Food wastes as feeds incorporated with Chinese herbs and prebiotic fibers on growth and non-specific immunity of grass carp, bighead, mud carp and Nile tilapia

Wing Yin Mo
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Food Wastes as Feeds Incorporated with Chinese Herbs and Prebiotic Fibers on Growth and Non-specific Immunity of Grass Carp, Bighead, Mud Carp and Nile Tilapia

MO Wing Yin

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy

Principal Supervisor: Prof. WONG Ming Hung

Hong Kong Baptist University

August 2014
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: August 2014
Abstract

Food waste accounts for about 1/3 of the municipal waste generated in Hong Kong. Using food waste as major ingredients to produce fish feed pellets could ease part of the disposal pressure on the existing landfill sites. The present study focused on the use of food wastes and feed supplements (prebiotic fibres and Chinese herbs) for rearing freshwater fish (grass carp, bighead, mud carp and Nile tilapia) in Hong Kong.

Two isonitrogenous formulations, Food Waste Diet A (FWA), consisted of 53% cereal, 10% fruit and vegetables, 8% bone meal, 4% other food waste, 10% fishmeal, 15% corn meal and Food Waste Diet B (FWB), consisted of 25% meat, 28% cereal, 10% fruit and vegetables, 8% bone meal, 4% other food waste, 10% fishmeal, 15% corn meal were manufactured by Kowloon Biotechnology Ltd, were used as feeds for rearing grass carp, bighead, mud carp and Nile tilapia.

The essential amino acid profiles and proximate compositions (crude protein, crude lipid and total phosphorous) of the two food waste based feed pellets were compared with other common feed items, including Napier grass, rice bran, breads, noodles, soybean dreg (remains of soybean after juicing for soybean milk) and the commercial feed pellets (Jiefeng® 613). Jiefeng® 613, FWA and FWB possessed 5.83%, 5.76% 5.79% (% protein) of lysine, which could satisfy the dietary requirements of both grass carp and Nile tilapia. Results indicated all the fish feed pellets possessed sufficient essential amino acids and suitable proximate compositions (crude lipid, crude protein, crude fibre and non-fibrous carbohydrate) for both grass carp and Nile tilapia.

A field trial was conducted using the three feed pellets (Jiefeng® 613, FWA and FWB) to study their effects on fish growth (grass carp, bighead and mud carp) as well as water quality. FWA that possessing a higher P content (2770 μg/g feed, while control= 967 μg/g feed and FWB= 1942 μg/g feed) favoured the growth of plankton and led to better growth of bighead carp (in terms of length gain, wet weight gain and productivity), while grass carp fed with FWB showed significant better growth (in terms of length gain, wet weight gain, productivity,
feed conversion ratio, specific growth rate and protein efficiency ratio), probably due to the relatively lower amount of carbohydrate (24.2%) and CHO:L ratio (1.83) than Control and FWA. Mud carp grew equally well in ponds fed with the three diets. FWB was subsequentially selected for further feeding experiments.

A laboratory feeding trial was conducted to study the effects of feeding grass carp and Nile tilapia with FWB on their growth performance in terms of relative weight gain (RWG), feed conversion ratio (FCR), specific growth rate (SGR) and protein efficiency ratio (PER), and protein digestibility. Both fish fed with FWB showed similar growth performances to groups fed with control diet (Jiefeng® 613), while grass carp showed impaired protein digestibility when compared to group fed with control diet. FWB supplemented with 0.3% of vitamin-mineral premix (VMP) significantly improved the growth performance of both fish species and protein digestibility for grass carp. Results suggested that FWB incorporation with VMP would be necessary as it significantly enhanced growth of the fish.

Four dietary supplements (inulin, mannan-oligosacharride, huangqi and goji, at the rates of 0.2% or 2%, w/w) were incorporated into FWB for further enhancing fish growth as well as non-specific immunity of grass carp and Nile tilapia. Grass carp fed with 0.2% and 2% inulin, 2% MOS and 0.2% goji, and Nile tilapia fed with 0.2% goji had significantly enhanced growth (RWG, FCR, SGR and PER). Both prebiotic fibres and Chinese herbs boosted the tested non-specific immune parameters (total serum immunoglobin, serum bactericidal activity and anti-protease activity) of both species. Among all the dietary supplements, 0.2% goji appeared to be the best supplement for both grass carp and Nile tilapia as it significantly enhanced the growth among all experimental diets. Grass carp and Nile tilapia fed with 0.2% showed about 10% and 30% higher RWG, 10% and 30% lower FCR value, respectively, than groups fed with other experimental diets. Moreover, all the tested non-specific immune parameters (total serum immunoglobin, serum bactericidal activity and anti-protease activity) were significantly enhanced when compared with the groups fed with control diets (Control and FWB without supplementation).

The present study demonstrated the feasibilities of using food wastes incorporated with feed
supplements (prebiotic fibres and Chinese herbs) to enhance fish growth and immunity, for the sustainable development of Hong Kong inland aquaculture.
Acknowledgement

I would like to express my deepest gratitude to my principal supervisor, Prof. Ming Hung Wong, for providing me with professional guidance, continuous support and encouragement throughout my study.

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# Table of content

Declaration......................................................................................................................................................... i

Abstract................................................................................................................................................................... ii

Acknowledgements............................................................................................................................................... v

Table of Contents................................................................................................................................................ vi

List of Tables......................................................................................................................................................... x

List of Figures......................................................................................................................................................... xii

Abbreviations and Acronyms................................................................................................................................. xv

# Chapter 1 General Introduction

1.1 Food waste as an environmental issue........................................................................................................... 1

1.2 History and current situation of inland fish culture industry in Hong Kong: problems experienced and opportunity ................................................................................................................................. 2

1.3 Farming practice adopted by local fish farmers................................................................................................. 4

1.4 The need for alternative protein source ........................................................................................................... 8

1.4.1 Increment in feed prices................................................................................................................................. 8

1.4.2 Replacing fishmeal with other protein sources.............................................................................................. 8

1.5 Using feed supplements for upgrading fish diets............................................................................................ 10

1.5.1 Alternatives to antimicrobial agents for promoting fish health and growth.................................................. 10

1.5.2 Prebiotic fibres............................................................................................................................................... 11

1.5.3 Chinese herbs................................................................................................................................................ 13

1.6 Aims and objectives......................................................................................................................................... 14

1.7 Contributions and significances of the present research.................................................................................. 17

# Chapter 2 Proximate compositions and essential amino acid profiles of common used fish feeds used by local fish farmers and food waste based feed pellets

2.1 Introduction......................................................................................................................................................... 18

2.2 Materials and Methods.................................................................................................................................. 20

2.2.1 Fish feed pellets production........................................................................................................................ 20

2.2.2 Common used feed preparation .................................................................................................................. 21
Chapter 3 Effects of food waste based feed pellets on polyculture of carps and water quality: A field trial

3.1 Introduction.................................................................................................................... 36
3.2 Materials and Methods................................................................................................... 38
   3.2.1 Fish diets............................................................................................................. 38
   3.2.2 Fish ponds, fish and feeding trial........................................................................ 38
   3.2.3 Proximate compositions of fish diets................................................................. 40
   3.2.4 Sampling scheme for fish and water quality monitoring.................................... 41
   3.2.5 Total plankton estimation................................................................................... 42
   3.2.6 Statistical analyses.............................................................................................. 42
3.3 Results and Discussion................................................................................................. 42
3.4 Conclusions..................................................................................................................... 54

Chapter 4 Upgrading food waste based diets with vitamin-mineral premix: effects on feed protein digestibility and fish growth

4.1 Introduction..................................................................................................................... 56
4.2 Materials and Methods.................................................................................................... 58
   4.2.1 Fish and fish tanks............................................................................................... 58
   4.2.2 Experimental diets............................................................................................... 59
   4.2.3 Growth performance of fish................................................................................ 59
   4.2.4 Chemical analyses of fish carcass and apparent protein digestibility of diets.... 62
   4.2.5 Statistical analyses.............................................................................................. 64
4.3 Results and Discussion................................................................................................. 64
4.4 Conclusions..................................................................................................................... 72
Chapter 5 Upgrading food waste based feeds with prebiotic fibres: effects on feed digestibility, growth and non-specific immunity of fish

5.1 Introduction ................................................................. 73
5.2 Materials and Methods .................................................. 75
   5.2.1 Fish, fish tanks and maintenance .................................. 75
   5.2.2 Experimental diets and diet preparation ....................... 75
   5.2.3 Growth performance of fish ................................... 76
   5.2.4 Fish immune parameters....................................... 78
   5.2.5 Apparent protein digestibility of diets .................... 80
   5.2.6 Statistical analyses ............................................. 80
5.3 Results and Discussion ............................................... 80
5.4 Conclusions .............................................................. 91

Chapter 6 Upgrading food waste based feeds with Chinese herbs: effects on feed digestibility, growth and non-specific immunity of fish

6.1 Introduction ................................................................. 93
6.2 Materials and Methods .................................................. 95
   6.2.1 Fish, fish tanks and maintenance .................................. 95
   6.2.2 Experimental diets and diet preparation ....................... 96
   6.2.3 Growth performance of fish ................................... 97
   6.2.4 Fish immune parameters....................................... 99
   6.2.5 Apparent protein digestibility of diets .................... 99
   6.2.6 Statistical analyses ............................................. 99
6.3 Results and Discussion ............................................... 99
6.4 Conclusions .............................................................. 112

Chapter 7 General discussion and major conclusions

7.1 Introduction ................................................................. 114
7.2 General Discussion ..................................................... 115
   7.2.1 Nutritional qualities of food waste based feeds and the growth performance of
fish fed with food waste based feeds................................................................. 115

7.2.2 Effects of incorporating dietary supplements into food waste based diets (FWB) on fish growth.............................................................. 119

7.2.3 Effects of incorporating dietary supplements into food waste based diets on fish non-specific immunity.................................................. 122

7.3 Major Conclusions...................................................................................... 125

7.4 Limitation of Study..................................................................................... 126

7.5 Future Work............................................................................................... 127

References......................................................................................................... 131

Publications and Conference Presentations.................................................... 151

Curriculum Vitae.............................................................................................. 152
# List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Fish species farmed by pond owners participated in Accredited Fish Farm Scheme (AFFS).</td>
<td>5</td>
</tr>
<tr>
<td>2.1</td>
<td>Essential amino acid contents of fish feeds.</td>
<td>26</td>
</tr>
<tr>
<td>2.2</td>
<td>Amino acid scores of the feed items comparing to the requirements of grass carp.</td>
<td>28</td>
</tr>
<tr>
<td>2.3</td>
<td>Amino acid scores of the feed items comparing to the requirement of Nile tilapia.</td>
<td>29</td>
</tr>
<tr>
<td>2.4</td>
<td>Proximate composition of fish feed items studied in this experiment.</td>
<td>30</td>
</tr>
<tr>
<td>3.1</td>
<td>Compositions of the food wasted based feed pellets.</td>
<td>39</td>
</tr>
<tr>
<td>3.2</td>
<td>Proximate compositions of the experimental diets.</td>
<td>43</td>
</tr>
<tr>
<td>3.3</td>
<td>Growth performance of three studied fish species after 6 months.</td>
<td>45</td>
</tr>
<tr>
<td>4.1</td>
<td>Detailed compositions of experimental diets.</td>
<td>60</td>
</tr>
<tr>
<td>4.2</td>
<td>Table 4.2 Amount of vitamins and minerals in DietB and suggested values.</td>
<td>61</td>
</tr>
<tr>
<td>4.3</td>
<td>Growth performance and protein digestibility of grass carp and Nile tilapia fed with various experimental diets after 4 weeks.</td>
<td>66</td>
</tr>
<tr>
<td>4.4</td>
<td>Proximate compositions of grass carp and Nile tilapia carcass that fed with various experimental diets after 4 weeks.</td>
<td>67</td>
</tr>
<tr>
<td>5.1</td>
<td>Detailed compositions of experimental diets.</td>
<td>77</td>
</tr>
<tr>
<td>5.2</td>
<td>Growth performance of grass carp fed with different experimental diets after 8 weeks.</td>
<td>81</td>
</tr>
<tr>
<td>5.3</td>
<td>Growth performance of Nile tilapia fed with different experimental diets after 8 weeks.</td>
<td>83</td>
</tr>
<tr>
<td>6.1</td>
<td>Detailed compositions of experimental diets.</td>
<td>98</td>
</tr>
<tr>
<td>6.2</td>
<td>Growth performance of grass carp fed with different experimental diets after 8 weeks.</td>
<td>100</td>
</tr>
<tr>
<td>6.3</td>
<td>Growth performance of Nile tilapia fed with different experimental diets after 8 weeks.</td>
<td>102</td>
</tr>
<tr>
<td>7.1</td>
<td>Summary of feeding trials on growth of fish fed with food waste diets</td>
<td>120</td>
</tr>
</tbody>
</table>
supplemented with vitamin-mineral premix, prebiotic fibres and Chinese herbs.

Table 7.2 Summary of feeding trials on non-specific immunity of fish fed with food waste diets supplemented with prebiotic fibres and Chinese herbs.
List of Figures

Fig. 1.1  Fig. 1.1 A schematic map of the response of a fish challenged by pathogen (Shoemaker et al., 2001) p.12

Fig. 1.2  Framework of research p.16

Fig. 3.1  Fig 3.1 Phosphorous compounds in fish ponds. Statistics for P compounds (2 way ANOVA): PO₄-P in water: treatment: p<0.0001, sampling month: P<0.0001, interaction: p<0.0001; Total P in water: treatment: p<0.0001, sampling month: P<0.0001, interaction: p<0.0001; Org P in water: treatment: p<0.0001, sampling month: P<0.0001, interaction: p<0.0001; TP in pond sediment treatment: p<0.0001, sampling month: P<0.0001, interaction: p<0.0001. Black = control, Grey = FWA, White= FWB. Bars that sharing at least one superscript do not differ at p< 0.05. p.46

Fig. 3.2a  Nitrogen compounds in fish ponds. Statistics for N compounds (2 way ANOVA): Organic N: treatment: p<0.0001, sampling month: P<0.0001, interaction: p<0.0001; NH₄-N: treatment: p=0.635, sampling month: P=0.533, interaction: p=0.01; TKN: treatment: p<0.0001, sampling month: P<0.0001, interaction: p<0.0001; Total N: treatment: p<0.0001, sampling month: P<0.0001, interaction: p<0.0001; p.47

Fig. 3.2b  Nitrogen compounds in fish ponds. Statistics for N compounds (2 way ANOVA): NO₃-N: treatment: p=0.733, sampling month: P=0.17, interaction: p=0.763; NO₂-N: treatment: p=0.112, sampling month: P<0.0001, interaction: p=0.001; TKN in sediment: treatment: p=0.214, sampling month: P<0.0001, interaction: p<0.0001; significantly different (p< 0.05). Black = control, Grey = FWA, White= FWB. p.48

Fig. 3.3  Plankton density in fish ponds. Statistics for plankton density (2 way ANOVA): NO₃-N: treatment: p=0.733, sample month: P=0.17, interaction: p=0.763 Black = control, Grey = Diet A, White= Diet B. p.49
Different superscripts (a,b,c) indicated significant difference ($p<0.05$) at the same sampling week. ContA = Control diet A, commercial diet based diet; ContB = control diet B, food waste based diet; Inu02 = 0.2% inulin and food waste based diet; Inu2 = 2% inulin and food waste based diet; MOS02 = 0.2% mannan-oligosaccharide and food waste based diet; MOS2 = 2% mannan-oligosaccharide and food waste based diet.

Different superscripts (a,b,c) indicated significant difference ($p<0.05$) at the same sampling week. ContA = Control diet A, commercial diet based diet; ContB = control diet B, food waste based diet; Inu02 = 0.2% inulin and food waste based diet; Inu2 = 2% inulin and food waste based diet; MOS02 = 0.2% mannan-oligosaccharide and food waste based diet; MOS2 = 2% mannan-oligosaccharide and food waste based diet.

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Values are mean ± SD. Different superscripts (a,b,c) indicate significant differences ($p<0.05$) at the same sampling week. #Growth performance of AM2 group was not recorded after 4 weeks only as most of the fish died 1 month after the commence of the feeding trial. ContA = Control diet A, commercial diet; ContB = food waste based diet with 2% cellulose; AM02 = 0.2% Huangqi and
food waste based diet; AM2 = 2% Huangqi and food waste based diet; LB02 = 0.2% Goji and food waste based diet; LB2 = 2% Goji and food waste based diet.

