 2% also showed a higher weight gain rate and specific growth rate (Table 6.2) compared to the control group, but the difference was not significant ($p>0.05$). The production cost of the TCM feed is listed in Table 6.3. The TCM costs involved in the total feed production cost were 7.3% and 13.8% for 1% and 2% TCM feeds respectively. The costs using the 2% TCM feed were 308.4 and 616.9 USD/ha, the profits of improved yields when using the TCM feed were 5400 and 10800 USD/ha, for low (9000 fish/ha) and high (18000 fish/ha) stocking density respectively. The profits were based on 20% of the reduced mortality in field trial when induced disease.

6.3.4 *In vitro* activation on plasma bactericidal activity

For the single herbs treatments, the bactericidal activity in heat inactivated plasma was significantly enhanced in the presence of Rsf and Ha at 20 and 60 mg/L ($p<0.01$; $p<0.05$ for Rsf 60 mg/L) (Fig. 6.4a). The activity in normal plasma was enhanced when

treated with Rc 60 mg/L, and Ha 20 and 60 mg/L ($p<0.01$), but inhibition was observed when treated with Rsf at 6 mg/L (Fig. 6.4b). The highest bactericidal activity (50.92 ± 2.25%) was observed in Rc 60 mg/L among all single TCM extracts.

In mixed TCM extracts, the activity in heat inactivated plasma was generally improved significantly ($p<0.05$ or <0.01), except Rc Rs at 20 mg/L, Rc Rs Ha (at ratio 1:1:2) at 6 mg/L, Rc Rs Rsf (1:1:2) at 60 mg/L, all four TCM mixed at equal quantity at 6 mg/L and all tested doses in Rs Ha (Fig. 6.5). Generally, extracts with 2 types of TCM showed significantly higher bactericidal activity than the normal plasma control ($p<0.05$ or <0.01), except Rc Rs and Rsf Ha at all doses, while Rc Rsf at 6 mg/L showed the highest bactericidal activity (52.10 ± 2.85%) in all single and mixed herbal extracts which was significantly higher than control ($p<0.01$), but the activity decreased with increasing concentration (Fig. 6.6). Surprisingly, no activation was found in normal plasma treated with extracts of 3 types of TCM and suppression of bactericidal activity was found in treatment Rc Rs Ha (1:1:2) at 6 mg/L ($p<0.05$) and Rc Rs Rsf (1:1:3) at 60 mg/L ($p<0.01$). In the combinations of 4 types of TCM, significant activations were found in TCM mixed with equal ratio (at 6 mg/L) and at the ratio 1:1:2:3 of Rc, Rs, Ha, Rsf at all tested doses ($p<0.01$).

Table 6.3 The product cost (in $USD) of 1% and 2% Traditional Chinese Medicine (TCM) feed

Item	Cost per kg ($USD/kg)	1% TCM feed			2% TCM feed		
		Quantity (kg)	Cost $USD	%	Quantity (kg)	Cost $USD	%
Raw material and processing cost (Control feed)	0.9	990	891	92.7	980	882	86.2
Rhizoma coptidis	27.69	1.43	39.60	4.1	2.86	79.19	7.7
Radix scutellaria	6.79	1.43	9.67	1.0	2.86	19.33	1.9
Herba andrographis	2.56	2.86	7.33	0.8	5.71	14.62	1.4
Radix sophorae flavescentis	3.21	4.29	13.77	1.4	8.57	27.51	2.7
Total	/	1000	961.37	100	1000	1022.65	100
Control feed cost = $0.891 USD/kg							
1% TCM feed cost = $0.961 USD/kg							
2% TCM feed cost = $1.02 USD/kg							

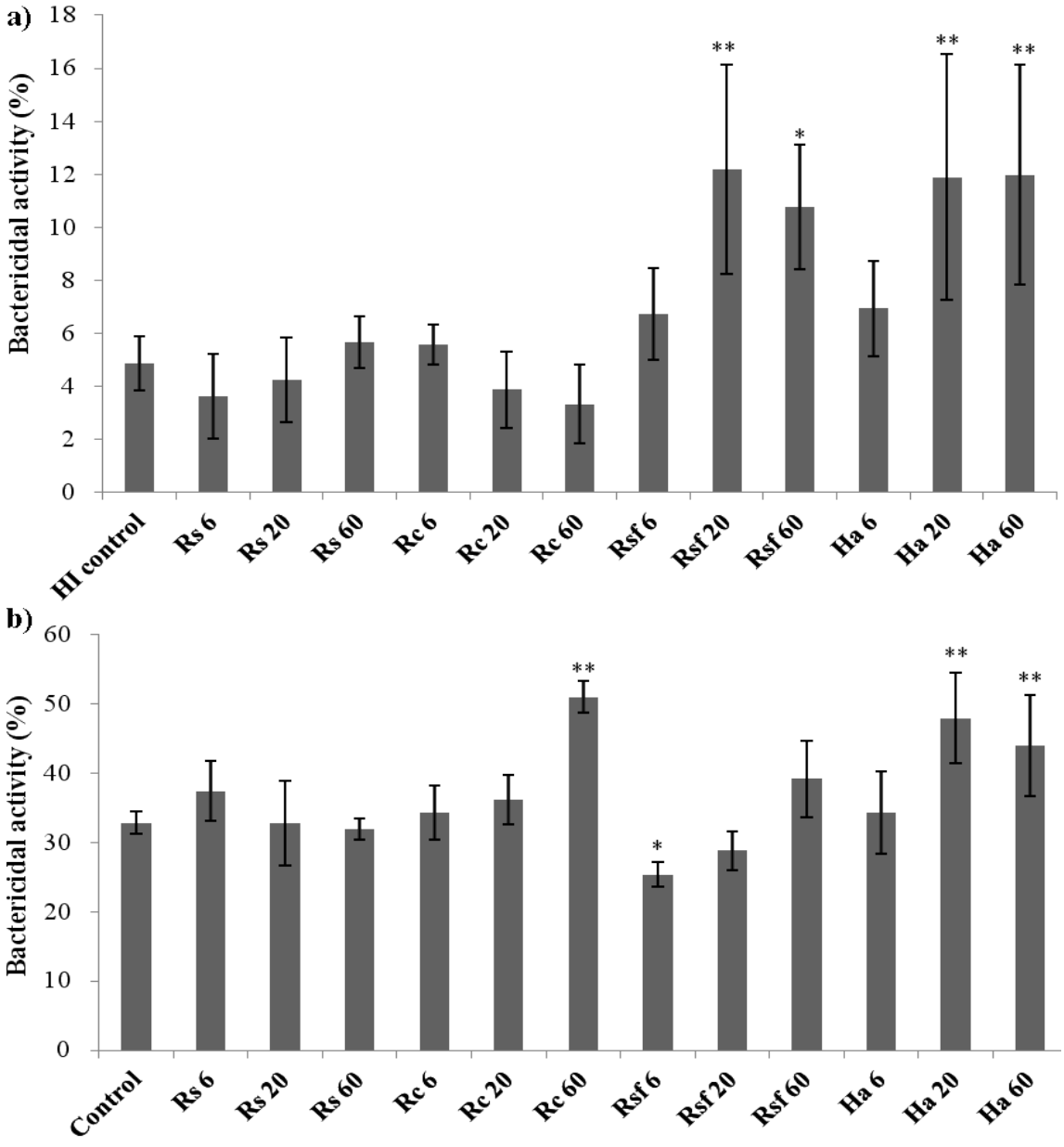

Fig 6.4 The *in vitro* effect of *Rhizoma coptidis* (Rc), *Radix scutellaria* (Rs), *Herba andrographis* (Ha) and *Radix sophorae flavescentis* (Rsf) extracts (6, 20 and 60 mg/L) on the bactericidal activity of grass carp in a) heat inactivated (HI) and b) non-heated plasma, treatments marked with asterisks showed significant difference to control (= $p<0.05$; ** = $p<0.01$*)**

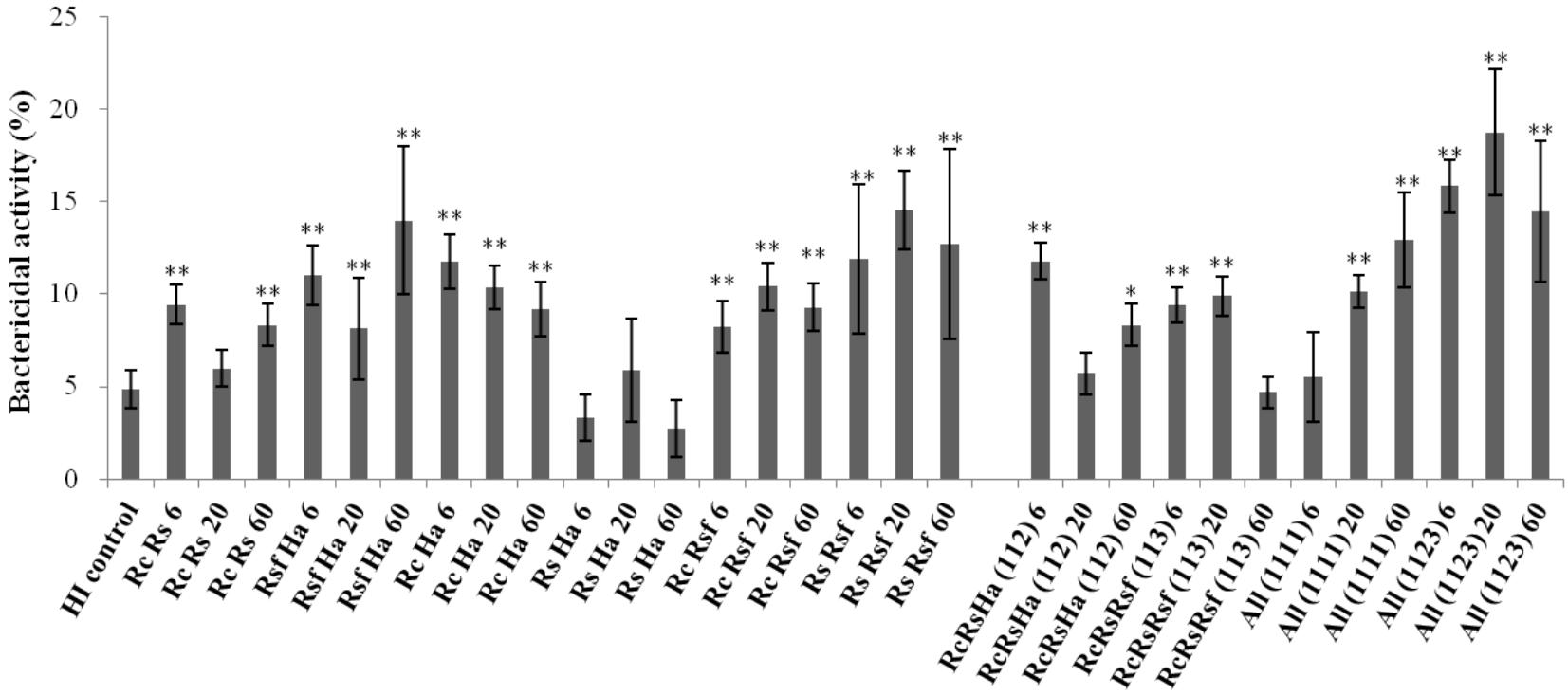

Fig 6.5 The *in vitro* effect of mixed TCM extracts (6, 20 and 60 mg/L) on the bactericidal activity of grass carp in heat inactivated (HI), treatments marked with asterisks showed significant difference to control (*= *p<0.05*; **= *p<0.01*)

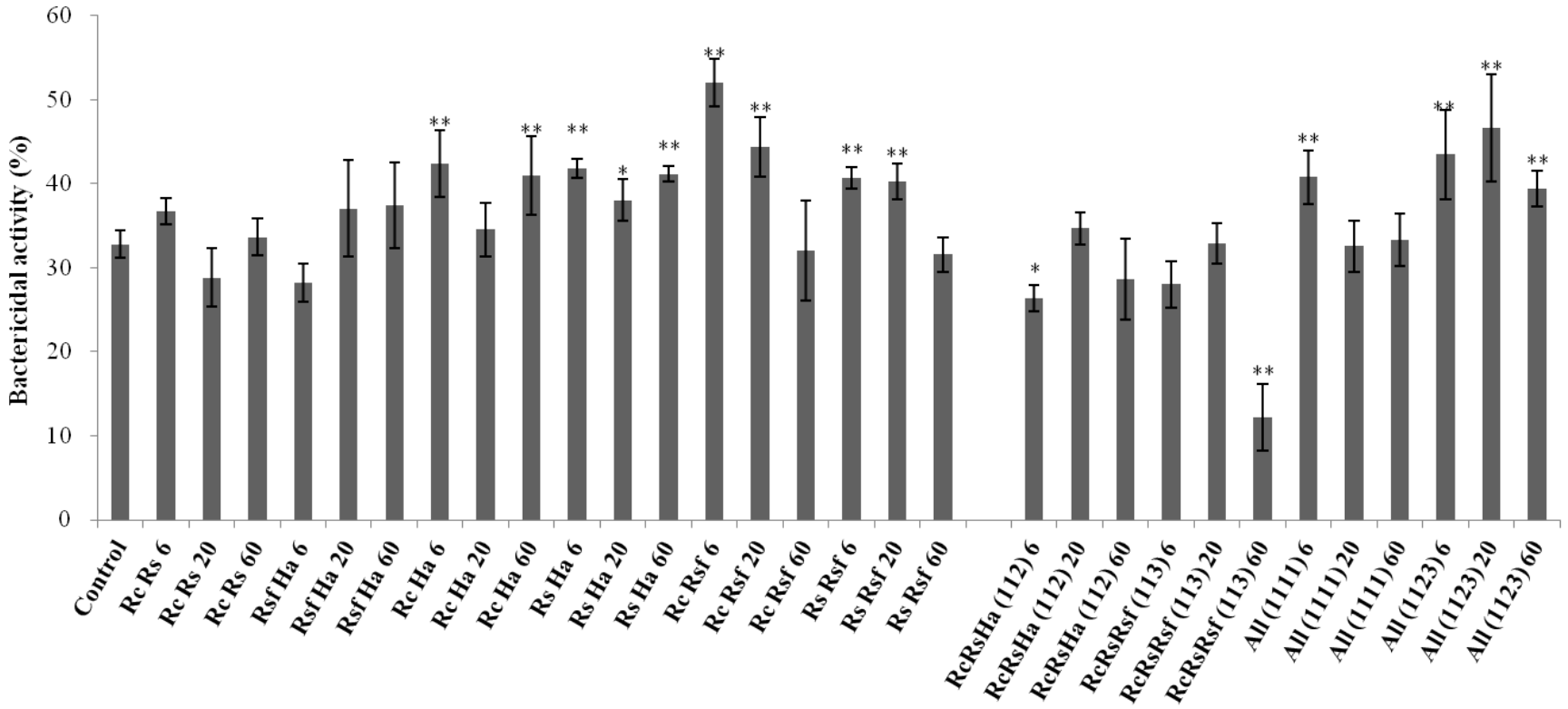

Fig 6.6 The *in vitro* effect of mixed Traditional Chinese Medicine extracts (6, 20 and 60 mg/L) on the bactericidal activity of grass carp in non-heated plasma, treatments marked with asterisks showed significant difference to control (*= *p<0.05*; **= *p<0.01*)

6.4 Discussion

6.4.1 Haematological parameters of grass carp fed with TCM formulation

In this study, the herbal extracts in feed did not show any effect on the protein levels in plasma, but 1% and 2% of TCM elevated the immunoglobulin levels at Day 21 (Fig. 6.1b). Serum proteins are responsible for innate immune response of fish and a higher level of serum protein provided stronger response (Wiegertjes *et al.,* 1996; Sahu *et al.,* 2007a). However, fish serum protein level is not generally altered by feed supplements like immuno-stimulants unless under nutritional deficiencies (Siwicki *et al.,* 1994). The addition of feed supplement may have no effect or even adverse effect on plasma protein levels, e.g. rainbow trout fed with *Laurus nobilis* had no effect on the protein level (Bilen and Bulut, 2010).

The oxidative radicals produced from neutrophils and monocytes is an important defense system, indicating the competence of fish immunity (Anderson *et al.* 1992). Hence the greater NBT reduction by oxidative radicals usually indicates stronger immunity. The production of intracellular superoxide radicals by leukocytes can be quantified by the NBT assay (Sahu *et al.,* 2007b; Ardó *et al.,* 2008). In this study, no significant change of NBT activity was noted in all TCM feeding groups. However, Yin *et al.* (2006) demonstrated that high doses of *Scutellaria* extract (0.5%

and 1%) reduced the phagocytic cell function in tilapia, while low dose (0.1%) activated the function.

Enhanced NBT activity was shown in 2% TCM group after feeding for 14 days but this not observed in Day 21, which may be related to the optimal time and dose of feeding (Yin *et al.*, 2006). Although administration of feed supplement excreted both positive and negative effects on NBT activity with different feeding period and dose, the use of this TCM formulation at 2% dose should be applicable as no inhibition of NBT activity was found after feeding for 21 days. In other studies, the respiratory burst activity of fish phagocytic cells was enhanced in large yellow croaker (*Pseudosciaena crocea*) and common carp (*Cyprinus carpio*) after feeding a mixture of *Astragalus membranaceus* and *Angelica sinensis* extracts (Jian and Wu, 2003 & 2004). Further investigation should be carried out in order to identify the causal relationship between immune responses and administration time and doses.

6.4.2 Plasma bactericidal activity of grass carp in feeding trial and *in vitro* study

The bactericidal activity of plasma in fish had been enhanced after 21 days of feeding in 2% TCM group, compared with the control group. The stronger plasma bactericidal activity indicated the stronger innate immune responses in fish (Das *et al.*, 2009). It has also been noted that the activities were enhanced after feeding with some

powdered dietary garlic (Sahu *et al.,* 2006 & 2007a) and mango kernel (Sahu *et al.,* 2007b) in rohu. A correlation between bactericidal activity of plasma and disease resistance against *A. hydrophila* was observed in a study on grass carp with cortisol injection (Wang *et al.,* 2005b). Several studies also stated that higher than 50% of bactericidal in fish activity indicating very good disease resistance, and 40 to 50% showing good resistance (Mikriakov and Silkin, 1978; Atanasova, 2003; Atanasova *et al.* 2008). Although the bactericidal activity of grass carp was below 25% in this study, the highest disease resistance and bactericidal activity (~ 25%) were also found in 2% TCM group.

The level of bactericidal activity of plasma is closely related to the disease resistances. The *in vitro* study on grass carp plasma also showed that the bactericidal activities could be enhanced by TCM extracts. Ji *et al.* (2012) also studied the activation by berberine, an active ingredient of *R. Coptidis*, and concluded that berberine could enhance bactericidal activity through activating complement system in grass carp serum. The complement system is capable to generate activated protein fragments which could initiate phagocytosis, microbial killing, inflammatory actions and antibody production. The alternative complement pathway activated by foreign bacteria is important defense system in fish (Holland and Lambris, 2002). In this

study, the plasma bactericidal activity in grass carp fed with *R. coptidis* containing feed was also activated.

Mixture of TCM extracts may be more effective in stimulating fish immunity than single herbs. Although *R. coptidis* and *H. andrographis* extracts also enhanced the bactericidal activity in *in vitro* study, the mixed TCM extracts showed stronger activation in both normal and heat inactivated plasma (Fig. 6.5 and 6.6). For example, activations were observed only at higher dose (20 or 60 mg/L) of Rc or Ha in non-heated plasma, while activations were found in various combinations e.g RcHa, RsHa, RcRsf and RsRsf at low dose (6 mg/L). The TCM mixtures may enhance the complement activity in plasma as a less extent of activations was found in heat inactivated plasma. The heat inactivation of plasma should destroy the complement activity and other heat liable factors e.g. lysozymes, but the heat stable factors i.e. immunoglobulins are remained (Leiro *et al.*, 2008). The immunoglobulin or complement could cause opsonization with bacteria which facilitate phagocytosis afterward, but the opsonization process in fish is still poorly understood (Holland and Lambris, 2002). The bactericidal activity in heat inactivated plasma could be due to the actions of immunoglobulins, but not the antimicrobial activity of herbal extracts as minimum inhibition concentrations (>320 mg/L) of TCM extracts on *A. hydrophila* determined in the previous experiment (Chapter 4) were much higher than the doses

(60 mg/L) adopted for the *in vitro* study. This is an encouraging result, calling for further investigations on the activation mechanisms in plasma.

6.4.3 Disease resistance to *A. hydrophila*

All TCM feeding groups showed lower mortalities compared to the control group. The highest disease resistance to *A. hydrophila* infection was observed in the highest tested dose (2%), whereas the highest plasma bactericidal activity was noted in the same group at Day 21 (Fig. 6.2a). A study using dried powder of *Andrographis paniculata* reduced the mortality of Nile tilapia (*Oreochromis niloticus*) after infected with *Streptococcus agalactiae* and a dose-mortality dependent relationship was also observed (Rattanachaikunsopon and Phumkhachorn, 2009). A similar dose response was also observed in this study. In general, the mixed herbal extracts with 4 mentioned TCMs could enhance the *in vitro* bactericidal activity of plasma, probably activating on complement system and opsonization with pathogens. It is also noted that the same TCM formulation enhanced the total immunoglobulin and bactericidal activity in fish plasma after feeding with the formulation, also with a reduced morality. All these results supported the effectiveness of the TCM formulation on stimulating grass carp immunity and disease resistance.