Fig. 6.2  
Serum bactericidal activity of (a) grass carp and (b) Nile tilapia fed with various experimental diets. Values are mean ± SD. Different superscripts (a,b,c) indicate significant differences (p<0.05) at the same sampling week. #Growth performance of AM2 group was not recorded after 4 weeks only as most of the fish died 1 month after the commence of the feeding trial. ContA = Control diet A, commercial diet; ContB = food waste based diet with 2% cellulose; AM02 = 0.2% Huangqi and food waste based diet; AM2 = 2% Huangqi and food waste based diet; LB02 = 0.2% Goji and food waste based diet; LB2 = 2% Goji and food waste based diet.

Fig. 6.3  
Serum anti-protease activity of (a) grass carp and (b) Nile tilapia fed with various experimental diets. Values are mean ± SD. Different superscripts (a,b,c) indicate significant differences (p<0.05) at the same sampling week. #Growth performance of AM2 group was not recorded after 4 weeks only as most of the fish died 1 month after the commence of the feeding trial. ContA = Control diet A, commercial diet; ContB = food waste based diet with 2% cellulose; AM02 = 0.2% Huangqi and food waste based diet; AM2 = 2% Huangqi and food waste based diet; LB02 = 0.2% Goji and food waste based diet; LB2 = 2% Goji and food waste based diet.

Fig 7.1  
Schematic diagram to show the potential applications of food waste based diets and their upgrades on fish.
### Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACH50</td>
<td>Alternative complement pathway activity</td>
</tr>
<tr>
<td>AFCD</td>
<td>Agriculture, Fisheries and Conservation Department</td>
</tr>
<tr>
<td>AFFS</td>
<td>Accredited Fish Farm Scheme</td>
</tr>
<tr>
<td>AHVLA</td>
<td>Animal Health and Veterinary Laboratories Agency</td>
</tr>
<tr>
<td>AM</td>
<td>Huangqi, <em>Astragalus membranaceus</em></td>
</tr>
<tr>
<td>AOAC</td>
<td>Association of Official Analytical Chemists</td>
</tr>
<tr>
<td>APAH</td>
<td>American Public Health Association</td>
</tr>
<tr>
<td>APS</td>
<td><em>Astragalus</em> polysaccharides</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>BAPNA</td>
<td>α-Benzoyl-L-arginine 4-nitroanilide hydrochloride</td>
</tr>
<tr>
<td>CFS</td>
<td>Center for Food Safety</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Units</td>
</tr>
<tr>
<td>CHO: L</td>
<td>Carbohydrates to Lipid ratio</td>
</tr>
<tr>
<td>ContA</td>
<td>Control Diet A</td>
</tr>
<tr>
<td>ContB</td>
<td>Control Diet B</td>
</tr>
<tr>
<td>Cys</td>
<td>Cystine</td>
</tr>
<tr>
<td>Cr$_2$O$_3$</td>
<td>Chromium Oxides</td>
</tr>
<tr>
<td>DI water</td>
<td>Deionized water</td>
</tr>
<tr>
<td>DM</td>
<td>Dry matter</td>
</tr>
<tr>
<td>DO</td>
<td>Dissolved oxygen</td>
</tr>
<tr>
<td>EAA</td>
<td>Essential amino acid</td>
</tr>
<tr>
<td>EPD</td>
<td>Environmental Protection Department</td>
</tr>
<tr>
<td>EU</td>
<td>European Union</td>
</tr>
<tr>
<td>FAO</td>
<td>Food and Agriculture Organization of the United Nations</td>
</tr>
<tr>
<td>FCR</td>
<td>Feed Conversion Ratio</td>
</tr>
<tr>
<td>FWA</td>
<td>Food Waste Feed A</td>
</tr>
<tr>
<td>FWB</td>
<td>Food Waste Feed B</td>
</tr>
<tr>
<td>GOS</td>
<td>Galactooligosaccharides</td>
</tr>
<tr>
<td>HARVEST</td>
<td>Helping Address Rural Vulnerabilities and Ecosystem</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>RWG</td>
<td>Relative Weight Gain</td>
</tr>
<tr>
<td>SGR</td>
<td>Specific Growth Rate</td>
</tr>
<tr>
<td>TAN</td>
<td>Total ammonia nitrogen</td>
</tr>
<tr>
<td>TCM</td>
<td>Traditional Chinese medicine</td>
</tr>
<tr>
<td>Thr</td>
<td>Threonine</td>
</tr>
<tr>
<td>TKN</td>
<td>Total Kjeldahl nitrogen</td>
</tr>
<tr>
<td>TIG</td>
<td>Total immunoglobin</td>
</tr>
<tr>
<td>TLR4</td>
<td>Toll-like receptor 4</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factors</td>
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<td>TP</td>
<td>Total phosphorous</td>
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<tr>
<td>Try</td>
<td>Tryptophan</td>
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<tr>
<td>USAID</td>
<td>United States Agency for International Development</td>
</tr>
<tr>
<td>USD</td>
<td>US Dollar (United States of America)</td>
</tr>
<tr>
<td>USEPA</td>
<td>United States Environmental Protection Agency</td>
</tr>
<tr>
<td>μl</td>
<td>Microliter</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>Val</td>
<td>Valine</td>
</tr>
<tr>
<td>VMP</td>
<td>Vitamin-mineral premix</td>
</tr>
</tbody>
</table>
Chapter 1 Introduction

1.1 Food waste as an environmental issue

In 2011, about 13,000 tonnes per day of solid waste were disposed at landfill sites (HKEPD, 2012b) and about 3,500 tonnes of the municipal waste were food waste (HKEPD, 2012a), and the volume of food waste doubled in the last 5 years. It is predicted that the existing landfills will be exhausted one by one by 2020 if waste levels continue to increase at current levels.

There are varieties of food waste sources such as leftover generated from restaurants, byproducts from food processing facilities and expired food products from retailing shops. According to USEPA (2012), food waste is any food substance, raw or cooked, which is discarded, or intended or required to be discarded. In general, food wastes are the organic residues generated by handling, storage, sale, preparation, cooking, and serving of foods.

In order to reduce the burden and extend the lifespan of local landfills, the policy framework of the Hong Kong Government on municipal solid waste management (2005-2015) mainly focused on waste reduction through landfill disposal bans, producer responsibility schemes and waste charging (EPD, 2005). Moreover, the reuse, recovery, and recycling of waste have been strongly recommended (EPD, 2005). Some trial facilities were established for recycling food waste. A pilot scale Waste Recycling Centre at Kowloon Bay, with a 4 tonnes/day capacity, was launched in 2008 to handle food waste generated from the Olympic equestrian events. A trial operation for the recycling of source-separated food waste generated was commenced and food waste was collected from selected commercial and industrial sources.
including hotels, wholesale markets, restaurants and generators of the catering, food production, bakery and bean curd industries (EPD, 2009).

Based on the results obtained from different pilot trials, larger facilities were proposed (locating in Siu Ho Wan, Lantau Island, expected to start operation in mid-2010s, and Shaling, North District expected to start operation in the late 2010s) with handling capacity of 200 tonnes of organic material per day (mostly food waste) for producing biogas and compost (20 tonnes) every day (EPD & ENB, 2008). In 2013, public consultation regarding waste charging scheme started. The objective of the charging scheme is to create an economic incentive for the general public to achieve waste reduction (Council for Sustainable Development, 2013).

Those preliminary investigations and works done by the Hong Kong Government suggested that recycling food waste is feasible, but those facilities were not permanent and the handling capacities were too small for coupling with the amount of food waste generated in Hong Kong. Thus, it is suggested to turn food waste generated in Hong Kong into fish feeds as an additional option.

1.2 History and current situation of inland fish culture industry in Hong Kong: problems experienced and opportunity

Inland fish farming activities in Hong Kong have a long history. Polyculture pond-fish farming has been practiced since the 1950s (Suen, 1955; Fung, 1965; Cheong, 1963; Sin and Cheng, 1977). Nowadays, the local inland ponds, covering an area of approximately 1,150 ha,
are mainly located in north-western Hong Kong (AFCD, 2012). In the past, pond-fish farming was a highly profitable industry and pond-fish farming expanded dramatically during the period from 1961 to 1984 (Lau et al., 2003). After that, Hong Kong's pond-fish farming had experienced a substantial decline. Annual production of pond fish decreased from 6,500 tonnes (HK$ 104 million) in 1984 to 1,977 tonnes (HK$ 33 million) in 2004 (by 56.3 and 29.5% of the quantity and monetary value of total fish production, respectively) (Chan, 2005). Traditional fish farming practice is labour intensive, and in January 2002, the China Government removed the export-quota system on freshwater fish leading to more freshwater fish imported into Hong Kong. The import of low-priced freshwater fish products from the mainland China rendered the local fishery less and less profitable.

A number of safety issues relating to the import freshwater fish were reported. For example, malachite green (used for treating parasitic, fungal and protozoan diseases in fish) is probably carcinogenic and harmful to human beings (Culp, 2004), was detected in turbot, eel, freshwater grouper, and mud carp (CFS, 2006a, b, c). The higher number of reported food safety incidences have drawn the attention of local consumers and they are more and more concerned over food safety issues rather than pricing of the fish products. Some consumers are more willing to purchase local products which are believed to have higher quality, although the prices of these local fish products may also be higher. Moreover, more local consumers are willing to pay more for fish labelled as chemicals free (Boulanger et al., 2008). In 2012, local fish farming operators produced 2,306 tonnes of freshwater fish (HK Yearbook, 2013).
1.3 Farming practice adopted by local fish farmers

Currently, about 98% of the local fish farms are engaged in polyculture (bighead carp, grass carp, common carp and silver carp in combination with tilapia or grey mullet), and waste-fed polyculture system is the dominated farming practice adopted (ACFD, 2014a).

Detailed information regarding to the fish species farmed by fish farmers participated in the Accredited Fish Farm Scheme (AFFS) was extracted from AFCD database (provided by Dr. Eric PM Yau, Fisheries Officier of AFCD) (Table 1.1). AFFS is a voluntary scheme, which aims to promote and enhance the quality and safety of locally produced fish products (AFCD, 2014b). Different species of fish were farmed by farmers participated in the scheme. Commonly farmed species include jade perch (*Scortum barcoo*), grey mullet (*Mugil cephalus*), bighead (*Hypophthalmichthys nobilis*), grass carp (*Ctenopharyngodon idella*), edible goldfish (*Carassius auratus*) and different species of tilapia.

Local fishpond farmers preferred using cheaper feeds and seldom used crab meal, fishmeal or dried milk that used in some European countries (Fung, 1965). Traditionally, poultry and swine waste, food waste and agriculture waste are used in polyculture ponds as feed stuff (Lau et al., 2003; Wong et al., 2004). Unconsumed food or agricultural by-product used by local fish farmers for feeding fish are soybean dreg (remains of soybean after juicing soybean milk), rice bran, wheat bran, bread, instant noodles or leftover from household kitchen. Other than the market value of the marketable fish, fish pond itself is important to the environment. Drainage activity during harvesting exposes the mud bottom of the fish pond, which makes fish ponds important feeding sites for both stationary and migratory birds that prey for
Table 1.1 Fish species farmed by pond owners participated in Accredited Fish Farm Scheme (AFFS) (provided by Dr. Eric PM Yau, Fisheries Officer of AFCD)

<table>
<thead>
<tr>
<th>Fish species</th>
<th>No. of pond owners</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2010</td>
</tr>
<tr>
<td><strong>Freshwater fish</strong></td>
<td></td>
</tr>
<tr>
<td>Jade perch (Scortum barcoo)</td>
<td>9</td>
</tr>
<tr>
<td>Grey mullet (Mugil cephalus)</td>
<td>8</td>
</tr>
<tr>
<td>Bighead (Hypophthalmichthys nobilis)</td>
<td>9</td>
</tr>
<tr>
<td>Grass carp (Ctenopharyngodon idella)</td>
<td>8</td>
</tr>
<tr>
<td>Edible goldfish (Carassius auratus)</td>
<td>6</td>
</tr>
<tr>
<td>Tilapia*</td>
<td>6</td>
</tr>
<tr>
<td>Silver carp (Hypophthalmichthys molitrix)</td>
<td>4</td>
</tr>
<tr>
<td>Mud carp (Cirrhinus molitorella)</td>
<td>2</td>
</tr>
<tr>
<td>Catfish#</td>
<td>2</td>
</tr>
<tr>
<td>Tambaqui (Colossoma macropomum)</td>
<td>1</td>
</tr>
<tr>
<td>Snakehead (Ophiocephalus maculatus)</td>
<td>1</td>
</tr>
<tr>
<td>Common carp (Cyprius carpio)</td>
<td>1</td>
</tr>
<tr>
<td>Bream (Abramis brama)</td>
<td></td>
</tr>
<tr>
<td>Black carp (Mylophyrynogodon piceus)</td>
<td>1</td>
</tr>
<tr>
<td><strong>Brackish water fish</strong></td>
<td></td>
</tr>
<tr>
<td>Marble goby (Oxyeleotris marmorata)</td>
<td>2</td>
</tr>
<tr>
<td>Sea bass#</td>
<td>2</td>
</tr>
<tr>
<td>Giant grouper (Epinephelus lanceolatus)</td>
<td>3</td>
</tr>
<tr>
<td>Yellow finned seabream (Acanthopagrus latus)</td>
<td>2</td>
</tr>
<tr>
<td>Spotted scat (Scatophagus argus)</td>
<td></td>
</tr>
<tr>
<td>Snapper#</td>
<td></td>
</tr>
<tr>
<td><strong>Other animals</strong></td>
<td></td>
</tr>
<tr>
<td>Shrimp</td>
<td>2</td>
</tr>
<tr>
<td>Softshell turtle</td>
<td></td>
</tr>
</tbody>
</table>

* Tilapia is a common name of a number of species of cichlid fish
# Species not specified.

No. of fish farm operators participated in the scheme:
2010: 17    2011: 24
2012: 30    2013: 31
In the old days, animals such as pigs and ducks were reared along with fish, as input of manure provided nutrients as pond fertilizer by providing nitrogen and phosphorous that supported growth of plankton which in turn served as food for fish in fish ponds (Wong et al., 2004). Combined fish and duck culture was also practiced in other countries such as Hungary (Advisory Committee on Technology Innovation, 1981). However, backyard poultry farming in Hong Kong was banned in 2006 in order to prevent Avian Flu (Legislative Council, 2013). Moreover, the number of terrestrial animal farmers gradually reduced in Hong Kong, which made animal manure less available. As a result, manure from animals is becoming less popular in local inland fish culture. In order to compensate the reduced availability of manure (as pond fertilizer), fish farmers tend to use excessive amounts of feeds to maintain the fertility of pond water as well as compensating the nutrient requirement of fish. This practice would lead to excessive deposition of organic matter on the pond bottom and breaking down of organic matter consumes a lot of oxygen. Moreover, excessive amounts of feeds will lead to excessive plankton growth in the pond. Although plankton biomass is a food source for filter feeders like bighead and silver carp, the pond water may be too rich in nutrients leading to excessive plankton growth. The plankton, in turn, would consume oxygen at night causing the fish stock to suffocate (Lau et al., 2003).

Pellet diet is formulated to meet the nutritional requirement of fish, thus the growth performance of fish fed with pellet diets is generally better (Lie, 2001). Yu (1989) reviewed the feed conversion ratio of some commonly used fish feeds. For grass carp, the feed
conversion ratio of bean dreg, terrestrial grass and pellet diets (consisting of grass powder, rapeseed cake, fishmeal, bean cake, silkworm pupae and barley flour, and the protein content is 22.6%) were 25, 40 and around 2.5 to 3, respectively (Yu, 1989), indicating pellet diets could be a better option over agriculture by-products. However, local fish farmers prefer cheap materials for feeding the fish for cost saving. The retail price for fish feed pellets for low trophic level fish like grass carp and tilapia is about HK$20-30/kg, while they could collect expired bread or soybean dreg to feed their fish for free. Price would be therefore the dominating factor for local fish farmers, governing the selection of fish feeds. Except for those with higher market value such as giant groupers farmed in brackish ponds near the coastal line, waste material remains the first choice of feed for inland pond fish farming.