A number of studies were conducted using herbal powders and/or extracts on disease prevention and treatment (Punitha *et al.*, 2008; Sahu *et al.*, 2006). However, there is a lack of information on the field application of herbal feed and its cost evaluation in real aquaculture situation. The field trial on this TCM formulation in Yuen Long demonstrated a better disease resistance to *A. hydrophila* in the grass carp fed with TCM, with significant reduction of about 20% of mortality ($p<0.05$), when compared to the control group in the field experiment (Fig. 6.3b). However, the mortality rates observed in the field experiment (69-89%) were much higher compared to those in the laboratory experiment (27-57%).This could be due to the fluctuation of environmental conditions such as temperature and dissolved oxygen encountered in the pond environment, but both the mortalities in the control groups were matched with previous mentioned mortality, about 50-90% mortality for *A. hydriophila* infection (Yang, 2008). Zheng *et al.* (2012) demonstrated a superior effect of antibiotic treatment on grass carp enteritis, reducing 39 to 74% mortality compared to the control (~90%) depending on dosages (1-2 g/kg feed) of norfloxacin. However, the use of antibiotics for disease treatment in aquaculture also raised concerns on the development of drug resistance in pathogens which threatens human food security (Ibrahim *et al.*, 2010; Zheng *et al.*, 2012). Besides, in both laboratory

and field experiments, no significant differences on the weight gain rate and specific growth rate were noted between the control and TCM feeding groups ($p>0.05$).

6.4.4 Cost evaluation of using TCM feed in aquaculture

Based on the TCM feed cost evaluation for this field trial, the costs of control feed, and 1% and 2% TCM feed were $0.891, $0.961 and $1.02 USD/kg respectively (Table 6.3), the TCM contributed 7.3% and 13.8% to the total feed production cost for 1% and 2% (w/w) TCM feed respectively. The use of this formulation in aquaculture industry seems to be cost effective based on the estimations in this study. It could be applied to young fish in hatchery farms and fish farms as the enteritis is prevalent in young fish when the water temperature is above 18˚C (Xu *et al.*, 1988). The disease could also be prevalent in one-year-old grass carp (NACA, 1989); hence, the TCM feed should be applied to grass carp 1-2 months before spring.

When assuming the young grass carp (0.028 kg/fish) were fed with 2% TCM feed for two months, the cost is only equivalent to approximately 83 kg of grass carp, which could be compensated by the higher survival rate and subsequently high yield. In addition, due to the short feeding period in preventing grass carp enteritis, the cost of TCM feed used was insignificant, compared to the overall feed used throughout the

whole growth out period. Therefore, the application of TCM feed may be profitable for grass carp culture, due to the low cost could be compensated by improved yield.

The optimal dose of the formulated TCM based on the laboratory experiment was 2% (w/w) for the grass carp fingerlings, as reflected by promoted bactericidal activity and total immunoglobulin in plasma and the enhanced disease resistance of grass carp against *A. hydrophila* after 21 days of feeding. The results were also consistent with *in vitro* study on bactericidal activity treated with TCM extracts. The field trial also demonstrated the practical use of TCM containing feed in aquaculture with cost effective outcome based on reasoned estimation. However, further studies are required to show the effectiveness of this formulation on the disease resistance against other bacterial diseases such as bacterial gill rot disease. TCM supplement could then be applicable to prevent and combat other diseases, replacing the usage of antibiotics in aquaculture.

6.5 Conclusion

The TCM formulation showed stimulatory effects on grass carp immunity and cost effectiveness in preventing the *Aeromonas* infection in fish, hence the hypothesis of this experiment was accepted. In this study, four TCMs, *Radix scutellaria* (Rs), *Rhizoma coptidis* (Rc), *Herba andrographis* (Ha) and *Radix sophorae flavescentis*

(Rsf) were selected to form a compound formulation in the ratio of 1:1:2:3. Supplementation with 2% formulation significantly improved ($p<0.05$) the bactericidal activity and total immunoglobulin in the plasma after feeding grass carp for 21 days. Compared to control, 1% and 2% TCM feeding groups also had a significantly reduced mortality after *A. hydrophila* challenge at the end of experiment and the similar result was also achieved in the field trial. The results were also consistent with the *in vitro* study on bactericidal activity treated with TCM extracts, where the formulation enhanced bactericidal activity of both normal and heat inactivated plasma more effectively than single herbs at a lower dose (6 mg/L). In the cost evaluation, it was noted that the cost of using TCM formulation in grass carp culture was insignificant during production and could be compensated by improved yield.

Chapter 7

Upgrading food waste feed using TCMs, baker's yeast and enzyme on the immune responses of grass carp (*Ctenopharyngodon idellus*) against *Aeromonas hydrophila*

7.1 Introduction

The addition of immuno-stimulants for enhancing the fish defense system is a very effective and convenient approach in dealing with aquaculturual diseases. Probiotics and herbal medicines have been adopted in the industry as growth promoters and immuno-stimulants to replace antibiotics and chemical drugs. Application of antibiotics should only be allowed for therapeutic purposes, and other prophylactic measures for fish diseases should take higher priority than post infection treatments (GESAMP, 1997; FAO 2005).

The Traditional Chinese Medicine (TCM) formulation contained *Radix scutellaria*, *Rhizoma coptidis*, *Herba andrographis* and *Radix sophorae flavescentis* (in the ratio of 1:1:2:3) had been supplemented in grass carp feed (Chapter 6) and showed affirmative effects on weight gain, plasma bactericidal activity and disease resistance to *A. hydrophila*. The baker's yeast, *S. cerevisiae* also showed similar effects on grass carp growth and immune system (Chapter 4).

The immune-stimulating and growth promoting properties of TCM and probiotics on fish have been extensively studied, but most of these studies focused either herbal medicines (Sahu *et al.*, 2006; Yin *et al*., 2009) or probiotics (Wang and Xu, 2004; Wang *et al*., 2005a). In the present study, the beneficial effects of TCMs (Chapter 6), baker's yeast (Chapter 4) and mixed enzymes in upgrading food waste (Chapters 3 and 4) on fish growth or immunity have been demonstrated individually. However, very few studies attempted to reveal the combined effects of TCM and probiotics as fish feed supplements. The supplements may interfere with each other, leading to potential undesirable or synergistic effects. Therefore, the combined effects of these supplements on fish should be fully investigated.

The present study attempted to investigate the combined effects of some tested immune-stimulants i.e. TCM and yeast on fish. It was hypothesis that the mixed TCM and yeast could show additive beneficial effects to the host immunity and growth, compared to the individual TCM or yeast added feed. The major objectives of the present experiment were to investigate 1) growth performance, 2) immune parameters and 3) disease resistance to *Aeromonas hydrophila* of grass carp by feeding mixed yeast (2.5%) and TCM (2%) in enzyme treated food waste feed.

7.2 Materials and Methods

7.2.1 Experimental setup and fish feed preparation

Food waste feed formulation A (FW A) contained 75% food waste which mainly consisted of plant materials such as cereals, fruit and vegetables (refer to Table 4.1 for details). The preparation of different types of fish feeds was described in Section 2.2.2. The fish feeds were supplemented with papain (0.5%) and bromelain (0.5%) (Section 3.2.1). In brief, the enzymes were added into the FW A by mixing with sodium dihydrogen phosphate (pH 8) at 37ºC for 5 h. The resulted substrates were heated to 95°C for 5 min to inactivate the enzymes (Wu *et al.*, 1998).

The dried enzyme treated FW A (A-E) were supplemented with 2.5 % w/w of baker's yeast (Y), *Saccharomyces cerevisiae* (Type II, YSC2, Sigma-Aldrich) and 2% of TCM formulation (refer to Section 6.3.1 for details), respectively. The TCM formulation comprised of 4 herbs, *Rhizoma coptidis*, *Radix scutellaria*, *Herba andrographis* and *Radix Sophorae flavescentis* in a ratio of 1:1:2:3. All feed supplements (enzymes, baker's yeast and TCMs) were added into FW A (named as Mixed) also, as one of the treatments for revealing the combined effects. Deionized water was added and the fish feed dough was pelletized with a meat grinder and dried at 50 ˚C for 24 h. The commercial fish feed without any supplement was used as

control. Five treatments (C, A-E, A-E-Y, A-E-TCM and Mixed) were conducted, each with triplicates.

7.2.2 Feeding trial and sample collections

Seven hundred fingerlings of grass carp *Ctenopharyngodon idella* (herbivore) were purchased from a local fish farm. Twenty individuals (~15-20g) were placed in each tank (~60L). The water temperature, pH and dissolved oxygen were monitored three times a week, using a portable Hanna pH meter and a YSI digital Dissolved Oxygen (DO) meter and the values ranged from 23.6-24.8°C, 6.4-7.0 and 6.2-6.7 mg/mL, respectively.

The control feed (Jinfeng®, 613 formulated commercial feed) was fed to fish at the rate of 2% body weight daily for 3 weeks before prior the start of experiment. The feeding trial lasted for a period of 28 days. Blood samples were collected by caudal venous puncture at Day 7, 14, 21 and 28. The bactericidal activity, neutrophil activity (Nitroblue tetrazolium (NBT) assay) in blood, total protein and total immunoglobulin of plasma were determined (refer to Section 3.2.4 and 4.2.3 for details).

The specific growth rate, relative weight gain, feed conversion ratio and protein efficiency ratio were calculated for monitoring the growth performance of different treatments (Section 2.2.5).

7.2.3 *A. hydrophila* injection to grass carp

Aeromonas hydrophila injection (same bacteria used for the infection in Section 4.2.4) was performed at Day 28 for investigating the disease resistance to the pathogen. A 0.1mL of suspension (1×10^8 cfu/mL) was injected into the peritoneal cavity of fish (11 fish for each tank) (refer to Section 4.2.3 for details). The mortality rate was recorded in the following 10 days after infection.

7.2.4 Statistical analysis

The results at each sampling day (Day 7, 14, 21 and 28) were compared using one-way ANOVA and Duncan's multiple range tests (SPSS Statistics 16.0, Chicago, Illinosis, USA). Significant differences between experimental groups were expressed at the significance level of $p<0.05$.

7.3 Results

7.3.1. Growth performance of grass carp fed with enzymes, baker's yeast and TCMs as supplements

After 28 days, the best growth performance was observed in grass carp supplemented with bromelain (0.5%), papain (0.5%) and baker's yeast (2.5%), i.e. A-E-Y (Table 7.1). Significantly higher ($p<0.05$) specific growth rate (SGR), relative weight gain (RGW) and protein efficiency ratio (PER) were noted in A-E-Y, when compared to enzyme supplemented FW A with or without 2% TCMs (A-E and A-E-TCM) and the control (C). The feed conversion ratio (FCR) of A-E-Y was also significantly lower than A-E, A-E-TCM and the control ($p<0.05$).