Food waste can be used to feed animals in some countries. For example, a local feed producer planned to export food waste based feeds to Vietnam (MingPao, 2013). However, the usage of food waste in animal diets is not always welcomed. In UK, it is illegal to use food waste or to feed livestock (including fish) with mammalian meat and bone meal (potentially infectious materials) to prevent new cases of bovine spongiform encephalopathy (AHVLA, 2014). In USA, feeding swine with meat or animal materials is regulated by the Federal Swine Health Protection Act (PL 96 468) to avoid transmission of diseases (USEPA, 2013).

As dumping food waste into landfill sites will definitely shorten the lifespan of the current landfill sites, making use of food waste in aquaculture can partially ease the disposal problem. Using food waste in food production, however, is not new to the industry. For example, food waste can be composted to produce fertilizer for crop and plant production. In Hong Kong,
unconsumed food stuffs have been used to feed swine. Moreover, fish farmers in the New Territories often feed fish with food stuff such as rice bran, soybean dregs, unconsumed or expired bread and noodles.

1.4 The need for alternative protein source

1.4.1 Increment in feed prices

Fishmeal is produced from whole fish or fish remains. Most of the commercial fish feed pellets contain fishmeal, which is widely used in aquaculture as protein source for fish. The global price of fishmeal increased from US$500 to $700/tonne in early 2010s to about $1,200 US$/per tonne in 2008 (Rana et al., 2009) and the price is expected to increase continuously. As per November 2013, the global fishmeal price is about US$1,550/tonne (Index Mundi, 2014a). Other than fishmeal, prices of other common ingredients also increase. For example, the price of soybean meal doubled from about US$250 in 2003 to about US$500 in 2013 (Index Mundi, 2014b), although plant proteins are still generally cheaper than fishmeal. It is expected that the inclusion rate of fishmeal in fish feed pellets will decrease in the long run (Tacon and Metian, 2008). Therefore, the increment in feed price could hinder the long-term development of the aquaculture society.

1.4.2 Replacing fishmeal with other protein sources

A number of studies attempted to replace fishmeal with different kinds of plant protein. Heinkken et al. (2006) used soybean meal for producing rainbow trout and found that the treatment group consuming soybean meal showed a higher food conversion ratio than the control group (fed with fishmeal diet). Carter and Hauler (2000) reported up to 33% of
Sitjà-Bobadilla et al. (2005) showed that gilthead sea bream fed with diets containing 50% of a mixture of plant proteins (corn gluten, wheat gluten, extruded peas, rapeseed meal and sweet white lupin) had significant improvement in feed efficiency, exerting an anti-oxidative effect with specific growth rates remained unchanged or slightly reduced in comparison to fish fed the fishmeal diet. These investigations showed that omnivorous or even carnivorous fish can be farmed with fishmeal partially replaced by plant protein in diet.

The presence of anti-nutritional factors (compounds that interfering nutrient absorption) in plant materials, such as Bowman-Birk trypsin inhibitor in soybean (Tan-Wilson et al., 1987), could affect growth of fish (Chubb, 1982). A study conducted by De Francesco et al. (2004) also found that completely replacing fishmeal with plant protein in fish diet affected growth of rainbow trout. Kissil et al. (2000) reported that feed intake and weight gain of sea bream were inversely related to the inclusion levels of both soy and rapeseed protein. The presence of anti-nutritional factors in feed ingredient could hinder the inclusion rate or require additional treatments.

About 80% of carps and 65% of tilapia worldwide are farmed without using modern compound feeds (Naylor et al., 2000). Herbivorous and omnivorous freshwater finfish utilize plant-based protein source better than carnivorous fish and require only a minimal amount of fishmeal to supply essential amino acids (De Silva & Anderson, 1994). On the other hand, omnivorous and herbivorous (such as Nile tilapia and grass carp) fish could tolerate a higher amount of carbohydrate. For example, tilapia could utilize 35-40% digestible carbohydrate
(FAO, 2014e), which makes them suitable choices to be fed with food waste based diets, as considerably amount of cereal products could be present in food waste.

1.5 Using feed supplements for upgrading fish diets

1.5.1 Alternatives to antimicrobial agents for promoting fish health and growth

Modern aquaculture systems adopt dense culture designs in order to increase productivity and profit. Fish reared in pond culture could be weakened by stressful conditions such as poor water quality and poor sanitation, with the fish more susceptible to microbial infections leading to the endemic of disease (Rottmann et al., 1992). In order to reduce loss due to diseases, use of antimicrobial agents became popular in the aquaculture society in the past decades. However, there are disadvantages of using antimicrobial agents such as the potential development of antibiotic resistance genes in bacteria, the presence of antibiotic residues in fish products, the destruction of microbial populations in the culture and nearby environment and the suppression of the immune system of aquatic animals (Smith et al., 2003; Cabello 2004; Sorum 2006; Sapkota et al., 2008). Various classes of antibiotics (including fluoroquinolones, tetracycline, sulfonamides and macrolides) as well as antibiotic resistance genes (coding for sulfonamide resistance) have been detected in some major rivers of China (Luo et al., 2010; Zhou et al., 2011). Thus, the uses of feed supplements as an alternative to the use of antimicrobial agents have attracted a lot of attention in aquaculture. There are many beneficial effects of using supplements. For example, prebiotic fibres (inulin) could be used for improving growth as well as serving as an immuno-booster for Nile tilapia (Ibrahim et al., 2010), while Chinese herbs supplement could improve the immune system of common carp (Yin et al., 2009) and grass carp (Choi et al., 2013).
A schematic map of the response of a fish challenged by pathogens is shown at Fig. 1.1. Non-specific immunity, or innate immunity, is the fundamental defence mechanism of fish (involving physical parameters, immune cells and humoral parameters) against pathogens (Magnadóttir, 2006). Specific immunity, or acquired immunity, is also present in fish. However, there are several limitations associated with the specific immune system, including their poikilothermic nature, limited repertoire of antibodies and the slow proliferation, maturation and memory of their lymphocytes (Whyte, 2007). Thus, majorities of research works focused more on improving the non-specific immune parameters of fish (Choi et al., 2013; Christy-bapita et al., 2007; He et al., 2003; Jian and Wu, 2003; Sitjà-Bobadilla et al., 2005; Yin et al., 2006).

1.5.2 Prebiotic fibres

A prebiotic fibre is an indigestible food ingredient that could beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the gut, and thus improves host health (Gilson and Roberfroid, 1995). Prebiotic fibres are usually administrated to fish by incorporating in diet, but it had also been tested via injection (Wang and Wang, 1997). The ultimate goal of using probiotic bacteria or prebiotic fibres in aquaculture is to improve the health and/or growth performance of fish by establishing beneficial bacterial community. Thus, prebiotic fibres are believed to be a more practicable supplementation in fish cultures to stimulate the growth of beneficial bacteria. However, there are several disadvantages of using probiotic bacteria, including low viability of the bacteria after pelletizing and during storage, leaching from the feed particles in rearing water, as well
Fig. 1.1 A schematic map of the response of a fish challenged by pathogens (Shoemaker et al., 2001)
as problems related with feed handling and preparation (Merrifield et al., 2010).

Prebiotic fibres promote the growth of beneficial lactic acid bacteria (LAB) which are capable for inhibiting the proliferation of pathogenic microorganisms, due to change in gut pH (Roberfroid and Slavin 2000). Non-specific immunity, growth performance of tested fish and/or digestability of diet could be improved with the inclusion of prebiotic fibres in diet. Ibrahim et al. (2010) reported that inclusion of inulin in fish diet (5 g/kg) positively affected growth, hematology, innate immunity, and resistance of Nile tilapia. Burr et al. (2008) reported that inclusion of prebiotic fibres in diet for red drum (*Sciaenops ocellatus*) enhanced protein digestibility of soybean meal based diet.

1.5.3 Chinese herbs

Chinese herbs have been used for thousands of years as immune booster or medicine. Because of the growth promoting and immune system boosting effects of Chinese herbs, Chinese herbs are attracting more attention for aquaculture.

Adding 1-2% of anthraquione extract from rhubarb (*Rheum rhabarbarum*) as feed supplement was found to be effective in preventing pathogenic infection, mitigating overcrowding stress and promoting growth of common carp (*Cyprinus carpio var. Jian*) (Xie et al., 2008). Zakęś et al. (2008) also reported that juvenile pikeperch (*Sander lucioperca*) fed diet with 0.1% supplement of huangqi (*Astragalus radix*) showed improved growth performance and food conversion ratio.
Other than stimulating fish growth, inclusion of Chinese herb (using single herb or compound formulations) in fish feed was found supporting the non-specific immune system of fish. Sahu et al. (2007) noted that rohu (*Labeo rohita*) fingerlings fed with diet containing garlic (1 or 5 g/kg) had improved immunity and improved resistance to *Aeromonas hydrophila* (a bacterium). Yin et al. (2006) reported that feeding tilapia with feed containing 0.1% and 0.5% extract of Chinese herbal medicine *Astragalus radix* enhanced lysozyme activity and phagocytosis by phagocytic white cells.

Multiple Chinese herbs are often used to improve the efficacy of the medicine. A number of studies attempted to apply compound formulations (combining 2 or more herbs) in fish diet. Jian and Wu (2003) found that feeding large yellow croaker (*Pseufosciaena crocea*) with 1.0% and 1.5% Astragalus Root (*Astragalus propinquus*) and Chinese Angelica Root (*Angelica sinensis*) (mixed at the ratio 5:1) increased the number of nitroblue tetrazolium (NBT) positive cells and lysoyme activities enhanced the complement activities of the fish. Moreover, fish fed with 1.0% and 1.5% TCM showed better survival rate than those fed with control diet or 0.5 % TCM mixture. Choi et al. (2013) also reported that grass carp juveniles fed with diets containing 2% mixture of *Radix scutellaria, Rhizoma coptidis, Herba andrographis*, and *Radix sophorae flavescentis* in a ratio of 1:1:2:3 resulted in significantly lower mortality in after *Aeromonas hydrophila* (bacterium) challenge.

### 1.6 Aims and objectives

This thesis focused on the use the food waste based diets and the supplements (prebiotic fibres and Chinese herbs) for culturing freshwater fish. It was hypothesized that food waste
could be used as major ingredients to produce fish feed pellets, without adverse effects on fish performance and the functional quality of food waste pellets could be improved with the addition of prebiotic fibres and/or Chinese herbs, for enhancing non-specific immunity, leading to better fish growth. A schematic diagram (Fig 1.2) shows the framework of this study.

The objectives of this thesis were:

1. To compare the proximate compositions and essential amino acid profiles of food waste based diets with some common fish feeds (Chapter 2);

2. To study the effects of food waste based diets on the growth of carp species (in polyculture) and water quality (Chapter 3);

3. To study the feed digestibility of food waste based diets and upgrades on the growth of grass carp and Nile tilapia (Chapters 4, 5, 6);

4. To investigate the effects of food waste based diets with or without vitamin supplements (Chapter 4) on the growth and proximate compositions of grass carp and tilapia; and

5. To investigate the effects of adding prebiotic fibres (Chapter 5) and Chinese herbs (Chapter 6) on the growth and non-specific immunity of fish (total immunoglobulin in serum, bactericidal activity of serum and anti-protease activity of serum).
Chapter 1 General introduction

Properties of food waste diets and their effect on fish growth

Chapter 2 Proximate compositions and essential amino acid profiles of common used fish feeds used by local fish farmers and food waste based feed pellets

Chapter 3 Effects of food waste based feed pellets on polyculture of carps and water quality: A field trial

Chapter 4 Upgrading food waste based diets with vitamin-mineral premix: effects on feed protein digestibility and fish growth

Chapter 5 Upgrading food waste based feeds with prebiotic fibres: effects on feed digestibility, growth and non-specific immunity of fish

Chapter 6 Upgrading food waste based feeds with Chinese herbs: effects on feed digestibility, growth and non-specific immunity of fish

Chapter 7 General discussion and major conclusions

Fig. 1.2 Framework of research
1.7 Contributions and significances of the present research

This thesis focused on using food wastes as major ingredients to produce fish feed pellets incorporated with dietary feed supplements for feeding low trophic level fish. The results from this study could provide information on dietary supplements (Chinese herb and prebiotic fibres) which may enhance fish immunity and their interaction on fish gut bacterial diversity.

Waste items have been used in aquaculture for a long time. As food waste generation is being one of the most significant environmental problems, using food waste to make fish feed pellets could help reducing the amount of waste dumped into landfill sites as well as retrieving useful energy resources for food production.

Feed supplements such as prebiotic fibres and Chinese herbs could enhance fish immunity and support fish growth, raising the yield of fishery products while reducing the usage of potentially harmful antimicrobial compounds and growth hormones.

It is hoped that the results from this study would provide information for marine aquaculture, which is another important sector of local fish farming industry. As freshwater fish ponds are also known as important feeding sites for both stationary and migratory birds, it would be beneficial to keep those sites actively managed and utilised.
Chapter 2 Proximate compositions and essential amino acid profiles of common used fish feeds used by local fish farmers and food waste based feed pellets

2.1 Introduction

Most of the fish farms in Hong Kong are engaged in polyculture (grass carp, bighead carp, common carp and silver carp in combination with tilapia or grey mullet), and it is the dominated farming practice (98%) adopted by local inland fish farmers (ACFD, 2014). Local inland fish farmers preferred using cheaper materials such as livestock manure and agricultural residues and seldom used crab meal, fishmeal or dried milk commonly used in some European countries (Fung, 1965). Examples of unconsumed food or agricultural by-products used by local fish farmers for feeding fish are rice bran, wheat bran, peanut cake, bread, expired instant noodles or leftover from household kitchens. Traditionally, excreta from poultry, food waste and agriculture waste are used in polyculture ponds as feed stuff (Lau et al., 2003; Wong et al., 2004). However, backyard poultry farming in Hong Kong was banned in 2006 in order to prevent Avian Flu (Legislative Council, 2013), which renders poultry excreta less available for fish farming.

Protein quality of fish feed is important for fish growth. Essential amino acids (EAA) would be important factors on determining the quality of a particular fish feed. Essential amino acids refer to the amino acid that cannot be synthesized by animal itself and have to replenish via diets. Ten essential amino acids are required by fish: including arginine, histidine, isoleusine, leusine, lysine, methionine, phenylalanine, threonine typtophan and valine (Li et al., 2008).
Other than protein and amino acids, other compositions of fish feed are also important for fish. Lipids and carbohydrates can be used as energy sources and they possess protein sparing effects (using carbohydrates or lipids instead of protein for energy generation) (De Silva et al., 2001; Skalli et al., 2004). Phosphorous is essential to fish for proper bone mineralization (Lim et al., 2001). As there are no published reports available on the essential amino acids contents of common fish feeds used by local inland fish farmers, the nutritional profiles and essential amino contents of the common fish feeds used locally were determined and compared to the requirements of both grass carp and Nile tilapia to evaluate their quality in the present experiment.

Grass carp (Ctenopharyngodon idella) and Nile tilapia (Oreochromis niloticus) are two commonly farmed fish species in Hong Kong (according to information provided by the Accredited Fish Farm Scheme database, Table 1.1). Grass carp is a herbivore in the natural habitats that primarily consumes aquatic macrophytes, but it prefers pellet diets in fish pond culture where pellet diets are given (Lopinot, 1972; Masser, 2002). Tilapia is an omnivore and consumes plankton, aquatic macrophytes, invertebrates, larval fish, detritus, and decomposing organic matter. It could digest animal protein more efficiently than plant protein (Popma, 1999). Both omnivorous and herbivorous fish require relatively lower protein (about 30%), compared with carnivorous fish (more than 40%) (Cai et al., 2005; Wang et al., 1985), and could tolerate higher amount of carbohydrates in their diets, making them suitable fish species fed with food waste based diets.

It is hypothesized that using food waste as major ingredients could produce fish feeds that
meet the nutritional requirements of grass carp and Nile tilapia. The objectives of this chapter were to estimate and compare the quality of fish feed items, including commercial fish feed pellets, breads, rice bran, instant noodles, soybean dreg and food waste based fish feeds, by (1) testing essential amino acid contents, (2) evaluating the first limiting essential amino acid, and (3) measuring the proximate compositions of the fish feeds.

2.2 Materials and Methods

2.2.1 Fish feed pellets production

Food wastes were collected from local sources. They were mainly food processing waste and partly post consumed waste, which were classified into 4 major categories; (1) fruit peel and vegetables, (2) meat, (3) bone meal and (4) cereal wastes. Fruit waste contained mainly peels with some flesh of various fruits, about 25% of pineapple, 25% watermelon, 15% cantaloupe, and 35% others, such as strawberry, banana, apple. Meat wastes included 60 to 70% of beef, pork and chicken, 30-40% of fish such as salmon and groupers. Vegetables comprised of various types of leaf vegetables, such as lettuce and spinach. Cereals usually included soy bean meal, rice grain and spaghetti.

Food waste based feeds were manufactured by Kowloon Biotechnology Ltd. Individual ingredients described above were chopped and surplus water was squeezed out. After drying for 6 h, the dried food waste was powdered and different ratios of food waste powders were mixed with starch and fishmeal for making fish feed pellets. Food waste consisted of 75% (w/w) of the final fish feed pellets. Food Waste Diet A (FWA) consisted of 53% cereal, 10% fruit and vegetables, 8% bone meal, 10% fishmeal, 15% corn meal and 4% others. Food
Waste Diet B consisted of 25% meat, 28% cereal, 10% fruit and vegetables, 8% bone meal, 10% fishmeal, 15% corn meal and 4% others.

2.2.2 Common used feed preparation

Common fish feeds adopted by local fish farmers include fish feed pellets, breads, rice bran, instant noodles and soybean dreg, according to Dr. Jim CW Chu and Dr Eric PM Yau of AFCD (personal communication, 3rd April, 2014). A commercial fish feed pellet (Jiefeng® 613) was purchased from a local animal feed supplier, rice bran was purchased from mainland China, and bread (white bread and hamburger bun) and soybean were purchased from a local market. Noodles and Napier grass were collected from a local fish farm. Fish feed pellets, bread and noodle were smashed to obtain fine powder. Soybean dreg was prepared by cooking soybean with water. Soybean (300 g) was soaked in tap water for 8 h before cooking with additional tap water (500 ml) and then mixed with a food blender. The soybean-water mixture was filtered for raw soybean dreg. Soybean dreg was then dried in an 65°C oven for 24 h and then thoroughly mixed in a food blender. All fish feed items were stored in a desiccator prior to chemical analyses.

2.2.3 Chemical analyses of fish feed items

Moisture and ash content determination

Moisture and ash contents in feeds were determined according to AOAC (1990a and 1990b). About 0.5 g of sample was weighed in a pre-weighed crucible. Samples and the crucible were dried at 105°C for 24 h. The crucible was cooled in a desiccator and weighed again. Afterwards, the crucibles with samples were combusted at 600°C for 2 h. The crucibles were
cooled in a desiccator and weighed again. The dry mass and ash content were calculated using
the following equations:

Dry mass (%) = 100*(weight of crucible/ dried sample - weight of crucible)/ (weight of
crucible w/ sample before drying - weight of crucible)

Ash (%) = 100*((weight of crucible/ combusted sample - weight of crucible)/ (weight of
crucible/ sample before combustion - weight of crucible)

**Acid digestion for total phosphorous and total Kjeldahl nitrogen**

Samples were digested using semi-micro Kjeldahl digestion apparatus. 500 mg of feed sample
was digested with 3 ml of concentrated sulphuric acid in the presence of 0.1 g Kjeldahl
catalyst. Samples were pre-digested at 98°C for 1 h and heated at 160°C for 30 min. Then the
temperature was increased to 250°C and heated for 30 min. Samples were further digested at
360°C until the sample turned clear. The resulted solution was diluted with 15 ml of DI water
and filtered through a filter paper (Whatman No. 1) into 25 ml volumetric flasks and marked
up with DI water.

**Total Kjeldahl nitrogen determination**

Total Kjeldahl nitrogen (TKN) of samples was determined using phenate method with
modification (APHA, 2002). Briefly, 1 ml of digested sample was neutralized to neutral pH
with 5N NaOH and then diluted to 4 ml with DI water. The sample was then mixed with 1 ml
of complexing reagent, 1 ml of colouring reagent and 1 ml of bleach solution. The solution
was further marked up to 10 ml and stood at room temperature (~22°C) for at least 1 h prior
to measurement. The absorbance was measured on a spectrophotometer at 626 nm (UV-1601,
Shimadzu, Tokyo, Japan). The concentration of TKN was checked against a series of standard solutions ranging from 0.1 to 0.7 µg/ml.

**Total phosphorous determination**

Total phosphorous of samples was determined using ascorbic acid method with modification (APHA, 2002). Briefly, 1 ml of digested sample was neutralized to neutral pH with 5N NaOH and then diluted to 7 ml with DI water. 1.6 ml of ascorbic acid reducing agent was added for colour development. The solution was further marked up to 10 ml and the absorbance was measured on a spectrophotometer at 880 nm (UV-1601, Shimadzu, Tokyo, Japan). The concentration of TP was checked against a series of standard solutions ranging from 0.02 to 0.08 µg/ml.

**Crude protein calculation**

The crude protein in fish feed was calculated by multiplying total Kjeldahl nitrogen by 6.25 (AOAC, 2002).

**Lipid determination**

The lipid content was determined using ultrasound assisted extraction method according to Metherel et al. (2009) and modified by Choi (2013). 0.1 g of sample was extracted with 12 ml of 3:2 (v:v) hexane: isopropanol mixture for 20 min with a sonicator (Branson Bransonic 5510). The organic layer was collected after centrifugation and the process was repeated twice. The pooled supernatant was dried in a pre-weighed tube under nitrogen and the tube was kept at 102°C for 30 min. The tube was weighed again after cooling in a desiccator for 3 h.
Crude fibre determination

The crude fibre was determined according to AOAC (1984). Briefly, 1 g of sample was digested with boiling in 1.25% of sulphuric acid, followed by digestion with boiling in 1.25% of potassium hydroxide. The weight of sample after digestion represented the crude fibre and ash content in comparison to the initial weight.

Nitrogen-free extract determination

The 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).

2.2.4 Essential amino acids profiles of the feed

The essential amino acid profile in feed items was determined according to GB/T18246-2000 (Standardization Administration of China, 2001). Briefly, the sample was digested with 6N hydrochloric acid at 110°C for 24 h. The digested sample was filtered through a 0.45 μm filter and the amino acid content of the sample was analysed by an amino acid analyser (Hitachi, 835-50).

2.2.5 Calculation of amino acid scores for feed items

Amino acid scores of feed items were calculated according to Tacon (1987), using the dietary essential amino acid requirements of grass carp and Nile tilapia as references (Santiago and Lovell, 1988; Wang et al., 2005), according to the following equation:
Amino acid score = \([\text{Amount of EAA in feed}]/[\text{EAA requirement of fish (grass carp or Nile tilapia)}]\)*100

Cystine and tyrosine were included in the calculation because they are involved in the metabolic pathway of methionine and phenylalanine (Ogino, 1980).

2.2.6 Statistical analyses

Normality and homogeneity of data were tested prior to ANOVA. Essential amino acid contents and proximate compositions of feed were analysed using one-way ANOVA and Duncan’s multiple range tests (SPSS Statistics 19.0, IBM, USA). Significant differences between feeds were expressed at the significance level of \(p<0.05\).

2.3 Results and Discussion

Table 2.1 shows the amino acid contents of the studied feed items. Amino acids are the building blocks of protein. Essential amino acids refer to the amino acids that cannot be synthesized by the animal itself and have to replenish via diets. Among all of the essential amino acids (EAA), lysine is the most important one, as it is the EAA found at the highest level in the carcass of many fish species (Wilson and Cowey, 1985; Wilson and Poe, 1985; Kim and Lall, 2000). Lysine is often one of the most limiting amino acids in the ingredients used for production of commercial fish feeds, especially in those with fishmeal replaced by plant proteins (Mai et al., 2006). Inadequate lysine in diet could seriously affect growth and health of fish (Li et al., 2008). Wang et al. (2005) observed that the dietary lysine requirement of grass carp juvenile should be 5.44% of the dietary protein, while Santiago and Lovell (1988) noted that Nile tilapia requires 5.12% of the dietary protein. In the present
Table 2.1 Essential amino acid contents of fish feeds

<table>
<thead>
<tr>
<th>Essential amino acid</th>
<th>Amino acid contents of feed items (% protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Napier grass</td>
</tr>
<tr>
<td>Arginine (Arg)</td>
<td>2.18 ± 0.05a</td>
</tr>
<tr>
<td>Histidine (His)</td>
<td>1.99 ± 0.10c</td>
</tr>
<tr>
<td>Isoleucine (Ile)</td>
<td>4.72 ± 0.13d</td>
</tr>
<tr>
<td>Leucine (Leu)</td>
<td>6.23 ± 0.13a</td>
</tr>
<tr>
<td>Lysine (Lys)</td>
<td>3.11 ± 0.33ab</td>
</tr>
<tr>
<td>Methionine (Met)</td>
<td>1.43 ± 0.20b</td>
</tr>
<tr>
<td>Phenylalanine (Phe)</td>
<td>7.27 ± 0.17d</td>
</tr>
<tr>
<td>Threonine (Thr)</td>
<td>3.73 ± 0.33c</td>
</tr>
<tr>
<td>Tyrosine (Typ)</td>
<td>0.52 ± 0.03a</td>
</tr>
<tr>
<td>Valine (Val)</td>
<td>6.00 ± 0.39f</td>
</tr>
</tbody>
</table>

Mean ± SD having the same letters in the same row are not significantly different (p<0.05) (n=3).
FWA= Food Waste Diet A; FWB= Food Waste Diet B
study, none of the common fish feeds (except Jiefeng® 613) contained sufficient lysine for both grass carp and Nile tilapia. Among the common fish feeds, Jiefeng® 613 had the highest amount of lysine (5.83%), followed by rice bran (4.65%), while the two bread samples had the lowest amount of lysine (about 2.8%). On the other hand, the lysine content of the 3 fish feed pellets (Jiefeng® 613: 5.83%; FWA: 5.76%; FWB: 5.79%) were all higher than the suggested value for both grass carp (5.44%) and Nile tilapia (5.12%) (Wang et al., 2005; Santiago and Lovell, 1988).

Tables 2.2 and 2.3 show the amino acid scores of the feed items for grass carp and Nile tilapia, respectively. The amino acid score of a particular EAA that equals 100 means that the diet contains sufficient amount of that particular amino acid. For grass carp, lysine is always the first limiting amino acid in all of the tested fish feeds, while lysine, methionine, cystine, and threonine could be all limiting to tilapia, with lysine being the most frequently limiting EAA.

Table 2.4 shows the proximate compositions of the studied fish feeds. All the feed items (except soybean dreg) had significantly lower (p<0.05) crude protein contents than all the 3 fish pellets. No significant differences (p>0.05) in the crude protein content of the 3 fish feed pellets were noted. Soybean dreg had the highest protein content among all tested fish feeds. It has been recommended that the optimal dietary protein level for grass carp should be about 22-30% (Cai et al., 2005), while for Nile tilapia 30% (Wang et al., 1985), with casein as the primary protein. If the principal fish feeds cannot satisfy the nutritional requirement, fish would also consume other food source for replenishing the insufficient nutrients. Pandit et al. (2005) conducted a study on the food utilization of grass carp and tilapia reared in polyculture.
Table 2.2 Amino acid scores of the feed items comparing to the requirements of grass carp

<table>
<thead>
<tr>
<th>EAA</th>
<th>Napier grass</th>
<th>Rice bran</th>
<th>Soybean dreg</th>
<th>Noodle 1</th>
<th>Noodle 2</th>
<th>White bread</th>
<th>Hamburger bun</th>
<th>Jiefeng® 613</th>
<th>FWA</th>
<th>FWB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg</td>
<td>61*</td>
<td>170</td>
<td>93</td>
<td>93</td>
<td>129</td>
<td>107</td>
<td>141</td>
<td>199</td>
<td>218</td>
<td>219</td>
</tr>
<tr>
<td>His</td>
<td>102</td>
<td>139</td>
<td>67*</td>
<td>93</td>
<td>151</td>
<td>117</td>
<td>130</td>
<td>136</td>
<td>150</td>
<td>151</td>
</tr>
<tr>
<td>Ile</td>
<td>165</td>
<td>204</td>
<td>73</td>
<td>112</td>
<td>155</td>
<td>139</td>
<td>172</td>
<td>156</td>
<td>164</td>
<td>165</td>
</tr>
<tr>
<td>Leu</td>
<td>121</td>
<td>130</td>
<td>66*</td>
<td>115</td>
<td>165</td>
<td>137</td>
<td>142</td>
<td>161</td>
<td>172</td>
<td>173</td>
</tr>
<tr>
<td>Lys</td>
<td>57*</td>
<td>83</td>
<td>58*</td>
<td>35*</td>
<td>59*</td>
<td>49*</td>
<td>45*</td>
<td>107</td>
<td>106</td>
<td>106</td>
</tr>
<tr>
<td>Met+Cys#</td>
<td>60*</td>
<td>128</td>
<td>79</td>
<td>38*</td>
<td>106</td>
<td>144</td>
<td>184</td>
<td>144</td>
<td>136</td>
<td>137</td>
</tr>
<tr>
<td>Phe+Tyr#</td>
<td>270</td>
<td>192</td>
<td>89</td>
<td>98</td>
<td>131</td>
<td>174</td>
<td>162</td>
<td>187</td>
<td>201</td>
<td>203</td>
</tr>
<tr>
<td>Thr</td>
<td>152</td>
<td>132</td>
<td>60*</td>
<td>108</td>
<td>128</td>
<td>119</td>
<td>98</td>
<td>172</td>
<td>172</td>
<td>174</td>
</tr>
<tr>
<td>Typ</td>
<td>83</td>
<td>278</td>
<td>63*</td>
<td>39</td>
<td>138</td>
<td>163</td>
<td>339</td>
<td>607</td>
<td>429</td>
<td>478</td>
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<tr>
<td>Val</td>
<td>182</td>
<td>166</td>
<td>68*</td>
<td>113</td>
<td>157</td>
<td>137</td>
<td>150</td>
<td>160</td>
<td>171</td>
<td>172</td>
</tr>
</tbody>
</table>


Asterisk (*) indicates the EAA that is insufficient for dietary requirements of grass carp. EAA requirement (expressed as % of total protein) of grass carp: threonine 2.50; valine 3.25; methionine+cystine 2.65; isoleucine 2.84; leucine 5.14; phenylalanine+tyrosine 4.52; lysine 5.11; histidine 1.87; arginine 3.47; and tryptophan 0.72 (Wang et al., 2005). #Cys and Tyr are included as they are involved in Met and Phe metabolism pathways, respectively (Ogino, 1980).
Table 2.3 Amino acid scores of the feed items comparing to the requirement of Nile tilapia. Asterisk (*) indicates the EAA that is insufficient for dietary requirements of grass carp. EAA requirement (expressed as % of total protein) of Nile tilapia: threonine 10.6; valine 9.5; methionine 5.4; cystine 2.7; isoleucine 7.5; leucine 13.5; phenylalanine 9.5; tyrosine 6.5; lysine 16.8; histidine 4.8; arginine 11.6; and tryptophan 1.7 (Santiago and Lovell, 1988). #Cys and Tyr are included as they are involved in Met and Phe metabolism pathway, respectively (Ogino, 1980).

<table>
<thead>
<tr>
<th>EAA</th>
<th>Napiergrass</th>
<th>Rice bran</th>
<th>Soybean dreg</th>
<th>Noodle 1</th>
<th>Noodle 2</th>
<th>White bread</th>
<th>Hamburger bun</th>
<th>Jiefeng® 613</th>
<th>FWA</th>
<th>FWB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg</td>
<td>50*</td>
<td>140</td>
<td>77</td>
<td>76</td>
<td>106</td>
<td>88</td>
<td>116</td>
<td>165</td>
<td>180</td>
<td>181</td>
</tr>
<tr>
<td>His</td>
<td>110</td>
<td>151</td>
<td>73</td>
<td>101</td>
<td>164</td>
<td>127</td>
<td>142</td>
<td>148</td>
<td>163</td>
<td>165</td>
</tr>
<tr>
<td>Ile</td>
<td>151</td>
<td>186</td>
<td>66*</td>
<td>102</td>
<td>142</td>
<td>127</td>
<td>157</td>
<td>143</td>
<td>150</td>
<td>151</td>
</tr>
<tr>
<td>Leu</td>
<td>183</td>
<td>198</td>
<td>99</td>
<td>174</td>
<td>250</td>
<td>208</td>
<td>216</td>
<td>244</td>
<td>260</td>
<td>263</td>
</tr>
<tr>
<td>Lys</td>
<td>61*</td>
<td>88</td>
<td>62*</td>
<td>38*</td>
<td>62*</td>
<td>53*</td>
<td>48*</td>
<td>114</td>
<td>113</td>
<td>113</td>
</tr>
<tr>
<td>Met+Cys#</td>
<td>50*</td>
<td>106</td>
<td>65*</td>
<td>32*</td>
<td>88</td>
<td>119</td>
<td>152</td>
<td>119</td>
<td>112</td>
<td>113</td>
</tr>
<tr>
<td>Phe+Tyr#</td>
<td>220</td>
<td>157</td>
<td>73</td>
<td>80</td>
<td>107</td>
<td>142</td>
<td>132</td>
<td>152</td>
<td>164</td>
<td>165</td>
</tr>
<tr>
<td>Thr</td>
<td>101</td>
<td>88</td>
<td>40*</td>
<td>72</td>
<td>85</td>
<td>79</td>
<td>65*</td>
<td>115</td>
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<td>Typ</td>
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<td>99</td>
<td>118</td>
<td>244</td>
<td>437</td>
<td>309</td>
<td>344</td>
</tr>
<tr>
<td>Val</td>
<td>211</td>
<td>193</td>
<td>79</td>
<td>131</td>
<td>183</td>
<td>159</td>
<td>174</td>
<td>186</td>
<td>199</td>
<td>200</td>
</tr>
</tbody>
</table>

First limiting AA Met+Cys Lys Thr Met+Cys Lys Lys Lys Lys Met+Cys Lys/L Met+Cys
Table 2.4 Proximate composition of fish feed items studied in this experiment.