7.3.2. Immunity of grass carp fed with enzymes, baker's yeast and TCMs

There was no significant difference of NBT activity between the supplemented feed and control groups at Day 7 and 14 (Fig. 7.1a). The NBT activities of A-E-TCM and all supplements mixed group were significantly higher than the control at day 21 ($p<0.05$). It was also noted that all supplements mixed group showed the highest NBT activity which was significantly higher than all other groups at Day 21 ($p<0.05$). At Day 28, the NBT activities of A-E-Y and all supplements mixed groups were significantly higher than the control ($p<0.05$).

There was no significant difference between supplemented feed groups and the control in bactericidal activity at Day 7 (Fig. 7.1b). Significantly higher activity was found in the Mixed group (with all supplements), compared to the control and food waste with enzyme (A) ($p<0.05$). All groups except food waste with enzyme (A) had significantly higher bactericidal activity than the control at Day 21 ($p<0.05$). A similar pattern was also observed at Day 28, except the bactericidal activity of the Mixed group ($p>0.05$).

In general, total plasma protein levels were not significantly different between supplemented feed groups and the control, at all sampling days ($p>0.05$) (Fig. 7.2a), except a significantly higher plasma protein level was obtained in grass carp fed with food waste with enzyme at Day 21 ($p<0.05$).

Total IgI level in A-E-Y was significantly higher than A-E-TCM and all other groups at day 7 and 14, respectively ($p<0.05$), but there were no significant difference in total IgI levels between each treatment ($p>0.05$) (Fig. 7.2b). Although the IgI levels in A-E-Y and A-E-TCM were significantly higher than A-E ($p<0.05$), the values were not significantly different from the control.

Table 7.1 Growth performance of grass carp feeding with enzymes upgraded food waste (A-E) supplemented with baker's yeast (Y) and Traditional Chinese Medicine (TCM)

Measurement	Control	A-E	A-E-TCM	A-E-Y	Mixed
Initial weight (g)	17.96 ±0.85a	18.44 ±1.00a	18.59 ±0.27a	18.68 ±0.59a	17.76 ±0.78a
Final weight (g)	21.67 ±0.86a	22.25 ±0.18ab	22.62 ±0.75ab	23.45 ±1.20b	21.84 ±0.51a
Feeding rate (% b.w./day)	2.09 ± 0.01a	2.07 ±0.01ab	2.05 ±0.02bc	2.02 ±0.04c	2.04 ±0.02bc
SGR (% b.w./day)	0.67 ±0.06a	0.67 ±0.03a	0.70 ±0.01a	0.81 ±0.01b	0.74 ±0.08ab
RWG (%)	20.71 ±1.96a	20.67 ±0.90a	21.69 ±0.24a	25.51 ±0.24b	23.03 ±2.68ab
FCR	3.13 ±0.25a	3.10 ±0.11a	2.93 ±0.02a	2.51 ±0.07b	2.79 ±0.30ab
PER	1.06 ±0.09a	1.04 ±0.04a	1.12 ±0.01a	1.30 ±0.03b	1.17 ±0.13ab

*Different superscripts (a, b) among feeding groups are significantly different ($p<0.05$)

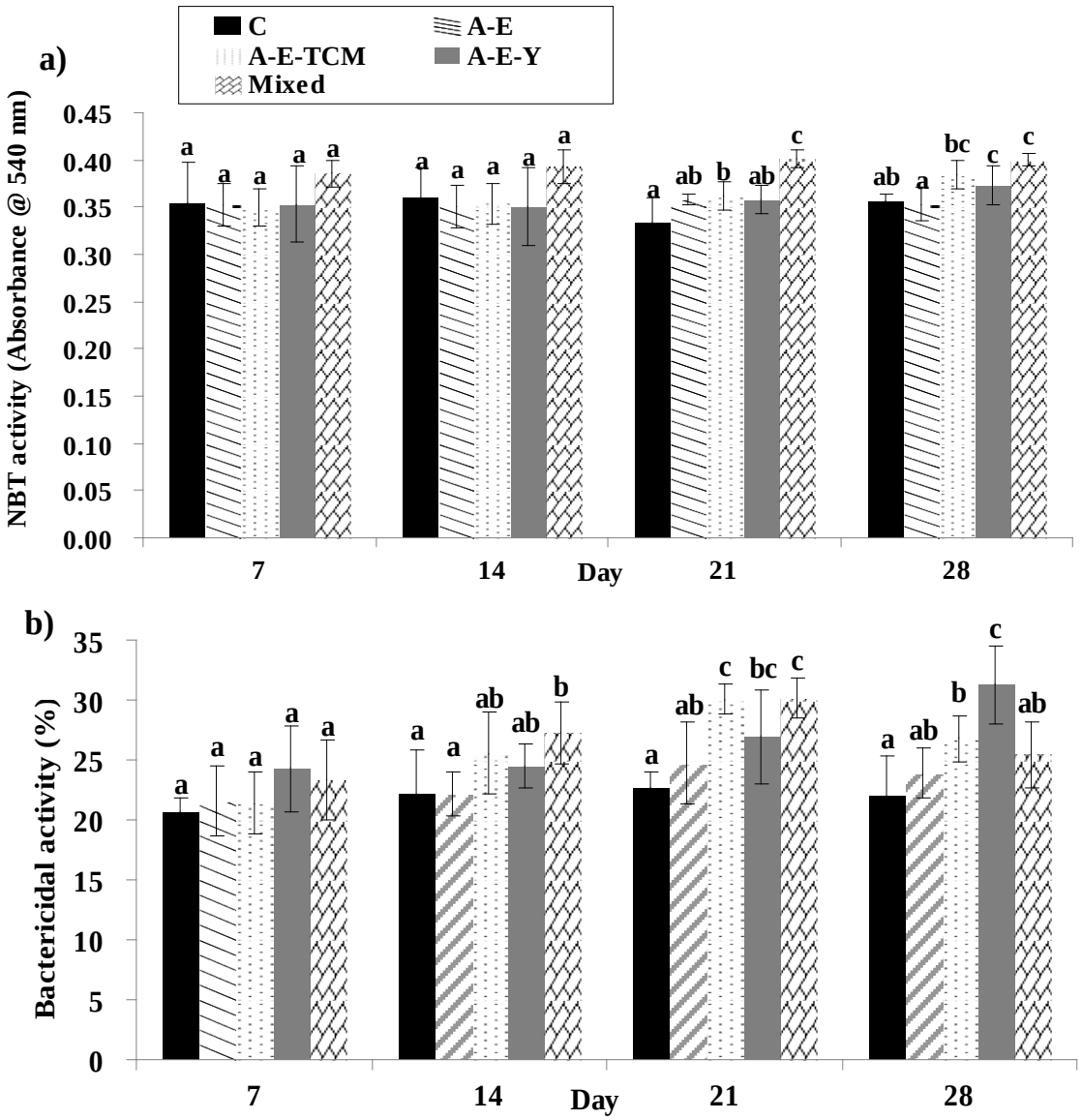

Fig. 7.1 a) NBT activity (absorbance at 540 nm) and b) plasma bactericidal activity (%) of grass carp fed with commercial feed (control, C) and enzymes upgraded food waste (A-E) supplemented with baker's yeast (Y) and Traditional Chinese Medicine (TCM).

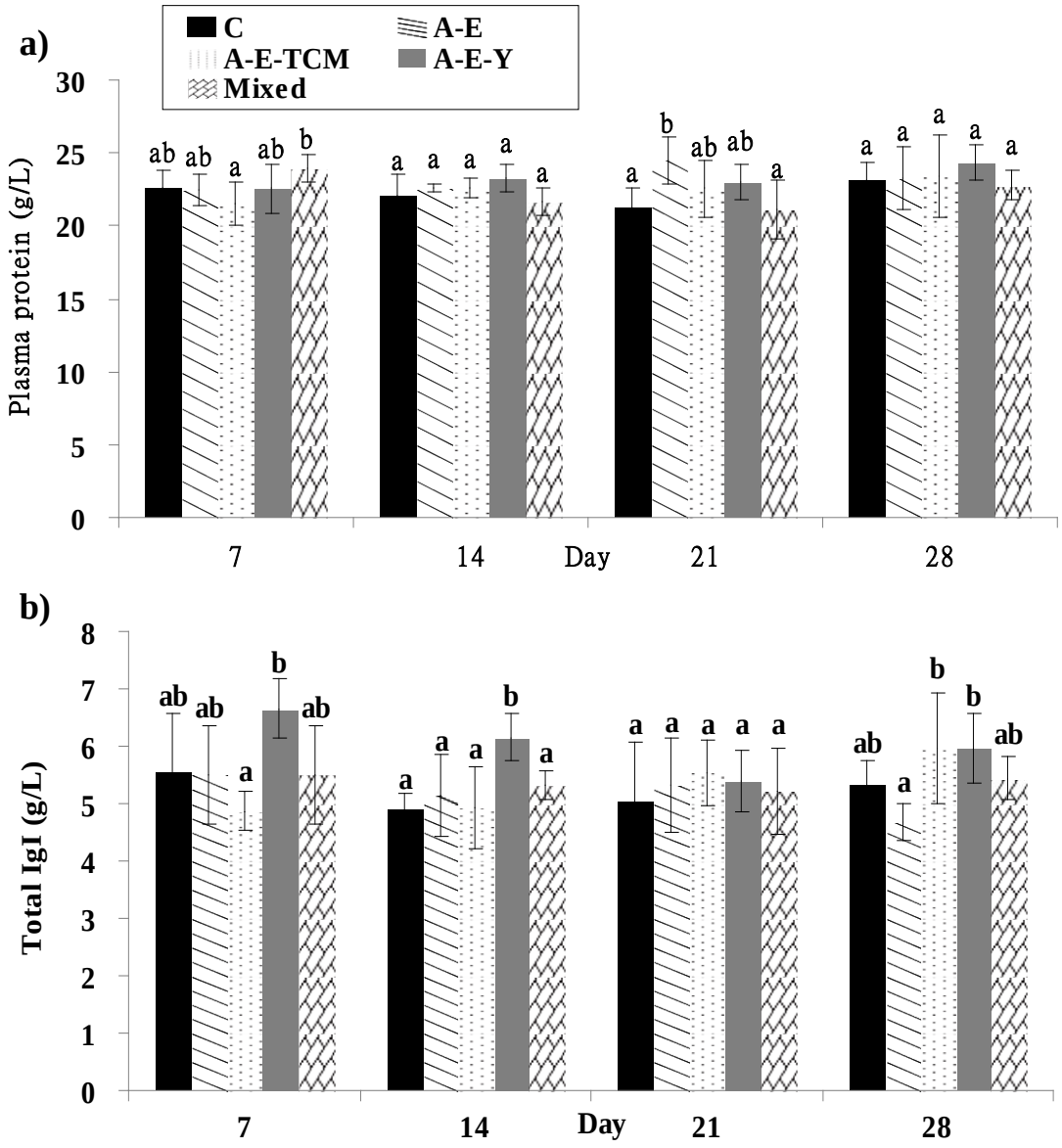

Fig. 7.2 a) Total plasma protein and b) total immunoglobulin, IgI (g/L) grass carp fed with commercial feed (control, C) and enzymes upgraded food waste (A-E) supplemented with baker's yeast (Y) and Traditional Chinese Medicine (TCM).