<table>
<thead>
<tr>
<th>Proximate compositions</th>
<th>Feed items</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Napier grass</td>
</tr>
<tr>
<td>Total phosphorous (mg P/g)</td>
<td>21.72 ± 2.49b</td>
</tr>
<tr>
<td>Crude protein (% DM)</td>
<td>12.75 ± 1.44b</td>
</tr>
<tr>
<td>Crude lipid (% DM)</td>
<td>2.3 ± 0.8a</td>
</tr>
<tr>
<td>Crude fibre (% DM)</td>
<td>32.4 ± 4.2d</td>
</tr>
<tr>
<td>Moisture (% DM)</td>
<td>5.05 ± 0.04b</td>
</tr>
<tr>
<td>Ash (% DM)</td>
<td>10.65 ± 0.88e</td>
</tr>
<tr>
<td>Non-fibrous carbohydrate (%)</td>
<td>36.9</td>
</tr>
</tbody>
</table>

Mean ±SD having the same letters in the same row are not significantly different (p<0.05)
environment, using Napier grass as the primary fish feed. Other than Napier grass, multicellular algae, detritus and sometimes copepods could be found in the gut content of fish.

Other than essential amino acids and crude protein, other crude compositions of the diets could also contribute to the quality of a fish feed. Phosphorous is essential to fish for proper bone mineralization (Lim et al., 2001). Phosphorous (0.4 or 0.85 mg/kg) could also reduce mortality in channel catfish when challenged with a bacterium, *Edwardsiella ictaluri* (Eya and Lovell, 1998). Common signs of phosphorous deficiency observed in other fish including reduced growth (rainbow trout, Atlantic salmon, channel catfish, common carp, Japanese eel and red sea bream), poor feed conversion (rainbow trout, Atlantic salmon, Chinook salmon, channel catfish and red sea bream) and reduced bone mineralization (rainbow trout, Atlantic salmon, channel catfish, common carp and red sea bream) (Lall, 2002). Phosphorous in mineral premix could significantly improve weight gain, food conversion ratio and protein efficiency ratio of Nile tilapia (Datocajegas and Yakupitiyage, 1996). Wang et al. (2002) revealed that phosphorous requirement of grass carp was 1.42-1.58% of diet, while tilapia requires 0.9% P/kg diet (Lall, 2002). Total phosphorous contents detected in feed items are listed in Table 2.4. Some of the feed items, including the 2 noodle samples, white bread, hamburger bun and Jiefeng® 613 fish feed pellets, all had total phosphorous contents lower than the dietary requirement of grass carp (1.42-1.58%), while all of the feed items contained sufficient phosphorous for tilapia (0.9%).

Lipid is an important energy source for fish. A sufficient amount of dietary lipid content in fish feeds could promote growth and food utilization, as protein in fish is spared (conserved
for other metabolic functions and building muscle) from energy generation and more energy is derived from other energy sources (such as carbohydrates or lipids) (De Silva et al., 2001; Phillips, 1972; Skalli et al., 2004). The dietary lipid requirement for grass carp is 4% (Du et al., 2004), while Nile tilapia 5% (preferably 10-15%) (Ng and Chong, 2004). In the present study, crude lipid contents of Napier grass, white bread and soybean dreg were 2.3%, 3.2% and 1.5%, respectively, which were lower than the dietary requirements of both grass carp and Nile tilapia. The two noodles samples, on the other hand, contain rather high crude lipid contents (21.3% and 19.5%) were higher than the requirements of both grass carp and Nile tilapia. FWB (13.3%) and rice bran (11.2%) possessed lipid contents higher than the requirement of grass carp, while suitable for Nile tilapia.

The exact requirement of carbohydrates of fish is unknown, as carbohydrates are not essential for fish (NRC, 1993). Although fish prefer using deaminated amino acids than glucose for energy more efficiently (Phillips, 1972; Lovell, 1988), carbohydrates could serve as the least expensive energy source for fish (NRC, 1993; Wilson, 1994) and spare protein from catabolism for energy generation for some fish species, such as grass carp and rainbow trout (Stone, 2003). Carbohydrate is also used as a binding agent in formulated diets (FAO, 2014b). It has been suggested that the carbohydrate inclusion rate of for warm water omnivorous fish should be 40% while carnivorous fish 20% (Allan and Rowland, 2002; Catacutan and Coloso, 1997; Ding, 1991; Hardy and Barrows, 2002; Lim, 1991; Satoh, 1991; Shimeno, 1991; Wilson, 1994). Nile tilapia could efficiently utilize up to 30-70% digestible carbohydrates (FAO, 2014b), while inclusion of 38% carbohydrates in the diet of grass carp had no adverse effects on specific growth rate, feed efficiency and protein retention efficiency (Li et al.,
In the present study, all common fish feeds (except Napier grass) contained higher than 40% non-fibrous carbohydrate, while FWA and FWB 39.9% and 24.2%, respectively.

Among all common fish feeds, soybean dreg contained the highest amount of crude protein (about 35%) and it is a popular feed used by local fish farmers. However, the non-essential amino acids consist of a large proportion of total protein in soybean. Aspartic acid and glutamic acid contributed to 11.4% and 17.9% of protein, respectively, of soybean meal (Feedipedia, 2014b). Even though soybean dreg contains high protein content, supplementation with lysine would be essential as lysine is inadequate in plant based protein sources (Mai et al., 2006). On the other hand, the method for cooking soybean milk (boiling by hot water followed by squeezing) is not adequate to inactivate the anti-nutritional factors such as protease inhibitors, because effective inactivation requires heating the soybean at 134°C for 1.5 min or at 102°C for 40 min (Qin et al., 1996). It has also been observed that completely replacing fishmeal with plant protein in fish diet affected growth of rainbow trout (De Francesco et al., 2004). Kissil et al. (2000) reported that feed intake and weight gain of sea bream were negatively affected by the inclusion levels of both soy (60% and 100% replacement) and rapeseed protein (100% replacement). To fully utilize the protein in soybean, removal of anti-nutritional factors present in soybean with heat treatment and supplementation with lysine, would be necessary, but these would involve a higher cost.

Fish meal was thought to be the best protein source for fish as it contains sufficient amount of lysine (7.5%) (Feedipedia, 2014a), as lysine is a major amino acid found in fish muscle (Wilson and Cowey, 1985; Wilson and Poe, 1985; Kim and Lall, 2000). However, protein is
the most expensive ingredient in fish feeds (Pillay, 1990). As per November 2013, the global fishmeal price is about US$1,550/tonne (Index Mundi, 2014a). Items with low protein content were adopted by local fish farmers as price is the major governing factor. Inadequate amounts of nutrients (such as lipid, protein, EAA and minerals) in fish feeds would hinder fish growth, which will in turn reduce the turnover rate of fish ponds and reduce the incomes of fish farmers.

In the present study, two types of food waste based fish diets, FWA and FWB, were formulated and their EAA profiles and proximate compositions were analysed and compared to common fish feeds. FWA mainly composed of plant based materials (53% cereals) as major protein sources, while FWB has a more balanced ratio of meat and cereal (25% meat products and 28% cereals). The lysine contents as well as the EAA profiles of the food waste diets were sufficient for both grass carp and Nile tilapia. In general, crude protein, crude lipid, carbohydrate and total phosphorous were suitable for both fish species (except crude lipid of FWB, about 3 times higher than the suggested value for grass carp, compared to other common fish feeds). These results indicated that food waste could be utilized to produce fish feed which containing satisfactory amino acids, protein, lipid, carbohydrate and phosphorous. Thus, the hypothesis was accepted.

2.4 Conclusions

Results from this experiment clearly demonstrated that the common fish feeds, including Napier grass, rice bran, soybean dreg, noodles and breads, are nutritionally insufficient, with insufficient lysine content (except Jiefeng® 613), crude protein (except Jiefeng® 613 and
soybean dreg) and lipid (except for the two noodle samples). All the fish feed pellets (Jiefeng® 613, FWA and FWB) contained sufficient amounts of essential amino acids, crude protein, crude lipid and total phosphorous for both grass carp and Nile tilapia, indicating that the fish feed pellets are more suitable fish feeds compared with the traditional feed items used by local fish farmers. The effects of the food waste based diets on growth performance of fish fed with the fish feed pellets will be investigated in Chapters 3 and 4, based on a field feeding experiment involving polyculture of carps, and a laboratory feeding experiment rearing grass carp and Nile tilapia, respectively.
Chapter 3 Effects of food waste based feed pellets on polyculture of carps and water quality: A field trial

3.1 Introduction

As dumping food waste into landfills will definitely shorten the lifespan of the existing landfill sites in Hong Kong, making use of food waste for farming fish may ease part of the pressure in disposing waste in landfills. Fish farms in Hong Kong currently are engaged in polyculture of low trophic level fish, such as grass carp, bighead carp, common carp and silver carp in combination with tilapia or grey mullet, and is the dominant farming practice (98%) adopted by local inland fish farmers (ACFD, 2014).

Traditionally, feed stuff such as poultry and swine waste, food waste and agriculture waste are used in polyculture ponds (Lau et al., 2003; Wong et al., 2004). Examples of unconsumed food or agricultural by-product used by local fish farmers for feeding fish are rice bran, wheat bran, peanut cake, bread, expired instant noodles or leftover from household kitchens. However, the growth performance of fish in waste-fed or grass-fed culture pond is generally inferior to fish reared in pellet-fed ponds as compound diets are made up with nutritious ingredients such as fishmeal, fish oil and oilseed and are designed to meet the nutrient requirements of fish. Pandit et al. (2004) reported the FCR values of grass carp fed with Napier grass were about 30-39 (wet weight basis). Moreover, the FCR values of grass carp fed with bean dreg (remains of soybean after juicing soybean milk) and commercial pellet diet (“320” green grass powder pellet feed, consisted of 40% green grass powder, 20% rapeseed cake, 5% fishmeal, 15% bean cake, 5% silkworm pupae and 15% barley flour) were 25 and
2.5-3, respectively (Yu, 1989), indicating grass carp showed a better growth when fed with the pellet diet. Afzal et al. (2008) observed that bighead reared in pond with supplementary feeding (composed of fishmeal, soybean meal, rapeseed meal, rice bran, wheat bran and vitamin remix) had a better weight gain than fish reared in pond with fertilizer (cow dung, urea or single super phosphate) only.

Historically, fish farmed in inland ponds are fed with waste material or agricultural by-products. However, there is a lack of information on the use of food waste on fish growth or water quality. Three species of carp, grass carp (*Ctenopharyngodon idella*), bighead (*Hypophthalmichthys nobilis*) and mud carp (*Cirrhinus molitorella*), possessing different feeding habits were used in this study. Grass carp primarily consumes macrophytes in natural environment, but it prefers pellet diets in fish pond culture where pellet diets are given (Lopinot, 1972; Masser, 2002). Bighead is a filter feeder that primarily consumes plankton in water. Mud carp is a bottom feeder and mainly feeds on organic matter deposited at the pond bottom as well as benthic organisms, when reared as a secondary fish species (FAO, 2014d). The species chosen represent a herbivore (which adapted to feed pellets containing needed ingredients), a filter feeder and a bottom feeder, which are considered as lower trophic level fish species. Although there are other fish species (e.g. grey mullet and tilapia) that are commonly farmed in polyculture ponds, they were not included because the fish fry were not available.

It was hypothesized that farming different species of low trophic level fish (carp species) using food waste based diets would be beneficial in fully utilizing the residual energy
contained in the food waste, as different fish species possess different feeding habits. The objectives of the present study were to (1) investigate the effects of food waste based diets on water quality and plankton density; and (2) estimate the growth performance of different fish species fed with food waste based diets.

3.2 Materials and Methods

3.2.1 Fish diets

Three fish feed diets were used in this experiment: Control (Jiefeng® 613), Food Waste Diet A and B (FW A and FW B). Detailed information of FWA and FWB were listed in Section 2.2.1. Prepared diets were then stored in a dry place. Jiefeng® 613 formulated feed was used as the control fish feed pellet (Control), which is a complete diet (contains flour, wheat middling, fishmeal, rapeseed meal, bean pulp, bean oil, fish oil and vitamin-mineral supplement). In general, all three fish feed pellets contained sufficient essential amino acids for grass carp (tested in Chapter 2, Table 2.1).

3.2.2 Fish ponds, fish and feeding trial

Three rectangular-shaped fish ponds with about 400 m² in surface area (18 m x 22 m) and 3 m in depth were dug during the summer of 2011. U-shaped pipes were installed in each pond to collect drain water from a nearby river via rainfall and mountain drainage. Fry of grass carp (Ctenopharyngodon idella), bighead carp (Hypophthalmichthys nobilis) and mud carp (Cirrhinus molitorella) were purchased from a fish farm in Dongguan, mainland China. One thousand individual fish in total were stocked at each pond with grass carp: bighead: mud
Table 3.1 Compositions of the food wasted based feed pellets

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Food waste (% w/w)</th>
<th>Non-food waste (% w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fruit and vegetables&lt;sup&gt;1&lt;/sup&gt;</td>
<td>Meat products&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>FWA</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>FWB</td>
<td>10</td>
<td>25</td>
</tr>
</tbody>
</table>

<sup>1</sup>Fruit and vegetables includes pineapple watermelon, cantaloupe, strawberry, banana, apple and leaf vegetables, like as lettuce and spinach.

<sup>2</sup>Meat products includes beef, pork, chicken, salmon and groupers

<sup>3</sup>Cereals includes rice bran, soy bean meal, rice grain and spaghetti

<sup>4</sup>Bone meals includes bone from beef, pork, chicken and grouper
carp ratio at 3:1:1. The feeding trial commenced on October, 2011 and finished on April, 2012. Each pond was fed daily with one of the experimental diets and fish were fed at a fixed feeding rate of 4% body mass (w/w) of grass carp twice a day at 9:00 am and 4:30 pm manually. The wet weight gain (g), length gain (cm) and productivity of fish (kg fish/ha) were monitored for all fish species and at least 10 fish of each species from each pond were collected by netting and fish weight and length was measured again at the end of feeding trial. The productivity of fish was estimated by the following equation according to William (1992):

\[
\text{Productivity (kg fish/ha) = Fish weight (kg) × no. of individuals /pond area (ha)}
\]

In addition, specific growth rate (SGR), feed conversion ratio (FCR) according to Bake et al. (2009) and protein efficiency ratio (PER) were calculated according to Kaushik et al. (2004) (for grass carp only). SGR, FCR and PER were respectively calculated by the following equations:

\[
\text{SGR (\%) = } \frac{\ln \text{final weight (g)} – \ln \text{initial weight (g)}}{\text{feeding period (day)}} \times 100
\]

\[
\text{FCR = feed provided (g) / } \left[ \text{Final biomass – Initial biomass (g)} \right]
\]

\[
\text{PER (\%) = } \frac{\text{wet body weight gain (g)/protein intake (g)}}{100}
\]

Unconsumed food was not included in the calculation, as it was not removed from the fish ponds.

### 3.2.3 Proximate compositions of fish diets

Total Kjeldahl nitrogen (TKN), total phosphorous (TP), crude protein, ash content, organic content and moisture content of the 3 kinds of experimental diets were determined. TKN and TP were determined following the methods described in APHA (2002). Crude protein was
calculated by multiplying total Kjeldahl nitrogen by 6.25, moisture content was determined by heating at 105°C for 24 h using an oven, and ash content was determined by heating at 550°C for 4 h using a furnace muffler (AOAC, 2002). Carbohydrate content and energy of diets were calculated according to the following equations:

Carbohydrates (%) = 100 - (crude protein % + crude lipid % + moisture% + ash % + fibre %) (Castell and Tiews, 1980)

Energy (KJ/g diet) = (% crude protein × 23.6) + (% crude lipids × 39.5) + (% carbohydrates × 17.3) (Chatzifotis et al., 2010)

Proximate compositions of the 3 experimental diets are listed in Table 3.2.

3.2.4 Sampling scheme for fish and water quality monitoring

During October 2011, December 2011, February 2012 and April 2012, 3 water samples and 3 sediment samples were collected from 3 edges of each fish pond, approximately 1 m apart from the pond edge. At least 10 fish of each species were collected during October 2011 and April 2012, with their weight and length being recorded. Water quality variables: dissolved oxygen (DO), pH, nitrite nitrogen (NO₂-N), nitrate nitrogen (NO₃-N), total ammonia nitrogen (TAN), total Kjeldahl nitrogen (TKN), phosphate phosphorus (PO₄-P), and total phosphorus (TP) were determined. Total alkalinity was determined by a titrimetric method (Stirling, 1985). Nitrate and nitrite nitrogen (cadmium reduction), reactive phosphate and total phosphorus (molybdenum blue method), total ammonical nitrogen and total Kjeldahl nitrogen were determined following the methods described in APHA (2002). TKN and TP of pond sediment were also monitored. Freeze-dried solid sediment samples were grounded and digested using concentrated sulphuric acid in the presence of Kjeldahl catalyst using micro-Kjeldahl
apparatus and then TKN and TP were measured according to the same methods mentioned above.

3.2.5 Total plankton estimation

Ten L of sub-surface water (0.5 m under water surface) were collected with a bucket from each fish pond and then passed through a 20-μm plankton net (Rahman et al., 2008). Plankton retained in the net were concentrated to 50 ml. Within 6 h, plankton samples were preserved and stained with Lugol's iodine. The count of plankton samples were performed with Sedgewick–Rafter (S–R) cell containing 1000 fields of 1 mm³. A 1-ml sample was put in the S–R cell and left for 10 min to allow the plankton to settle. The number of plankton in each of the 10 randomly selected fields in the S–R cell was counted.

3.2.6 Statistical analyses

Normality and homogeneity of data were tested prior to ANOVA. The growth results of fish were compared at each sampling using one-way ANOVA. Two-way ANOVA was used to test the effect of diets and sampling time on pond water nutrients and Duncan's multiple comparison test was used to compare means. Significant differences between experimental groups were expressed at the significance level of \( p < 0.05 \). All statistics were calculated using SPSS Statistics 19.0 (IBM, USA).

3.3 Results and Discussion

Amounts of dry matter, moisture content, crude protein and TKN were not significantly differed (\( p > 0.05 \)) (Table 3.2). On the other hand, there were
Table 3.2 Proximate compositions of the experimental diets

<table>
<thead>
<tr>
<th>Formulation</th>
<th>Control</th>
<th>FWA</th>
<th>FWB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry matter (%)</td>
<td>93.7±0.20a</td>
<td>95.7±0.02a</td>
<td>93.2±0.05a</td>
</tr>
<tr>
<td>Ash (%)</td>
<td>8.24±0.09a</td>
<td>9.18±0.46a</td>
<td>18.9±0.03b</td>
</tr>
<tr>
<td>Total phosphorous (µg P/g fish feed)</td>
<td>967±313a</td>
<td>2770±470c</td>
<td>1942±478b</td>
</tr>
<tr>
<td>Total Kjeldahl nitrogen (µg KN/g fish feed)</td>
<td>2470±47a</td>
<td>2350±215a</td>
<td>2299±232a</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>30.2±1.55a</td>
<td>31.4±0.44a</td>
<td>31.1±3.36a</td>
</tr>
<tr>
<td>Lipid (%)</td>
<td>5.17±0.94a</td>
<td>6.12±1.66a</td>
<td>13.3±1.81b</td>
</tr>
<tr>
<td>Fiber (%)</td>
<td>9.57±0.21 a</td>
<td>5.72±0.87 b</td>
<td>10.1±0.63 a</td>
</tr>
<tr>
<td>Carbohydrates (%)&lt;sup&gt;1&lt;/sup&gt;</td>
<td>40.5</td>
<td>39.9</td>
<td>24.2</td>
</tr>
<tr>
<td>Energy (kJ/g diet)&lt;sup&gt;2&lt;/sup&gt;</td>
<td>16.2</td>
<td>16.8</td>
<td>16.6</td>
</tr>
<tr>
<td>CHO/L ratio&lt;sup&gt;3&lt;/sup&gt;</td>
<td>7.84</td>
<td>6.36</td>
<td>1.83</td>
</tr>
<tr>
<td>P/E (mg/kJ)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1865</td>
<td>1857</td>
<td>1898</td>
</tr>
</tbody>
</table>

Note: Different letters (a, b, c) between feeding groups are significantly different (p< 0.05) (n=3).

<sup>1</sup>Carbohydrates (%) = 100 − (crude protein % + crude lipid % + moisture% + ash %+ fibre %) (Castell and Tiews 1980)

<sup>2</sup>Energy (KJ/g diet) = (% crude protein × 23.6) + (% crude lipids × 39.5) + (% carbohydrates × 17.3) (Chatzifotis et al., 2010)

<sup>3</sup>Carbohydrates to lipid (CHO: L) ratio = % wt. in CHO/ % wt. in lipid

<sup>4</sup>Protein to energy (P/E) (mg/kJ) = crude protein (%) / Energy
significant differences (p<0.05) in ash content and TP among the 3 diets, which indicated that incorporating more cereal would lead to a higher amount of phosphorous in the fish diet.

Table 3.3 shows the growth performance indices of fish (including length gain, wet weight gain and productivity for all 3 species fish); feed conversion ratio, specific growth rate and protein efficiency ratio of grass carp only. Bighead carp in pond fed with FWA showed significantly higher wet weight gain and length gain (p<0.05), while grass carp fed with FWB significantly higher wet weight gain, length gain, productivity, FCR, SGR and PER (p<0.05). No significant difference was noted in the growth of mud carp among the 3 treatments.

The bi-monthly changes in concentrations of soluble nitrogen and phosphorous compounds are shown in Fig. 3.1 and Fig. 3.2, respectively. In general, concentrations of nitrogenous compounds in all fish ponds were elevated (except for NH$_4$-N and NO$_2$-N). On the other hand, except for the pond fed with FWA, no significant differences in the concentrations of phosphorous compounds in the 3 fish ponds were observed. During April, nutrient concentrations of the 3 fish ponds generally decreased, possibly caused by the changes of plankton biomass. From October to February, the biomass of plankton was low, as the low atmospheric temperature resulted in lower photosynthetic rate (Coles and Jones, 2000; Robarts and Zohary, 1987). In addition, introduction of bighead into fish ponds could control the amount of plankton (Lieberman, 1996). As temperature increased in spring, the photosynthetic rate of phytoplankton increased and thus more nutrients were incorporated (Coles and Jones, 2000; Robarts and Zohary, 1987), leading to the decrease of soluble nutrients in April.
Table 3.3 Growth performance of three studied fish species.

<table>
<thead>
<tr>
<th></th>
<th>Grass carp</th>
<th></th>
<th>Bighead carp</th>
<th></th>
<th>Mud carp</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>FWA</td>
<td>FWB</td>
<td>Control</td>
<td>FWA</td>
<td>FWB</td>
</tr>
<tr>
<td>Length (cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>21.13±0.99a</td>
<td>21.33±0.82a</td>
<td>23.70±1.30b</td>
<td>16.4±1.26a</td>
<td>19.6±2.50b</td>
<td>16.0±1.19a</td>
</tr>
<tr>
<td>Length gain (%)</td>
<td>47.7±6.9a</td>
<td>49.2±5.7a</td>
<td>65.7±9.1b</td>
<td>44.2±11.5a</td>
<td>40.3±10.2a</td>
<td>71.7±22.6b</td>
</tr>
<tr>
<td>Weight (g)</td>
<td>102.58±6.52b</td>
<td>83.02±8.98a</td>
<td>139.32±25.80c</td>
<td>39.4±9.46c</td>
<td>64.7±24.2a</td>
<td>36.4±9.28c</td>
</tr>
<tr>
<td>Weight gain (%)</td>
<td>76.4±11.2b</td>
<td>42.7±15.4a</td>
<td>139.6±44.4c</td>
<td>60.6±39.4a</td>
<td>164±99.0b</td>
<td>49.3±38.8a</td>
</tr>
<tr>
<td>Productivity (Kg fish/ha)</td>
<td>1539±98b</td>
<td>1245±135a</td>
<td>2344±387c</td>
<td>197±47a</td>
<td>324±121b</td>
<td>182±49a</td>
</tr>
<tr>
<td>Feed conversion ratio</td>
<td>2.41±0.36b</td>
<td>4.76±1.82a</td>
<td>2.02±0.47b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific growth rate (%)</td>
<td>24.68±3.62b</td>
<td>13.81±4.99a</td>
<td>45.09±14.34c</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein efficiency ratio (%)</td>
<td>13.17±1.93b</td>
<td>7.37±2.66a</td>
<td>29.08±7.65c</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Different superscripts (a, b, c) between feeding groups are significantly different (p<0.05) (n>10). Feed conversion ratio, specific growth rate and protein efficiency ratio were calculated for grass carp only as grass carp is the only member consuming fish feed pellets.

1 Weight gain (%) = \( \frac{\text{final weight (g)} - \text{initial weight (g)}}{\text{initial weight (g)}} \times 100 \)

2 Productivity (Kg fish/ha) = Amount of fish produced (Kg) \times area of pond (ha)

3 Feed conversion ratio = \( \frac{\text{feed intake (g)}}{\text{Final biomass} - \text{Initial biomass (g)}} \)

4 Specific growth rate (%) = \( \frac{\ln \text{final weight (g)} - \ln \text{initial weight (g)}}{\text{feeding period (day)}} \times 100 \)

5 Protein efficiency ratio (%) = \( \frac{\text{wet body weight gain (g)}}{\text{protein intake (g)}} \times 100 \)
Fig 3.1 Phosphorous compounds in fish ponds (n=3). Statistics for P compounds (2 way ANOVA): PO₄-P in water: treatment: p<0.0001, sampling month: P<0.0001, interaction: p<0.0001; Total P in water : treatment: p<0.0001, sampling month: P<0.0001, interaction: p<0.0001; Org P in water: treatment: p<0.0001, sampling month: P<0.0001, interaction: p<0.0001; TP in pond sediment treatment: p<0.0001, sampling month: P<0.0001, interaction: p<0.0001. Black = control, Grey = FWA, White= FWB. Bars that sharing at least one superscript do not differ at p< 0.05.
Fig 3.2a Nitrogen compounds in fish ponds (n=3). Statistics for N compounds (2 way ANOVA): Organic N: treatment: p<0.0001, sampling month: P<0.0001, interaction: p<0.0001; NH₄-N: treatment: p=0.635, sampling month: P=0.533, interaction: p=0.01; TKN: treatment: p<0.0001, sampling month: P<0.0001, interaction: p<0.0001; Total N: treatment: p<0.0001, sampling month: P<0.0001, interaction: p<0.0001;
Fig 3.2b Nitrogen compounds in fish ponds (n=3). Statistics for N compounds (2 way ANOVA): NO$_3$-N: treatment: p=0.733, sampling month: P=0.17, interaction: p=0.763; NO$_2$-N: treatment: p=0.112, sampling month: P<0.0001, interaction: p=0.001; TKN in sediment: treatment: p=0.214, sampling month: P<0.0001, interaction: p<0.0001; significantly different (p< 0.05). Black = control, Grey = FWA, White= FWB.
Fig 3.3. Plankton density in fish ponds. Statistics for plankton density (2 way ANOVA): NO₃-N: treatment: p=0.733, sample month: P=0.17, interaction: p=0.763 Black = control, Grey = Diet A, White= Diet B.
The roles and feeding habits of carp species in polyculture ponds have been reviewed (Wong et al., 2004). Grass carp is a herbivore which mainly consumes aquatic plants in the natural habitat, but it prefers pellet diets than aquatic macrophytes in pond culture (Lopinot, 1972; Masser 2002). Bighead carp is a filter feeder and primarily consumes plankton, although it has been reported that the productivity of bighead could be enhanced with supplement diet (consisted of 20% fishmeal, 10% soybean, 5% rapeseed meal, 40% rice bran, 23% wheat bran and 2% vitamin premix, having 22.76% crude protein) (Afzal et al., 2008). Mud carp is a bottom feeder which consumes organic matter and benthic organisms deposited at the pond bottom. The growth performance of the 3 fish species could reflect the effects of different diets on fish with different feeding habits.

FCR value is a measure of the effectiveness of animal converting feed stuff into biomass. A low FCR value is a good indicator of a high quality of feed (USAID-HARVEST, 2011). More grass carp was stocked (600 individuals per pond) in this experiment. Grass carp fed with FWB showed a better (lower) FCR value (2.04), followed by the group fed with Control diet (2.41) and then FWA (4.60). This indicated that grass carp could utilize FWB better than the other diets. However, the FCR values in the present study might be over-estimated, as unconsumed feed was not removed from the ponds, the FCR value of FWB was better than other diets (“320” green grass powder pellet feed, consisted of 40% green grass powder, 20% rapeseed cake, 5% fishmeal, 15% bean cake, 5% silkworm pupae and 15% barley flour, FCR = 2.5-3) investigated by Yu (1989) and Napier grass (FCR = 30.6-39.1, wet weight basis) (Pandit et al., 2004).
The total productivity of the 3 species was also estimated (Table 3.3). The pond fed with FWB resulted in a higher productivity of grass carp (2344 kg/ha) than ponds fed with Control (1539 kg/ha) and FWA (1245 kg/ha). On the other hand, the pond fed with FWA had a better productivity of bighead (324 kg/ha) than ponds fed with Control (197 kg/ha) and FWB (182 kg/ha). The better performance of FWB for grass carp could be explained by the carbohydrate content of fish diets. Carbohydrate content of FWA and Control were 40.5% and 39.9%, respectively, which were much higher than FWB (24.2%). It has been observed that inclusion of low levels (≦10%) of digestible carbohydrates in diets improved feed utilization and protein retention in rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*) European eel (*Anguilla anguilla*), cod (*Gadus morhua*) and different carp species (*Catla catla*, *Labeo rohita* and *Cirrhinus mrigala*) than with diets lacking carbohydrates (Bergot, 1979; Degani et al., 1986; Degani and Viola, 1987; Erfanullah, 1998; Hemre, 1992; Hemre et al., 1995).

No previous studies regarding the optimal amount of carbohydrates in the diets of grass carp are available. The present results showed that the growth performance of grass carp fed with the low carbohydrate diet (about 24% in FWB) would be more suitable than the higher carbohydrate diet (about 40% in FWA). Further studies on the optimal amount of carbohydrates in diets would be necessary.

On the other hand, Gao et al. (2010) noted that grass carp could utilize lipids slightly better than carbohydrates. The ratio of carbohydrate to lipid (CHO:L) of FWA and FWB were 6.83 and 1.83, respectively. Sufficient amount of dietary lipid content in fish feed could promote
growth and food utilization, as protein in fish is spared from oxidation (conserved for other metabolic functions and building muscle) by carbohydrates and lipids (De Silva et al., 2001; Skalli et al., 2004). FWB contains a higher lipid content (about 13%) led to better growth performance of grass carp.

Moreover, Das and Tripathi (1991) also observed that high amylase and protease activities in grass carp from culture pond and inclusion of animal protein in the diets was suggested, in order to suit its digestive enzyme pattern. The better growth performance of grass carp obtained from this study was in line with the finding that grass carp fed with FWB containing 25% of meat products gave rise to significantly higher wet weight gain and length gain, better PER, FCR and SGR.