7.3.3. Disease resistance to *A. hydrophila* of grass carp fed with enzymes, baker's yeast and TCMs

The mortality of grass carp was recorded for 10 days after the injection, which ranged from 42.4 to 60.6% (Fig. 7.3), with the lowest mortality obtained in A-E-Y, while the highest in A-E. Significantly lower mortalities were observed in A-E-Y and A-E-TCM than control and A-E ($p<0.05$).

7.4 Discussion

7.4.1 Growth and feed utilization of grass carp fed with enzyme upgraded food waste with baker's yeast and TCM

In the presence study, the food wastes comprised of yeast supplement (2.5%) and enzyme upgraded food waste feed which promoted fish growth significantly, reflected by the higher specific growth rate (SGR), relative weight gain (RWG), feed conversion ratio (FCR) and protein efficiency ratio (PER). The result was in agreement with the previous experiment described in Chapter 4 and various other studies on different carp species e.g. catla carp (*Catla catla* Ham.) (Mohanty *et al.*, 1996), mrigal carp (*Cirrhina mrigala*) (Swain *et al.*, 1996) and rohu (*Labeo rohita*) (Misra *et al.*, 2006).

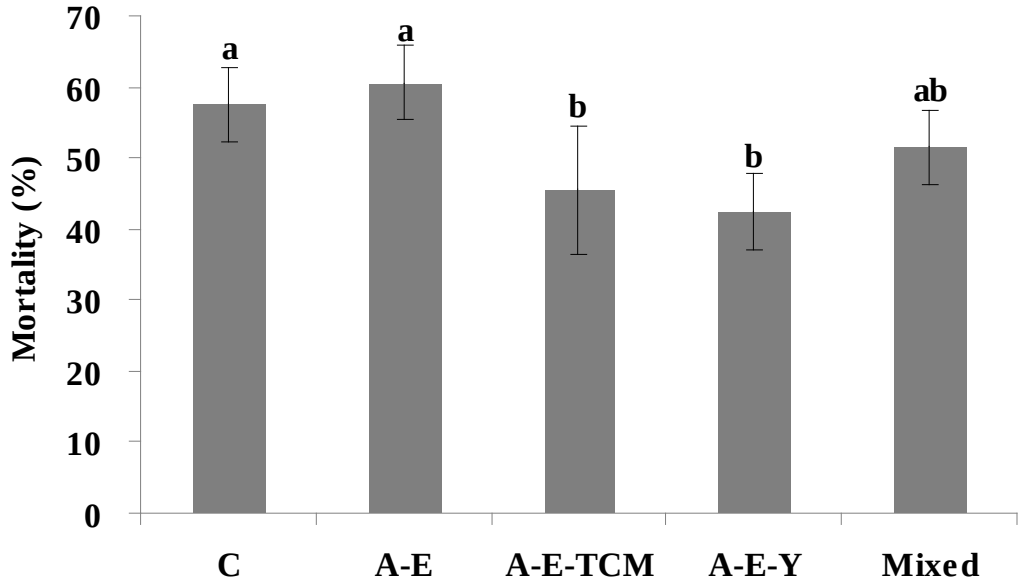

Fig. 7.3 Mortality (%) of grass carp fed with commercial feed (control, C) and enzymes upgraded food waste (A-E) supplemented with baker's yeast (Y) and Traditional Chinese Medicine (TCM), after 10 days after the intra-peritoneal injection of *A. hydrophila*

The promoted growth, protein utilization and feed conversion were due to the enhanced digestibility and utilization of proteins and lipids. The yeast assisted the establishment of beneficial intestinal microbial communities and enhanced the enzymes activities in gut (Tovar *et al.*, 2002; Waché *et al.*, 2006). Microbial communities are important for the digestion, uptake and utilization of nutrients such as amino acids and fatty acids (Merrifield *et al.*, 2010; Nayak, 2010). Yeast attaching on the gut wall could also stimulate enzyme (such as amylase) secretion (Tovar *et al.*, 2002; Irianto and Austin, 2002). Higher protein digestibility and utilization resulted in a higher growth rate. The same results were observed on Nile tilapia (*Oreochromis niloticus*) (Lara-Flores *et al.*, 2003; Abdel-Tawwab *et al.*, 2008) and sea bass (*Dicentrarchus labrax*) (Tovar *et al.*, 2002), feeding with yeast (*S. cerevisiae*).the previous study (Chapter 4) on grass carp also showed similar improvements on weight gain, protein utilization and retention when feeding with yeast supplemented feeds.

7.4.2 Immunity parameters of grass carp fed with enzyme upgraded food waste with baker's yeast and TCM

The present experiment revealed that both baker's yeast and TCM could stimulate the immune parameters (plasma bactericidal and NBT activities) at the dose of 2.5% and 2%, respectively, which were in line with the results obtained in Chapters

4 and 6. Significant increases of total IgI, bactericidal activity and NBT activity were noted after feeding baker's yeast for 28 days, while only NBT activity was enhanced in Mixed group. The neutrophil oxidative radical production could be measured by NBT assay and it is an important immune parameter in fish (Siwicki *et al.*, 1994). Stimulations on fish immune system due to feeding with probiotics or TCM are commonly found, with the same activations on NBT activity and IgI levels observed in fish feeding with *S. cerevisiae* (Siwicki *et al.*, 1994; Anderson *et al.*, 1995; Li and Gatlin 2003) and herbs (Jian and Wu, 2003; Sharma *et al.*, 2010)

Up regulations of the bactericidal activity and the total IgI levels were observed when feeding with TCM formulation, which agreed with the results presented in Chapter 6. Based on the experiment described in Chapter 6, the mixed TCM extracts showed a stronger activation on the *in vitro* bactericidal activity in both normal and heat inactivated grass carp plasma, than the single herb. Ji *et al*. (2012) also showed that berberine, an active ingredient of *R. coptidis* could enhance the bactericidal activity through activating complement system in grass carp serum. Mixed TCM feed could also motivate the complement system and lysozyme activities and increase the number of NBT positive cells in Jian carp (*Cyprinus carpio* var. jian), after feeding for 20 days (Jian and Wu, 2004).

The baker's yeast (2.5%) enhanced the plasma bactericidal and NBT activities in the present study. He *et al.* (2009) reported that *S. cerevisiae* fermented products (commercial product, DVAqua®) enhanced the serum alternative complement activity of hybrid tilapia (*Oreochromis niloticus* ×*O. aureus*), which is one of the major pathways for serum bactericidal activities. β-glucans could be the responsible component in activating fish immune systems, e.g. turbot (*Scophthalmus maximus*) (Toranzo *et al.*, 1995) and common carp (*Cyprinus carpio*) (Selvaraj *et al.*, 2005), contained in DVAqua® (He *et al.*, 2009) and *S. cerevisiae* (White *et al.*, 2002).

7.4.3 Effects of baker's yeast and TCM on fish disease resistance against *A. hydrophila*

The disease resistance of grass carp to *A. hydrophila* was the highest in yeast supplemented feed, followed by TCM group. About a 25% and 30% reduction in mortalities were observed in TCM and yeast supplemented groups, respectively, compared to the control. The nucleotides extracted from *S. cerevisiae* promoted the blood phagocytic and serum lysozyme activity of common carp (*Cyprinus carpio*), and hence increased the disease resistance to *A. hydrophila* (Sakai *et al.*, 2001). The enhanced plasma bactericidal activity also upheld the disease resistance to pathogens,

which showed a correlation between this activity and disease resistance against *A. hydrophila* in a study conducted on grass carp (Wang *et al*., 2005b).

Bacteria colonization in gut is another possible mode of action to enhance the disease resistance in fish. As mentioned above, probiotics modulated the host intestinal community and the pathogens were excluded in the gut during the competition of adhesion sites (Chabrillon *et al.,* 2005; Vine *et al.,* 2004). Gibel carp (*Carassius auratus*) fed with fermented product of *S. cerevisia*e possessed higher populations of lactic acid bacteria in the intestine (He *et al.,* 2011), the beneficial communities found in fish guts and could inhibit growth of fish pathogens, e.g. *A. hydrophila* and *Streptococcus iniae* by producing lactic acids (Aly *et al.,* 2008; Perdigon *et al.,* 2002) TCM also showed similar actions in gut communities which could promote growth of beneficial bacteria and inhibit the proliferation of opportunistic pathogens, such as *Aeromonas* sp., *Vibrio* sp. (Liu *et al.,* 2004). Therefore, the number of pathogen colonization and hence proliferation on the grass carp intestinal wall could be greatly reduced due to yeast and TCM in feed, the disease incidence and mortality were also reduced afterward.

However, the positive effects of yeast and TCM were less prominent when two supplements were mixed. The combined supplementation impaired the beneficial

effects of yeast on fish growth, immunity and disease resistance to pathogens. There are studies focused on the growth promoting and immune-stimulating effects of herbal medicines (Minomol, 2005; Harikrishnan *et al*., 2009) and probiotics (Oliva-Teles and Goncalves, 2001; Li and Gatlin 2003 & 2005) separately, but not on the combined effects of both supplements. An exceptional study focused on the supplementation of black cumin seeds (*Nigella sativa*) and *Bacillus subtilis* PB6 (CloSTAT) in Nile tilapia diet, and the results revealed the fish resistance to *A. hydrophila* and serum globulins, white blood cell counts and phagocytic activities were substantially enhanced (Elkamel and Mosaad, 2012).