The approximate amounts of phosphorous compounds loaded (via feeding) to pond fed with Control diet, FWA and FWB pond were 0.2 kg, 0.6 kg and 0.4 kg, respectively. The pond fed with FWA had the highest amount of P loading and showed a significantly higher density (p<0.05) of plankton and FWA contained a significantly higher phosphorous content (p<0.05) than those of control diet and FWB. Consequently, phosphorous would be released due to degradation of fish excretion and unconsumed feed. Brabrand et al. (1990) revealed that the excreted phosphorous compounds from fish can be readily utilized by phytoplankton, which in turn provide more food to bighead, leading to a better growth of bighead in pond fed with FWA. In fact, it is commonly observed that a higher density of plankton could enhance weight gain of bighead (Cooke et al., 2009).
However, excessive growth of plankton may consume a substantial amount of dissolved oxygen, causing the fish stock to suffocate (Lau et al., 2003). Moreover, although a higher density of plankton can promote the growth of filter feeders (such as bighead and silver carp), filter feeders are usually the minority members included in polyculture system for controlling plankton populations. Filter feeding fish could also consume other food sources such as pellets when they are stocked at a high stock density (Stone et al., 2000).

It is worth to note that the inclusion of more cereals in fish feed pellets could lead to higher ash and phosphorous contents in the fish feeds (FWA). It would be important to control the amount of phosphorous in fish feeds. Excessive amounts of phosphorous used in fish feed such as FWA employed in this experiment, resulted in excessive amounts of plankton although no significant elevation (p>0.05) was observed in concentrations of phosphorous in the ponds. It is commonly noted that an elevated concentration of TP (relative to TN) in water system could support the growth of plankton (Hessen et al., 2007; Liess et al., 2009; Main et al., 1997). Phosphorous is also important for body metabolism as well as immunity of fish (Jokinen et al., 2003). On the other hand, the higher inclusion rate of cereal resulted in inferior growth performance of grass carp (fed with FWA). Thus, it would be desirable to include about 25% cereal in fish feed pellet production.

The use of food waste in fish ponds could also reduce the use of pond fertilizer. It was indicated in Chapter 2, the proximate composition analysis of fish feeds commonly used by local fish farmer generally contained low contents of crude protein and total phosphorous. In order to maintain the fertility of pond water for providing more nutrients, pond fertilizers such
as poultry manure or peanut cake are used, but often in excessive amounts. In the present study, the resulted soluble nutrient concentrations (total N: 0.26-0.56 mg/l; nitrite: 1.5-2.5 μg/l; nitrate: 3.5-12.5 μg/l; and total P: 0.08-0.24 mg/l) detected in the pond water were comparable to the findings of Mischke and Zimba (2004), who conducted pond fertilization experiments to study the effects of various fertilization regimes using cottonseed meal or inorganic N and P compounds. The soluble nutrients in pond water seemed to be suitable for rearing fish and could replace the use of animal manure as pond fertilizers.

In the old days, animals such as pigs and ducks are reared along with fish, as input of manure from these animals provide nutrients as pond fertilizer by providing nitrogen and phosphorous that support growth of plankton which will in turn serve as food for fish in fish ponds (Wong et al., 2004). However, backyard poultry farming in Hong Kong was banned in 2006 in order to prevent Avian Flu (Legislative Council, 2013). The reduction in numbers of terrestrial animal farmers also makes animal manure less available. As there are a large amount of food waste generated in Hong Kong, using food waste in fish ponds as pond fertilizers could be a feasible and attract option to ease the disposal pressure. The chances for diseases transmission could also be reduced.

3.4 Conclusions

In general, FWA and FWB favoured the growth of different fish species. FWA that possessing a higher P content favoured the growth of plankton, leading to better growth of bighead carp, while FWB favoured the growth of grass carp, probably due to the relatively lower amount of carbohydrates (24.2%) and CHO:L ratio (1.83) than Control and FWA. The choice of
ingredients for producing fish feed pellets is therefore important in order to strike the balance of fish growth, plankton growth and water quality. The more balanced ratio of meat products and cereals in FWB seems to be better than FWA which contained over 50% of cereals, in terms of yielding a higher overall productivity, better food conversion ratio and the effects on an optimal plankton density. Using food waste based diets could also reduce the amount of pond fertilizer used (animal manure) as food wastes are full of nutrients and readily available. Due to the fact that FWB was more superior than FWA based on fish growth performance, it will be further upgraded by incorporating vitamins, prebiotic fibres and Chinese herbs, to improve the digestibility of diets which associated fish growth performance.
Chapter 4 Upgrading food waste based diets with vitamin-mineral premix: effects on feed protein digestibility and fish growth

4.1 Introduction

The effects of food waste based diets on the growth performance of three carp species (grass carp, bighead and mud carp) in polyculture ponds were investigated (Chapter 3). However, the combination of fish species used could affect the accurate estimation of growth performance parameters. Thus, this chapter focused on the growth performance and protein digestibilities of grass carp and Nile tilapia.

Grass carp (*Ctenopharyngodon idella*) has the second largest production in freshwater aquaculture globally after silver carp (FAO, 2014a). Grass carp is considered to be a herbivore that primarily consumes aquatic plants, but would also consume other food items such as copepods, insects and their larvae, crustaceans and small fish (Chilton and Muoneke, 1992). In fact, grass carp feeds on anything when vegetation is scarce, including fish, worms and insects, but it prefers pelleted feed to vegetation in pond culture (Lopinot, 1972). Masser (2002) reported that grass carp would consume pelleted diet if it is available.

Tilapia (common name for almost a hundred species of cichlid fish, such as Nile tilapia, *Oreochromis niloticus*, blue tilapia, *Oreochromis aureus* and Mozambique tilapia, *Oreochromis mossambicus*) is the second most important group of fish farmed worldwide after carps (FAO, 2014b). Nile tilapia (*Oreochromis niloticus*) is considered to be an omnivorous fish. It is one of most frequently farmed fish species worldwide due to its fast
growth rate, tolerance to high stocking density and various diet preferences. The annual production of Nile tilapia was 3,197,330 tonnes in 2012 (FAO, 2014b). The feed items commonly consumed by tilapia include phytoplankton, periphytons, aquatic plants, small invertebrates, benthic fauna, detritus and bacterial films associated with detritus manures, agricultural by-products and formulated feeds (FAO, 2014b).

Inclusion of vitamins and minerals were not considered during the production of the food waste based diets. Incorporating minerals and vitamins in feeds is a common practice in animal production using vegetable-based diets, for compensating the low mineral availability and enhancing the nutritional qualities of the feeds (Deyhim et al., 1995). Vitamin-mineral premix refers to supplements that contain vitamins and minerals, to be included in diets. It has been noted that inclusion of vitamin-mineral premix in the diet for broadhead catfish (Clarias macrocephalus) fry could enhance their survival rate and weight gain than the control group (without supplement) (Taechajanta and Sitasit, 1981). Phosphorous in mineral premix could enhance the weight gain, FCR and PER of tilapia (Datocajegas and Yakupitiyage, 1996). However, other studies observed that reduced amounts or without vitamin mineral premix had no effects on improving the growth performance in shrimp and crab (Triño and Sarroza 1996; Triño et al., 2001). Thus, it would be worthwhile to study the effects of adding vitamin-mineral premix on fish growth.

This experiment focused on the protein digestibility, growth performance and the resulted body composition of fish that fed with food waste based diets. Nile tilapia and grass carp were used as model in this experiment. The effects of incorporating vitamin-mineral premix on the
aforementioned parameters were also studied. It was hypothesized that both fish species could effectively utilize food waste based diets and inclusion of vitamin-mineral premix could further enhance the growth of fish. The objectives of this experiment were to study the effects of diets (with or without vitamin-mineral premix) on (1) growth performance of grass carp and tilapia; (2) the digestibility of the diets; and (3) the whole carcass crude ash, crude lipid and crude protein of fish.

4.2 Materials and Methods

4.2.1 Fish and fish tanks

Two separate feeding trials were conducted involving grass carp (*Ctenopharyngodon idella*) and Nile tilapia (*Oreochromis niloticus*). Thirty individuals of each species were obtained from local fish farms in both Hong Kong and mainland China, respectively. Fish were treated with 3% of salt solution for 60 s for eliminating parasites before randomly dividing into 12 200 L plastic fish tanks. Each tank was connected to an external filter and continuously aerated. Fish were acclimatized for at least 2 weeks before introducing into 18 experimental tanks (60L). Jiefeng® 613 formulated feed (containing flour, wheat middling, fishmeal, rapeseed meal, bean pulp, bean oil, fish oil and vitamin-mineral supplement) was provided to fish twice per day at approximately 2% body mass of fish. One-third of water was replaced with de-chlorinated tap water twice a week. The salinity of water was maintained at about 4 ppt, using raw salt. Three days prior to experiment commenced, three fish were weighed and divided into each experimental tank (60 L), with each diet groups with 3 replicates. The light-dark cycle was set at 12L:12D, using florescent lamps as a primary lighting source. Initial weights of grass carp and Nile tilapia were 59.4±3.2 g and 56.2±5 g, respectively, and
they were approximately 12 cm long.

4.2.2 Experimental diets

Food waste diet formulation B fish feed pellets (FWB, composed of 10% fruits and vegetables, 25% meat products, 28% cereal, 8% bone meal, 10% fishmeal, 15% corn starch and 4% other food waste) were used for this feeding experiment. DietB contained 0.5% (w/w) chromium(III) oxide (as an inert indicator of protein digestibility) and 0.3% (w/w) vitamin-mineral premix (VMP) (Centrum, USA) (contained Vitamin A, B1, B3, B5, B6, B7, B9, B12, C, D, E, K1, calcium, chromium, copper, iodine, iron, magnesium, manganese, molybdenum, potassium, selenium and zinc) were mixed thoughtfully with grounded diets. The detailed contents of the VMP are listed in Table 4.1. Ingredients were mixed with water and re-pelletized using a meat grinder. Re-pelletized feeds were placed at 60°C in an oven, until completely dried. Two control diets, Jiefeng® 613 feed pellets with 0.3% α-cellulose (ContA) and FWB with 0.3% of α-cellulose (ContB), were also produced similarly to other experimental diets, except no vitamin-mineral premix was added. Fish were fed daily twice per day manually at 2.5% body mass of fish. The formulations of each experimental diet are listed in Table 4.2.

4.2.3 Growth performance of fish

Fish from the same fish tank were measured in bulk at day zero and then once every 2 weeks for 4 weeks (for calculating the amount of feed provided and fish weight gain). Prior to weighing, all fish were anesthetized with 100 mg/L MS222. Fish were weighed to nearest 0.1g using a balance and then returned to the tanks. The growth performance of fish was
Table 4.1 Amounts of vitamins and minerals in DietB and suggested values for trout, carp, tilapia and catfish (Chow, 1982)

<table>
<thead>
<tr>
<th>Vitamins</th>
<th>Amount (unit)</th>
<th>Suggested requirements (0.6% w/w inclusion)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A</td>
<td>10000 IU*</td>
<td>1000 IU*</td>
</tr>
<tr>
<td>Thiamine</td>
<td>4.5 mg</td>
<td>4 mg</td>
</tr>
<tr>
<td>Vitamin B3</td>
<td>40 mg</td>
<td>20 mg</td>
</tr>
<tr>
<td>Vitamin B5</td>
<td>10 mg</td>
<td>10 mg</td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>6 mg</td>
<td>4 mg</td>
</tr>
<tr>
<td>Vitamin B7</td>
<td>0.045 mg</td>
<td>0.02 mg</td>
</tr>
<tr>
<td>Vitamin B9</td>
<td>0.4 mg</td>
<td>1 mg</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>0.018 mg</td>
<td>0.004 mg</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>180 mg</td>
<td>40 mg</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>800 IU*</td>
<td>200 IU*</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>60 IU*</td>
<td>10 IU*</td>
</tr>
<tr>
<td>Vitamin K1</td>
<td>0.05 mg</td>
<td>2 mg</td>
</tr>
<tr>
<td>Calcium</td>
<td>324 mg</td>
<td>--</td>
</tr>
<tr>
<td>Chromium</td>
<td>0.2 mg</td>
<td>--</td>
</tr>
<tr>
<td>Copper</td>
<td>4 mg</td>
<td>3 mg</td>
</tr>
<tr>
<td>Iodine</td>
<td>0.3 mg</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>Iron</td>
<td>54 mg</td>
<td>50 mg</td>
</tr>
<tr>
<td>Magnesium</td>
<td>200 mg</td>
<td>--</td>
</tr>
<tr>
<td>Manganese</td>
<td>10 mg</td>
<td>20 mg</td>
</tr>
<tr>
<td>Molybdenum</td>
<td>50 mg</td>
<td>--</td>
</tr>
<tr>
<td>Potassium</td>
<td>60 mg</td>
<td>--</td>
</tr>
<tr>
<td>Selenium</td>
<td>0.05 mg</td>
<td>0.1 mg</td>
</tr>
<tr>
<td>Zinc</td>
<td>30 mg</td>
<td>30 mg</td>
</tr>
</tbody>
</table>

*IU = International unit
Table 4.2 Detailed compositions of experimental diets

<table>
<thead>
<tr>
<th>Ingredients (%w/w)</th>
<th>Experimental diets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ContA</td>
</tr>
<tr>
<td>Jiefeng® 613*</td>
<td>99.2</td>
</tr>
<tr>
<td>Food waste diet B</td>
<td>99.2</td>
</tr>
<tr>
<td>α-cellulose</td>
<td>0.3</td>
</tr>
<tr>
<td>Chromium(III) oxide</td>
<td>0.5</td>
</tr>
<tr>
<td>Vitamin-mineral premix</td>
<td></td>
</tr>
</tbody>
</table>

* Jiefeng® 613 formulated feed is a complete diet (contains flour, wheat middling, fishmeal, rapeseed meal, bean pulp, bean oil, fish oil and vitamin-mineral premix)
calculated according to the following equations:

Relative weight gain, RWG (%) = \frac{[\text{final weight (g)} - \text{initial weight (g)}]}{\text{initial weight}} \times 100

Specific growth rate, SGR (%/day) = \frac{[\ln \text{final weight (g)} - \ln \text{initial weight (g)}]}{\text{feeding period (day)}} \times 100

Feed conversion ratio FCR = \frac{\text{feed provided (g)}}{[\text{Final biomass} - \text{Initial biomass (g)}]}

Protein efficiency ratio, PER (%) = \frac{\text{wet body weight gain (g)}}{\text{protein intake (g)}} \times 100

### 4.2.4 Chemical analyses of fish carcass and apparent protein digestibility of diets

Six fish from each treatment were sacrificed for proximate analysis and assessing the digestibility of fish feeds. Internal organs in the abdominal cavity of fish were removed and faecal matters were collected from intestine of fish 12 h post feeding using dissection scissors and forceps. Faecal matters were placed in oven and dried at 60°C for 24 h. Fish carcass was then cut into several smaller pieces and wrapped individually with aluminium foil. Fish carcass was frozen and then freeze-dried for up to 2 weeks.

**Crude protein contents of fish feeds and fish faecal matter**

Samples were digested using semi-micro Kjeldahl digestion procedure (APHA, 2002). About 300 mg of feed sample or faecal matter was digested with 3 ml of concentrated sulphuric acid in the presence of 0.1 g Kjeldahl catalyst. Samples were pre-digested at 98°C for 1 h and heated at 160°C for 30 min. The temperature was then increased to 250°C and maintained for 30 min. Samples were further digested at 360°C until the sample turned clear. After cooling,
the resulted solution was diluted with 20 ml of DI water and filtered through a filter paper (Whatman #1) into a 25 ml volumetric tube and marked up with DI water. Total Kjeldahl nitrogen was measured using phenate method. Crude protein in sample was calculated by multiplying total Kjeldahl nitrogen by 6.25 (McKinney et al., 2004).

**Determination of chromic oxide in fish feeds and fish faecal matter**

Chromic oxide (Cr₂O₃) was used as the indicator of protein digestibility. Cr₂O₃ in samples were digested and determined (Furukawa and Tsukahara, 1996). About 100 mg of finely grinded samples (weighed to 0.0001 g) were placed in a boiling tube (75 ml). 5 ml of nitric acid was added to the tube and digested at 125°C for at least 1 h for oxidizing organic matter in the sample until the solution turned clear and greenish. After cooling, 3 ml of perchloric acid was added to the tube. Samples were further digested at 220°C for at least 15 min until the solution turned to yellow permanently. The solution was diluted with DI, transferred to a volumetric tube and marked up to 25 ml. The absorbance of solution was measured using a spectrophotometer at 350 nm (UV-1601, Shimadzu, Tokyo, Japan). The amount of chromic oxide in samples was calculated according to the following equation:

\[ X = \frac{(Y-0.0032)}{4/0.2089} \]

Where \( X \) = weight of chromic oxide in mg, \( Y \) = absorbance at 350 nm, 0.0032 and 022089 are constants.