The TCM formulation itself also affected the microbial communities in fish gut, and therefore potential antagonistic effects on modulating microflora may be existed between TCM and yeasts. It was noted that 1% supplementation of TCM formulation containing *Scutellaria baicalensis*, *Astragalus membranaceus*, *Poria cocos*, *Houttuynia cordata* and *Isatis indigotica* could increase the quantity of microflora in common carp intestine (Liu *et al.*, 2004). Moreover, berberine, the active ingredient of *R. coptidis* also inhibited *Staphylococcus* bacteria and coliforms, but did not affect some beneficial species such as lactobacilli and bifidobacteria (Domadia *et al.*, 2008). The inhibition may due to obstruction of the adhesions of

bacteria to intestinal epithelial cells (Sun *et al.*, 1988). As a result, competitions for the colonization between bacteria existed, and the two supplements, TCM and yeast may interfere with each other when modulating gut microflora. More investigations should be performed to reveal and validate the possible interactions between the yeast and TCM to the host, in particular, the effects on microbial community in gut.

7.5 Conclusion

The hypothesis was rejected based on the experimental results, no additive beneficaial effects of mixed yeast and TCM were observed on fish immunity and growth. In general, this study was the first attempt to investigate the effects of mixed TCM and baker's yeast on the immune parameters, growth performance and disease resistance to *A. hydrophila* of grass carp. The baker's yeast, *S. cerevisiae* at the dose of 2.5% promoted the growth rate and the levels of IgI, bactericidal activity and NBT activity in grass carp after feeding for 28 days, while 2% of TCM stimulated the bactericidal activity and total IgI level. The disease resistance to *A. hydrophila* was also promoted after baker's yeast or TCM feeding. However, only enhanced NBT activity was observed in the Mixed group, the combined supplementation of TCM and baker's yeast impaired the positive effects of yeast on grass carp growth, immunity

and disease resistance to pathogens. Therefore, supplementation of either yeast (2.5%) or TCM (2%) is recommended for enhancing immunity and against *A. hydrophila* infections in grass carp.

Chapter 8 General Discussion and Major Conclusions

8.1 Introduction

The fast growing aquaculture industry accompanied with increasing prevalence of fish diseases and declining availability of traditional feed materials (e.g. fish meal and soy bean meal), has urged the need to search alternative sources for medication and feed stuff. In addition to from fish nutrition and immunity, economic and environmental concerns should be considered when seeking for the alternatives. In this study, food waste was used as feed stuff in rearing fish, with meat and fish wastes good protein and lipid sources, while fruit and vegetable wastes rich in non-fibrous carbohydrate for fish growth (García *et al.*, 2005).

Grass carp and grey mullet are herbivorous and omnivorous fish, respectively, both have fewer requirements on dietary protein than carnivorous species (NRC, 1993). They are ideal species for investigating the feasibility of using food waste as fish feeds, as they are major species reared in Hong Kong with lower nutritional requirements. In Chapter 2, the cereal dominant feed (FW A) was found to be most suitable for feeding grass carp and grey mullet with superior growth performance observed (in terms of SGR, RWG and PER), when compared to feeds (FWB & C) which contained meat products. The supplementation of bromelain and papin further enhanced the efficient use of food waste by fish, without lowering fish immunity

(Chapter 3). Subsequent experiments on the addition of baker's yeast and TCM as feed supplements were conducted. The grass carp immunity e.g. bactericidal activity and growth was enhanced after feeding with TCMs (Chapter 6) and baker's yeast (Chapter 4) in enzymes upgraded food wastes.

The results obtained in different experiments demonstrated the feasibility of using food wastes incorporated with feed supplements (e.g. bromelain, papain, TCMs and baker's yeast) for rearing fish. The aims of this Chapter were to summarize and discuss the overall results previous chapters in three aspects; 1) the effects of adding enzymes in enhancing food waste utilization by fish, 2) the effects of adding TCMs and baker's yeast to cope with fish infections; and 3) the concerns on using feed supplements for application in aquaculture. Finally, overall conclusions and comments on future studies were included.

8.2 General Discussion

8.2.1 The effects of adding enzymes in enhancing food waste utilization by fish

Based on the results of previous experiments, food waste feed (FW A) without any supplements impaired fish growth (Chapters 2 and 3) and immunity (Chapters 3 and 4) (Table 8.1). The poor growth response could be due to two reasons: improper feeding ingredients and excessive dietary lipid, giving rise to poor immunity. After

incorporating with papain, bromelain and baker's yeast, the food waste feed (FW A) showed a similar growth performance as the commercial feed.

Chapter 2 showed that the cereal dominant food waste feed (FW A) was more suitable for the growth (in terms of SGR, RWG, FCR and PER) of grass carp and grey mullet, compared to the FW B and FW C which contained meat products (Table 8.1). Grass carp (herbivorous) preferred plant protein and its growth is susceptible to dietary lipid (Javed & Watanabe, 2000; Du *et al.*, 2009). The feed digestibility and utilization further confirmed these. The protein digestibility of FW A in grass carp was 83.29%, compared to 78.64% and 73.05% of FW B and FW C respectively. The ANPU of grass carp and grey mullet feeding with FW A were 43.39% and 14.45%, respectively. The carcass protein contents of grass carp feeding with three food waste feeds were also lower than the control (Jinfeng®, 613 formulated feed), especially in FW C which was significantly lower than all other groups ($p<0.05$). Lower carcass protein contents were retained in grey mullet feeding with food waste feeds, but not significantly lower than the control ($p>0.05$). In general, although all the parameters: protein digestibility, ANPU, SGR, RWG, FCR, and PER of FW A were lower than the control feed, FW A had the best performance among the three types of food waste feed.

The high lipid contents in food waste feeds (13.3% in FW B and 19.0% in FW C) hindered the growth of grass carp and grey mullet (Chapter 2). The lipid digestibility of FW A in grass carp was 86.15%, compared to 75.50% and 80.64% of FW B and FW C, respectively. A higher ANLU was observed in grass carp feeding with FW B and FW C than FW A, though not significantly different ($p>0.05$). The lower lipid digestibilities of FW B and FW C indicated poor digestion and adsorption by grass carp. Gao *et al.* (2010) also demonstrated a positive correlation between the lipid content of grass carp carcass and dietary lipid levels in food wastes, same as observed in the present study. Gao *et al.* (2010) also confirmed that the fish grew best when fed with CHO: L ratio of 7.5 in the ratio ranged from 1.74 - 202.5 (feed energy and protein levels were maintained at 16.2 kJ/g and 39%, respectively). However, such correlation was not found in the lipid content of grey mullet carcass. In general, grass carp and grey mullet utilized FW A with 30% protein and 6% lipid most efficiently among three types of food waste feeds.

As a result, attempts were made to upgrade FW A by adding bromelain and papain (1% and 2% of mixtures) for enhancing feed utilization by grass carp and grey mullet (Chapter 3) (Table 8.1). The growth of grass carp and grey mullet was indeed enhanced by supplementing mixture of bromelain and papain, with superior growth of both species feeding with 1% mixture, compared with the control ($p<0.05$). Addition

of enzyme mixture also significantly enhanced ANLU in grass carp and ANPU in grey mullet ($p<0.05$). Bromelain and papain could hydrolyze proteins and release shorter peptides in feed, leading to better enhancement on feed digestion and utilization by fish (Fennema, 1996; Singh *et al.* 2011). The enzyme mixture not only assisted feed protein utilization, but also reduced the lipid retention in grass carp. The enzymes could facilitate conversion of energy from lipid than from protein, and provide more energy to sustain growth and metabolic activities. However, there is no specific study related to the effects of exogenous enzymes on lipid utilization in fish, further experiments should be conducted to understand whether dietary lipid could be used as an energy source in carnivorous fish, such as salmon and groupers which are popular species cultured in Europe and Hong Kong, respectively. These species are more capable to utilize lipids, compared with grass carp and grey mullet (Du *et al.*, 2009), and therefore the lipids contained in food waste feeds could be utilized more efficiently.

Fish require sufficient energy for growth, which is usually converted from carbohydrate and lipid, if these are insufficient, more dietary protein is required. If lipid metabolism could be modulated by exogenous enzymes, the dietary protein level may be reduced, leading to lower feed costs. Better lipid metabolism may also favour

Table 8.1 Summary of feeding trials on growth and feed utilization of food wastes supplemented with enzymes, TCM and baker's yeast by fish

Feed types	Feed supplements	Feeding duration (Days)	Fish species	Major findings
Chapter 2 Control (Jinfeng®, 613 formulated feed) and FW A, B & C	--	35 days (grass carp), 30 days (grey mullet)	grass carp, grey mullet	- Both species performed the best growth (in terms of SGR, RWG, FCR and PER) fed with FW A, among all food waste feeds, but the growth was lower than control. - Both species utilized plant proteins (FW A) better than animal proteins (FW B & C) (in terms of ANPU). - Protein digestibilities of control, FW A, B and C (by grass carp) were 95.6, 86.2, 75.5 and 80.6% respectively. - Higher levels of lipid were observed in feeds contained meat products (FW B & C) which also affected fish growth.
Chapter 3 Control(without supplements), FW A and FW D	Mixture of bromelain & papain (1% & 2%)	56	grass carp, grey mullet	- Growth performance of both species was the best when fed with FW A supplemented with 1% enzyme mixture (FW A 1%), with impaired growth if without enzymes. - Addition of 1% and 2% enzyme mixtures significantly enhanced ANLU in grass carp and ANPU in grey mullet. - Decreased carcass lipid was significantly correlated with the increase of enzymes in FW A (r=0.630) & FW D (r=0.669).
Chapter 4 Control, FW A and FW D	Baker's yeast, *Saccharomyces cerevisiae* (2.5% & 5%)	56	grass carp	- Better growth rates in yeast supplemented feed groups, in terms of FCR, PER, SGR and RGW. - ANPU of yeast supplemented feed groups were generally higher than groups without yeast.
Chapter 6 Control	TCM formulation (ratio: 1:1:2:3), *Radix scutellaria*, *Rhizoma coptidis*, *Herba andrographis* and *Radix sophorae flavescentis* (0.5%, 1% & 2%)	21	grass carp	- Higher growth rates (SGR & RGW) in TCM feed groups, but not significantly different compared with control. - Cost of using TCM formulation was insignificant during production and could be compensated by improved yield (lowered mortalities to *A. hydrophila* injection).

the fish flesh quality, as increase in lipid content could affect flesh quality in terms of taste, smell, sight and touch, which generally named as organoleptic properties

Fish require sufficient energy for growth, which is usually converted from carbohydrate and lipid, if these are insufficient, more dietary protein is required. If lipid metabolism could be modulated by exogenous enzymes, the dietary protein level may be reduced, leading to lower feed costs. Better lipid metabolism may also favour the fish flesh quality, as increase in lipid content could affect flesh quality in terms of taste, smell, sight and touch, which generally named as organoleptic properties (Lopparelli *et al.*, 2004). Therefore, chemical compositions such as protein, lipid and fibre in flesh are also the concerns of fish farmers, which could affect fish product prices.

The food waste feeds impaired the growth and the immunities of both species (Table 8.2). Poor nutrient conversion from food waste feeds in fish resulted in lower fish plasma protein levels, total IgI and NBT activities in blood (Chapter 3). Significant reductions of total IgI and total serum protein levels were found in grass carp fed with FW A, without enzyme mixture at different sampling days ($p<0.05$). However, the enzyme mixture (1% or 2%) could compensate the negative impacts of food wastes on growth and immunity of fish, and elevate the levels of NBT activity, total protein and total IgI levels in grey mullet and grass carp after feeding for 56 days.

Table 8.2 Summary of effects of TCM and baker's yeast on fish immunity

Feed types	Feed supplements	Experimental period (Days)	Fish species	Major findings
Chapter 3 Control (without supplements), FW A and FW D	Mixture of bromelain and papain (1% & 2%)	56	grass carp, grey mullet	- Enzyme mixture (1% or 2%) minimized the negative impacts of food waste feeds on blood NBT activity, total plasma protein and total IgI levels.
Chapter 4 Control, FW A (with and without 1% enzyme mixture)	Baker's yeast, *Saccharomyces cerevisiae* (2.5% & 5%)	56	grass carp	- Fish immunity (higher NBT activity and bactericidal activity) was stimulated when fed yeasts (2.5% & 5%). - Enhanced disease resistance (to *A. hydrophila*) in fish when fed with 2.5% yeast in enzyme supplemented FW A, with 33.3% mortality, compared to 66.7% of control.
Chapter 6 Control	Formulation of *R. scutellaria* (Rs), *R. coptidis* (Rc), *H. andrographis* (Ha) and *R. sophorae flavescentis* (Rsf) (in a ratio of 1:1:2:3, 0.5%, 1% & 2%)	21	grass carp	- Enhanced disease resistance (*A. hydrophila*) of fish when fed 1% and 2% TCM, with 43.3% and 26.7% mortality, compared to 60% of control. - 2% TCM significantly increased the plasma bactericidal activity and total IgI.
Chapter 7 Control (without supplements), FW A with 1% enzyme mixture	TCM formulation (2%), baker's yeast (2.5 %) and mixed both	28	grass carp	- 2.5% baker's yeast promoted the growth and the IgI levels, bactericidal activity and NBT activity. - 2% TCM stimulated the bactericidal activity and total IgI levels. - Higher disease resistance to *A. hydrophila* when fed with baker's yeast or TCM, with mortalities of 42.4% and 45.5% respectively, compared to control (57.6%). - Combined both supplements impaired the positive effects of yeast on fish growth, immunity and disease resistance to pathogen.

Protein is responsible for the formations of hormone, enzyme and immunoglobulin which are important for metabolic functions. Serum proteins are responsible for innate immune response and a higher level of serum protein usually indicates stronger immunity in fish (Sahu *et al.*, 2007a). The growth and immunity of fish would be affected if the nutrients in food wastes cannot be utilized and absorbed fully by fish. It is noted that the impaired immunity was less obvious in grey mullet than that of grass carp. This further suggested different fish species possess different requirements on feed nutrients and utilization mechanisms for their growth.

8.2.2 The effects of adding of TCMs and baker's yeast to cope with fish infections

The probiotics and TCMs could be applied as feed supplements for enhancing feed conversion, pathogen inhibition, growth and immune response of fish (Citarasu *et al.*, 2006; Wang *et al.*, 2005a). The results described in Chapters 2 and 3 showed that food waste feeds with enzyme mixture did not substantially enhance fish immunity, so to further boost immunity, TCMs and baker 's yeast were applied to enhance grass carp immunity and resistance to bacterial infection (Table 8.2). Supplementation of baker's yeast (*S. cerevisiae*) in feeds could enhance growth, immunity and disease resistance to *A. hydrophila* in grass carp (Chapter 4). The immunity parameters such as oxidative radical production activity in blood (NBT

activity) and plasma bactericidal activity were promoted, especially 56 days after feeding with 5% of baker's yeast in various types of feeds: commercial feed, FW A with and without enzyme mixture. The reactive oxygen radicals (quantified by NBT assay) are harmful to bacterial pathogens and are important for killing bacteria in fish (Hardie *et al.*, 1996; Itou *et al.*, 1996). The stronger plasma bactericidal activity indicated the stronger innate immune responses and could enhance disease resistance to pathogens (Das *et al.* 2009; Wang *et al.*, 2005b). The baker's yeast contained β-glucan could also stimulate the growth and immunity of fish and enhance the survival of rohu (*L. rohita*) fingerlings (Misra *et al.*, 2006).

These enhanced parameters could boost the disease resistance of grass carp and reduce the mortality of fish injected by *A. hydrophila* ($p<0.05$). The mortality of 2.5% yeast in FW A with 1% enzyme mixture was almost halved (33.3%), compared to the commercial feed without any supplements (66.4%). Other treatments with 2.5% yeast in FW A without enzymes and 5% yeast in commercial feed also reduced the mortalities (44.4% and 52.8%, respectively).

The addition of TCM formulation which comprised of *R. scutellaria*, *R. coptidis*, *H. andrographis* (Ha) and *R. sophorae flavescentis* (Rsf) in the ratio of 1:1:2:3 also promoted immunity of grass carp (with 2%TCM). Significant enhancements ($p<0.05$) on the plasma bactericidal activity, total immunoglobulin and

disease resistance to *A. hydrophila* were found after feeding with 2% TCM feed for 21 days (Chapter 6). The extracts of TCM formulation stimulated the plasma bactericidal activity in the *in vitro* study (Chapter 6) (Table 8.3), further confirmed the same positive results observed in the feeding trial. In addition, the formulated TCM was more effective in enhancing the bactericidal activities of both normal and heat inactivated plasma than single herbs, at a lower dose (6 mg/L). This suggested that synergetic effects exist in the mixed herbs.

In general, both TCM and baker's yeast could enhance grass carp immunity, growth and disease resistance to *A. hydrophila*. A further experiment mixing both supplements was conducted in order to investigate the combined effects (Chapter 7). No addictive effects from TCM and yeast were demonstrated on both growth and immunity of grass carp. Mortalities were reduced by 15% and 20% in the grass carp feeding with TCM and yeast supplemented groups respectively, compared to control group (~59%), while the mortality in the group with mixed yeast and TCM was only lower by 6%. Similar effects in the group with mixed TCM and yeast on NBT activity in blood, plasma bactericidal activity and immunoglobulin level were also revealed.

The modulation of gut microbial community is a possible mode of action of TCM or yeast in enhancing fish growth, immunity and disease resistance. Baker's yeast attached to the gut wall could stimulate enzyme secretion such as amylase

(Tovar *et al.*, 2002; Irianto and Austin, 2002), while the competitions by exogenous microorganisms in feed could eliminate some pathogens e.g. *Vibrio* spp. in gut (Vine *et al.*, 2004).

TCM showed prominent antimicrobial activities on various bacteria, particularly *R. coptidis* which inhibited the isolated pathogens from diseased fish, e.g. *L. garvieae*, *V. cholreae* and *A. hydrophila* with MIC of 0.08, 0.08 and 0.32 mg/mL, respectively (Chapter 5) (Table 8.3). TCM also showed similar actions to gut community which could promote growth of beneficial community (e.g. lactobacilli and bifidobacteria) and inhibit the proliferation of opportunistic pathogens such as *Aeromonas* spp., *Vibrio* spp. (Liu *et al.*, 2004). The results presented in Chapter 5 showed that *R. coptidis* and *R. scutellaria* strongly inhibited the growth of *A. hydrophila*, with MICs 0.32 and 2.56 mg/mL, respectively. The presence of both TCM and yeast may interfere with each other, when modulating gut mircoflora leading to competitions between bacteria for the colonization in fish gut. However, these deductions should be confirmed by direct enumeration on the microbial diversity in fish gut, feeding with different supplements.

Based on the present results, the addition of yeast was the best on stimulating growth, immunity and disease resistance in grass carp, compared to TCM and mixed

Table 8.3 *In vitro* studies of antimicrobial activities and immuno-stimulating properties of TCM

Tested TCMs	Extract type & concentrations	Method	Major findings
Chapter 5 *R. coptidis, R. astragali, H. andrographis, H. houttuyniae, R. scutellariae, R. angelicae sinensis, A. capillaries, C. monnieri, R. isatidis, F. isatidis, R. glycyrrhizae, R. rhei, C. phellodendri, S. sinapis, F. forsythiae, F. gardeniae jasminoidis* and *R. sophorae flavescentis*	Boiled aqueous extract, 0.04-40.96 mg/mL	MIC determined by Micro-dilution method (NCCLS, 2001)	- *C. phellodendri, R. scutellaria, R. coptidis* and *F. forsythiae* showed stronger antimicrobial activities among tested TCMs. - *R. coptidis* showed strongest inhibitions, especially on fish pathogens, *L. garvieae, V. cholreae* and *A. hydrophila* (0.08, 0.08 and 0.32 mg/mL respectively). - *S. aureus* was the most susceptible pathogen (inhibited by 64.7% of TCM extracts), while *S. marcescens* was the most tolerant bacteria species (0%).
Chapter 5 *C. phellodendri, R. scutellaria, R. coptidis* and *F. forsythiae*	Hexane, ethanol, DCM and aqueous extracts, 0.04-40.96 mg/mL	Micro-dilution method (NCCLS, 2001)	- ethanol and DCM extracts of *R. scutellaria* possessed stronger antimicrobial activities on bacteria than aqueous extracts.
Chapter 5 Combinations of *C. phellodendri, R. scutellaria, R. coptidis* and *F. forsythiae*	Boiled aqueous extract, 0.04-40.96 mg/mL	Micro-dilution method (NCCLS, 2001), FIC indices (Schelz *et al.*, 2006)	- No synergetic effect on antimicrobial activity when *R. coptidis* combined with other TCMs - Enhanced inhibition when mixed with TCM with low antimicrobial activities e.g. *F. forsythiae* and *C. phellodendri* on *A. hydrophila* (MIC= 2.56mg/mL, FIC = 0.38) and *F. forsythiae and R. scutellaria* on *V. cholerae* (MIC= 1.28mg/mL, FIC = 0.50)
Chapter 5 *C. phellodendri, R. scutellaria, R. coptidis* and *F. forsythiae*	Boiled aqueous extract, 0.04-40.96 mg/mL	21 consecutive passages of bacteria at 1/2 MICs of TCMs or antibiotics and then 7 passages without drugs, MICs tested at 1, 7, 14, 21, 28 passages	- MICs of *V. cholerae, L. gariveae* and *A. hydrophila* against TCM extracts did not increase dramatically (<4 folds) - *R. coptidis* strongly inhibited fish pathogens e.g. *V. cholerae* and *L. garvieae* but with less drug resistance developed
Chapter 6 *R. scutellaria, R. coptidis, H. andrographis* and *R. sophorae flavescentis*	Boiled aqueous extract, 6, 20 and 60 mg/L	Grass carp's plasma bactericidal activity on *A. hydrophila*	- TCM formulation enhanced bactericidal activity of both normal and heat inactivated plasma more effectively than single herbs at a lower dose (6 mg/L)

FIC: Fractional inhibitory concentration indices, MIC: Minimum inhibition concentration, DCM: Dichloromethane

both supplements. The baker's yeast (2.5%) increased the growth rate and levels of IgI, bactericidal activity, NBT activity and disease resistance in grass carp after feeding for 28-56 days. TCM (2%) also stimulated the bactericidal activity, total IgI level and disease resistance after 21-28 days, but the effects were less prominent (Chapter 7). Supplementation of either yeast (2.5% with 28-56 days) or TCM (2% with 21-28 days) in feeds, could be used for immune-stimulating purpose, against *A. hydrophila* infections.

8.2.3 Concerns on using feed supplements for application in aquaculture

Fish feed supplements and their applications in aquaculture have been widely studied. It is believed that herbal and probiotics supplements could enhance fish immunity and growth performance. They can be easily applied in the form of feed supplements, with minimal side effects. The herbs can be powdered and directly incorporated into fish feeds, or applied as herbal decoction. Probiotics supplements can also be applied similarly or as additive to the culture system.

Various positive effects (e.g. higher feed conversion, stimulated plasma bactericidal activity and enhanced resistance to pathogens) by feeding fish with TCM and baker's yeast, and enzyme upgraded feeds (results summarized in Table 8.1 and 8.2) were demonstrated in different experiments (Chapters 3, 4, 6 and 7). However, more studies should be required before the actual application of immuno-stimulants in aquaculture practices. Based on the results obtained in Chapter 4 and 7, yeast supplement (2.5%) showed enhanced bactericidal activity after feeding for 28 and 56 days. The study period

of 56 days is considered short when compared to the production period of ~10 to 24 months for grass carp (depending on the initial size). Most relevant studies showed that the feed supplements (e.g. herbs or probiotics) are beneficial to fish within the feeding period of 1 to 10 weeks (Nayak, 2010; Harikrishnan *et al.*, 2010).

Nevertheless, the growth promoting and immuno-stimulating effects of feed supplements may be affected by the dose and duration, which are the important factors to be considered in the actual application. Grass carp showed lower mortalities when feeding with various doses of TCM formulation, when compared to the control group. Decreased fish mortality (against *A. hydrophila* infection) was observed with increased doses (from 0.5% to 2%) (Chapter 6). Dose and duration of feeding with supplements would affect responses of fish, and the effectiveness of immune-stimulants usually decreased over time, if orally administrated (Sakai, 1999).

The overdoses of TCM may suppress fish immunity, and long-term exposure to single herb or formulation may also show similar suppression. Past studies on rainbow trout fed with peptidoglucan showed increased protection to *V. anguillarum* infection, but the increased resistance observed after feeding for 28 days and disappeared after 56 days (Matsuo and Miyazano, 1993). Yoshida *et al.* (1995) also noted that the increased number of NBT-positive cells due to oral administration of glucan or oligosaccharide to African catfish lasted for 30 days only, and the effects disappeared after 45 days. However, it is still difficult to conclude the effects of feed supplements on growth and immunity of fish, as the effects of immuno-stimulants are varied in different development stages and

species of fish, stocking density, feed composition, concentration and species of probiotics and the feeding regime (Nayak, 2010; Welker and Lim, 2011). In other words, the effects of immuno-stimulants are specific to fish species and doses (Harikrishnan *et al.*, 2011). The optimum dose and duration of applied herbal medicine should be investigated more in depth.

The mixture of TCM extracts was more effective in stimulating plasma bactericidal activity than single herbs, with strong activation in normal and heat inactivated plasma under a wider range of doses (6-60 mg/L). Combinations of RcHa, RsHa, RcRsf and RsRsf were effective at the low dose (6 mg/L), while the mixture of RcRsHaRsf in ratio of 1:1:2:3 showed stimulations on both normal and heat inactivated plasma at all doses (6, 20 and 60 mg/L).

Finally, specific dose and duration should be considered when using TCM or baker's yeast for fish culture. Based on the present results, the optimal dose and duration of the formulated TCM were 2% (w/w) and 21 to 28 days, respectively for grass carp fingerlings. Furthermore, baker's yeast could be applied in feed at 2.5% for 28 to 56 days as immuno-stimulant and growth promoter.

8.3 Major conclusions

The present study focused on using food waste for rearing freshwater fish: grass carp and grey mullet with the supplementation of enzymes mixture (bromelain and papain), baker's yeast and Traditional Chinese Medicine (TCM). Based on the results, the

following conclusions could be drawn:

1) The cereal dominant food waste feed (FW A) was more suitable for grass carp and grey mullet, with the best growth performance (in terms of SGR, RWG, FCR and PER) and higher protein digestibility (in grass carp), compared to FW B and FW C which contained higher proportions of meat products (Chapter 2). The high lipid contents in FW B (13%) and FW C (19%) were also a possible reason for hindering fish growth. In addition, the NBT activities in blood and plasma protein levels were decreased in the grass carp, cultured with food waste feeds without any supplements (Chapter 3).

2) Upgrading FW A by the addition of 1% and 2% mixture of bromelain and papain could significantly increase the feed protein solubility and subsequent to growth (SGR, RWG) and feed utilization (FCR, ANPU, PER and ANLU) in both fish species (Chapters 3 and 4).

3) The utilization of feed with enzyme mixtures by grass carp and grey mullet were different, with grass carp showing a reduced lipid retention in body when feeding with enzyme supplemented feed. The results suggested that grey mullets may possess different utilization efficiency of lipid, and are more capable to utilize dietary lipid as energy source, especially feeding with diets containing low protein and high lipid contents.

4) The protein and feed utilizations by grass carp were promoted by the yeast supplements (2.5 or 5%) (Chapter 4). The yeast supplemented feed groups generally showed better growth rates than the feed without yeast, in terms of FCR, PER, SGR and RWG, and the optimal dose was 2.5% yeast (*S. cerevisiae*) adding to FW A upgraded by

bromelain and papain. This showed that yeast could further enhance nutrient utilization contained in feeds after addition of bromelain and papain.

5) *R. coptidis* and *R. scutellaria* possessed the strongest antimicrobial activity on fish pathogens (such as *A. hydrophila, L. garvieae* and *V. cholerae*) among the 17 tested TCMs, and TCM extracts were less likely for developing drug resistant pathogens than antibiotics (Chapter 5). The *in vitro* study on the grass carp's plasma treated with TCM extracts also showed that TCM extracts could stimulate plasma bactericidal activity (on *A. hydrophila*) through enhancing complement activity. The formulation with *Radix scutellaria*, *R. coptidis*, *H. andrographis* and *R. sophorae flavescentis* in the ratio of 1:1:2:3 was more effective in enhancing plasma bactericidal activity than single TCM extracts (Chapter 6).

6) Grass carp immunity (NBT activity in blood, plasma bactericidal activity, and total immunoglobulin level) was boosted by the addition of TCM formulation and baker's yeast (*S. cerevisiae*) (Chapters 4, 6 and 7). The disease resistance to pathogen (*A. hydrophila*) was also enhanced, with significantly lower mortalities observed in groups feeding with TCM (1 and 2% for 21 to 28days) and baker's yeast (2.5 and 5% for 28-56 days).

7) No synergetic or additive effects of mixed TCM and baker's yeast on growth, immunity and disease resistance (to *A. hydrophila*) in grass carp were noted (Chapter 7). The enhancement on the growth rate and immunity of grass carp was less effective when compared with TCM formulation or yeast. The combined use of both supplements may

impair the effects of TCM formulation or yeast in the modulation of gut mircoflora, and upset the balance of beneficial communities. Further investigations are needed to reveal the interactions of supplements in fish guts.

8) The use of yeast and TCMs led to positive effects on growth, immunity and disease resistance to pathogens in fish. Suitable dose and duration are crucial in promoting fish growth. Supplementations of either 2.5% yeast, and feeding of 28 to 56 days or 2% TCM and 21 to 28 days are effective in stimulating growth and immunity and enhancing resistance to *A. hydrophila* infection in grass carp.

8.4 Comments for future studies

Biological experiments involving fish (i.e. feeding trials) are expensive, laborious and time consuming. In various procedures such as maintaining fish culture setup, taking fish blood sampling and conducting chemical analyses. However, these feeding experiments could evaluate fish growth more precisely and directly.

In general, a fish feeding experiment requires at least 4 to 6 months from the preparation of the fish tank filter system to laboratory chemical analyses, but any errors or accidents within the fish culture period (e.g. unstable electricity, fish infected by diseases and worn-out filter system) could induce stress to fish and sometimes need to terminate the whole experiment. More effective and time saving methods are necessary in order to evaluate fish growth using different feeds and the effects of immuno-stimulants.

In vitro study on feed quality, the method used for protein digestibility method is less expensive and, time conserving for determining protein quality of feed ingredients. This method utilized fish digestive enzyme to evaluate the digestibility of nutrients contained in fish feeds, and could reveal the breakdown protein from raw materials (Dimes and Haard, 1994). It is a promising tool to identify the protein quality (i.e. protein digestibility and availability to fish) in different fish feeds and then select proper feed ingredients for specific fish species. However, validations by *in vivo* study, using the traditional method of feeding Cr_2O_3 added feed are needed. More investigations on strengthening the correlation between results generated by *in vivo* and *in vitro* studies should be performed.

The feasibility of using TCMs as immuno-stimulants could also be investigated through preclusive *in vitro* tests e.g. plasma bactericidal activity tests as performed in Chapter 4. This test could reduce the time needed in screening plentiful combinations of TCM, but again further investigations on the linkage between results derived from *in vitro* study and feeding trials (*in vivo*) are essential, with the effects confirmed by feeding trials. A single TCM formulation may also be effective in preventing other diseases based on the theory of TCM. This multi-target strategy for disease treatment is a novel trend in the drug design paradigm in modern pharmacology (Csermely *et al.*, 2005). However, more investigations on the same formulation for combating other diseases e.g. bacterial, viral and parasitic diseases are crucial in future studies.

As numerous types of immuno-stimulants e.g. probiotics, herbs and prebiotics are being investigated and applied in aquaculture, the combined effects of multiple supplements should be known. It is expected that co-existence of several types of immuno-stimulants in the same aquaculture zone are probable as different fish farmers may apply different immuno-stimulants. In this study, combined supplements impaired effects of TCM and yeast on fish growth and immunity of grass carp, which may be due to interference on the modulation of host gut mircoflora. Further investigations are needed to confirm the interacting effects before actual application of combined supplements.

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