Percentage of chromic oxide in the sample was calculated with the equation:

\[ \% \text{ chromic oxide} = 100 \times \frac{X}{A} \]

Where \( X \) = weight of chromic oxide in mg, \( A \) = weight of sample.

Apparent digestibility of the diets was calculated according to the following equation:
Apparent digestion coefficient, ADC of protein (\%) = 100 × [1-(dietary Cr₂O₃/fecal Cr₂O₃) × (fecal nutrient/dietary nutrient)]

4.2.5 Statistical analyses

Normality and homogeneity of data were tested prior to ANOVA. The growth parameters, proximate compositions of fish carcass of fish and protein digestibility were compared using one-way ANOVA, and Duncan's multiple comparison test was used to compare means. Significant differences between experimental groups were expressed at the significance level of \( p < 0.05 \). All statistics were calculated using SPSS Statistics 19.0 (IBM, USA).

4.3 Results and Discussion

Table 4.3 shows the growth performance and protein digestibility of grass carp consuming the 3 experimental diets (ContA, ContB and DietB). Grass carp consuming DietB showed significantly better growth (RWG, SGR, FCR and PER) \((p<0.05)\) than groups fed with ContA and ContB. No significant differences were noted between the groups fed with ContA and ContB. There were no significant differences in growth performance of grass carp fed with ContA and ContB, without addition of vitamin-mineral premix added. Grass carp fed with diet supplemented with vitamin-mineral premix (DietB) showed approximately 10% higher RWG than groups fed with ContA and ContB. The group fed with ContA DietB showed significantly better protein digestibility than the groups fed with ContB \((p<0.05)\).

Table 4.3 also shows the growth performance of Nile tilapia that fed with the 3 experimental diets. Similar to grass carp, Nile tilapia fed with DietB showed significantly better RWG,
SGR, FCR and PER (p<0.05), followed by the two control diets. However, no significant differences (p>0.05) in protein digestibility were noted among the 3 treatment groups. Table 4.4 shows the carcass proximate compositions of grass carp and Nile tilapia. There were no significant differences (p>0.05) in dry matter, ash, crude protein and crude lipid among the 3 treatment groups in both grass carp and tilapia.

One of the objectives of this experiment was to study the necessity of providing extra supplements to the fish diets. According to results of essential amino acid contents obtained in Chapter 2, both the commercial and the food waste based feed pellets contained sufficient and comparable essential amino acids, thus the next step would be studying the necessity of adding micro-nutrients. The importance of vitamin and minerals in diets for cultured animals has been investigated by several scientists. Taechajanta and Sitasit (1981) conducted a feeding trial to assess the effects of vitamin and mineral premix supplementation on broadhead catfish (Clarias macrocephalus) fry, and noted that the group fed with supplementation had better survival rate and weight gain than the control group, with results compared to the present study. Datocajegas and Yakupitiyage (1996) revealed that phosphorous in mineral premix was found to significantly enhanced tilapia weight gain, FCR and PER. On the contrary, Barrows et al. (2008) observed that vitamin premix did not exert significant effects on the weight gain and FCR value of rainbow trout (Oncorhynchus mykiss), but exerted significant effects on survival, feed intake, protein retention efficiency, energy retention efficiency, hematocrit and hepatosomatic index of the fish, indicating the importance of the vitamins on fish health.

Addition of the vitamin-mineral premix to the formulated diet is a common practice in order
Table 4.3 Growth performance and protein digestibility of grass carp and Nile tilapia fed with various experimental diets.

<table>
<thead>
<tr>
<th>Growth performance</th>
<th>Diet</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ContA</td>
<td>ContB</td>
<td>DietB</td>
</tr>
<tr>
<td>Grass carp</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative weight gain (%)</td>
<td>52.5 ± 1.0a</td>
<td>51.3 ± 4.3a</td>
<td>60.5 ± 2.2b</td>
</tr>
<tr>
<td>Feed conversion ratio</td>
<td>1.59 ± 0.05a</td>
<td>1.56 ± 0.05a</td>
<td>1.29 ± 0.04b</td>
</tr>
<tr>
<td>Protein efficiency ratio</td>
<td>2.1 ± 0.06a</td>
<td>2.14 ± 0.07a</td>
<td>2.59 ± 0.07b</td>
</tr>
<tr>
<td>Specific growth rate (%)</td>
<td>1.51 ± 0.02a</td>
<td>1.48 ± 0.10a</td>
<td>1.69 ± 0.05b</td>
</tr>
<tr>
<td>Protein digestibility (%)</td>
<td>82.5 ± 7.4b</td>
<td>65.2 ± 1.6a</td>
<td>79.0 ± 8.4b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nile tilapia</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Relative weight gain (%)</td>
<td>39.6 ± 10.8a</td>
<td>40.6 ± 4.3a</td>
<td>61.6 ± 2.2b</td>
</tr>
<tr>
<td>Feed conversion ratio</td>
<td>1.66 ± 0.39a</td>
<td>1.5 ± 0.18a</td>
<td>1.04 ± 0.04b</td>
</tr>
<tr>
<td>Protein efficiency ratio</td>
<td>2.09 ± 0.56a</td>
<td>2.24 ± 0.25a</td>
<td>3.21 ± 0.12b</td>
</tr>
<tr>
<td>Specific growth rate (%)</td>
<td>1.42 ± 0.35a</td>
<td>1.45 ± 0.14a</td>
<td>2.1 ± 0.07b</td>
</tr>
<tr>
<td>Protein digestibility (%)</td>
<td>86.4 ± 2.9a</td>
<td>84.0 ± 1.0a</td>
<td>85.7 ± 1.0a</td>
</tr>
</tbody>
</table>

Mean ±SD having the same letters in the same row are not significantly different (p<0.05) (n=9).
Table 4.4 Proximate compositions of grass carp and Nile tilapia carcass that fed with various experimental diets after 4 weeks.

<table>
<thead>
<tr>
<th>Proximate compositions</th>
<th>Diet group</th>
<th>ContA</th>
<th>ContB</th>
<th>DietB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Grass carp</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry matter (% w/w)</td>
<td></td>
<td>93.5 ± 0.7a</td>
<td>94.9 ± 0.2a</td>
<td>96.1 ± 0.5a</td>
</tr>
<tr>
<td>Ash (% DM)</td>
<td></td>
<td>13.0 ± 0.8a</td>
<td>14.2 ± 0.4a</td>
<td>13.1 ± 1.1a</td>
</tr>
<tr>
<td>Crude protein (% DM)</td>
<td></td>
<td>54.9 ± 2.6a</td>
<td>58.6 ± 4.0a</td>
<td>57.1 ± 0.6a</td>
</tr>
<tr>
<td>Crude lipid (% DM)</td>
<td></td>
<td>15.3 ± 0.2a</td>
<td>14.6 ± 0.3a</td>
<td>15.7 ± 3.2a</td>
</tr>
<tr>
<td>Nile tilapia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dry matter (% w/w)</td>
<td></td>
<td>97.6 ± 1.1a</td>
<td>96.1 ± 0.5a</td>
<td>98.6 ± 0.5a</td>
</tr>
<tr>
<td>Ash (% DM)</td>
<td></td>
<td>18.0 ± 1.5a</td>
<td>17.7 ± 0.7a</td>
<td>19.6 ± 0.5b</td>
</tr>
<tr>
<td>Crude protein (% DM)</td>
<td></td>
<td>59.1 ± 5.6a</td>
<td>57.6 ± 3.4a</td>
<td>59.8 ± 1.7a</td>
</tr>
<tr>
<td>Crude lipid (% DM)</td>
<td></td>
<td>12.7 ± 0.5a</td>
<td>12.2 ± 1.2a</td>
<td>13.3 ± 0.6a</td>
</tr>
</tbody>
</table>

Mean ±SD having the same letters in the same row are not significantly different (p<0.05) (n=3). Note: DM= dry matter.
to provide sufficient micro-nutrients to the farmed animals (Hardy and Barrows, 2002). Although there were 10% of vegetables and fruits included in the food waste based feeds, the superior fish growth in groups fed with VMP supplemented diet indicated that food waste did not contain enough micro-nutrients. Inclusion of vitamin supplements to Nile tilapia is necessary in order to achieve optimal growth and health when limited natural food sources are available (FAO, 2014b). In the present study, vitamin mineral premix appears to be essential for including in the food waste based diets, because of the significant better growth of both fish species in terms of RWG, FCR, PER, SGR, and improved protein digestibility (observed in grass carp only).

The differences in protein digestibilities of food waste based diets without VMP supplement between grass carp and Nile tilapia could be related to their different digestive systems. Similar to other cyprinidae, grass carp lacks a stomach and the gut length is only about 1.6-2 times of its standard length (a measurement of fish length: from mouth to the end of tail) (Hoa and Thi, 1973). It has been noted that grass carp could only digest about half of the plant materials ingested daily, due to its short gut (Fedorenko and Fraser, 1978). The intestine evacuation time of grass carp is about 12 h (Du et al., 2009). Although grass carp itself could secrete various enzymes (other than microbial enzymes) to digest the food ingested (Das and Tripathi, 1991), the shorter length of grass carp intestine could make it less efficient in digesting food. On the other hand, tilapia has a stomach with pH value as low as 1.4 (Moriarty, 1973), which could provide an acidic environment for digesting protein (Maier and Tullis, 1984). The long digestive tract of Nile tilapia, at least 6 times longer than its standard length, provides abundant surface area for digestion and absorption of nutrients (Opuszynski and
Although there were no previous reports available on the intestinal evacuation time of Nile tilapia, its highly acidic stomach could facilitate protein digestion and longer length of its whole gut could hold the food items for a longer period of time, thus resulting better digestibilities.

Addition of VMP into the diets enhanced digestive function of grass carp. Some of the ingredients are known for improving digestion. It has been observed that deficiency in thiamin (vitamin B1) resulted in poor growth and anorexia for grass carp (Aoe et al., 1969), while the inclusion of thiamin significantly enhanced the activities α-amylase, lipase, trypsin, Na+, K+-ATPase, alkaline phosphatase and gamma-glutamyl transpeptidase in intestine of juvenile Jian carp (Cyprinus carpio var. Jian) (Huang et al., 2011). Moreover, Halver (2002) summarized that fish would lose its of appetite if it lacks vitamins B3, B5, B6, B7 or B12. Other than vitamins, minerals could also enhance digestive enzyme activities. Li et al. (2007) revealed that hybrid tilapia (Oreochromis niloticus × Oreochromis aureus) fed with diets supplemented with copper (75 mg/kg), iron (50 mg/kg) and zinc 50 (mg/kg) significantly increased the activities of amylase in hepatopancreas homogenates, amylase in intestine and lipase in intestine, although protease activities were not affected with the supplements.

One of the objectives of this study was to investigate the feed conversion ratio of fish consuming different experimental diets. FCR value is a measure of the effectiveness of an animal converting feed stuff into biomass. Thus, a low FCR value indicated a high quality feed (USAID-HARVEST, 2011). Different kinds of diets could result in various FCR values. It has been revealed that the FCR values of grass carp consuming different kinds of non-pellet
diets and the FCR values of bean dregs, Sudan grass and cotton seed cake were 25, 40 and 6, respectively, while the range of FCR values of formulated feed pellets for grass carp (“320” green grass powder pellet feed, consisted of 40% green grass powder, 20% rapeseed cake, 5% fishmeal, 15% bean cake, 5% silkworm pupae and 15% barley flour) were 2.5-3 (Yu, 1989), and pellet diet (formulation not disclosed) for tilapia 1.5-2.0 (FAO, 2014c), respectively. The FCR values resulted from this experiment indicated that the food waste based diets, even without vitamin-mineral supplement could generally satisfy the needs of both grass carp and tilapia. With vitamin-mineral supplement, the FCR values of both grass carp and Nile tilapia were further improved from 1.56 to 1.29 and 1.50 to 1.04, respectively.

Lin et al. (2001) studied the protein digestibility of different protein sources for grass carp. For grass carp, similar digestibilities ranging from 79.21% to 87.54% were observed for animal proteins (fishmeal, crab meal and meat meal), while digestibilities of plant proteins (yellow rapeseed meal, soybean meal and expanded soybean meal) were all higher than 85%. In the present study, vitamin-mineral premix enhanced the protein digestibility of food waste based diets for grass carp (from to 65% to about 80%) and the digestibility of DietB was more comparable to common feed ingredients mentioned above. Maina et al. (2002) studied the digestibility of protein in some fish feed ingredients for Nile tilapia and the results showed that the apparent protein digestibility coefficient (APDC) for fibre-reduced sunflower cakes, omena fishmeal, anchovy fishmeal and wheat bran were 88.6%, 89.7%, 90.0% and 75.2%, respectively, and digestibilities of the food waste based diets for Nile tilapia used in this experiment were about 85%, which were slightly lower than those common feeds mentioned above.
The results of proximate analysis (Chapter 3, Table 3.2) and the analysis of amino acids profile (Chapter 2, Tables 2.1, 2.2 and 2.3) show that Jiefeng® 613 and FWB are isonitrogenous (same nitrogen contents), while having sufficient essential amino acids for satisfying the dietary requirements of both grass carp and Nile tilapia, although they were consisted of different materials (fishmeal based diets vs. food waste based diets). The better growth performance of fish fed with DietB could be related to the higher lipid content. Fish could use both carbohydrates and lipids as energy sources. Sufficient amount of dietary lipid content in fish feed could promote growth and food utilization, as protein in fish is spared from and more energy is derived from other energy sources (carbohydrates or lipids) (De Silva et al., 2001; Skalli et al., 2004), protein is conserved for growth. It has been noted that grass carp could utilize lipid slightly better than carbohydrates (Gao et al., 2009). Even with a higher lipid content than the control diet, no adverse effects on RWG, FCR, SGR and PER were noted in both grass carp and tilapia fed with ContB (no VMP supplemented). The suggested dietary lipid requirement of grass carp (4%) was tested based on diets containing 40% crude protein (Du et al., 2004). It is possible that grass carp require more lipid when fed with diets with a lower protein content (30%). In the present study, the lipid content of ContB or DietB (about 13%) did not affect RWG, FCR, SGR and PER (p>0.05), indicated that the fish grass carp could tolerate such a high lipid content of the diet. Due to the fact that there were no significant differences in growth of fish fed with ContA and contB, and addition of vitamin-mineral premix significantly enhanced growth of both fish species, the hypothesis was accepted.
4.4 Conclusions

Growth performances of both grass carp and Nile tilapia and their protein digestibilities were determined in this laboratory feeding trial. Feed conversion ratio and protein digestibility of food waste based diet formulation B (25% meat, 28% cereal, 10% fruit & vegetables, 8% bone meal, 10% fishmeal, 15% corn meal) and the resulted whole carcass composition for grass carp and Nile tilapia were determined. The FCR values and protein ADC of ContB (non-supplemented) for grass carp and for Nile tilapia were 1.56, 1.5; 65.2% and 84%, respectively. Inclusion of vitamin-mineral premix in the food waste based diet significantly improved FCR for both fish (grass carp = 1.29; Tilapia = 1.04) and protein ADC of grass carp (79%). Whole carcass proximate compositions (crude lipid, crude protein and ash) of both grass carp and Nile tilapia were not significantly differed among all treatment groups in both grass carp and Nile tilapia. With the fact that growth performances of both grass carp and Nile tilapia were significantly improved with the inclusion of vitamin-mineral premix, vitamin-mineral premix will be included in the food waste based diet in the later experiments, involving the uses of prebiotic fibres (Chapter 5) and Chinese herbs (Chapter 6).
Chapter 5 Upgrading food waste based feeds with prebiotic fibres: effects on feed digestibility, growth and non-specific immunity of fish

5.1 Introduction

The results of Chapter 4 showed that adding vitamin-mineral premix to Food Waste Diet B (FWB) improved fish growth as well as diet digestibility. In order to further improve the diet, prebiotic fibre was incorporated into the diet to study its effects on fish growth. A prebiotic is a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health (Gilson and Roberfroid, 1995, Ringø et al., 2010).

Prebiotic fibres have been suggested as an alternative to replace antibiotics, for improving the health of aquatic animals (Ringø et al., 2010), as there are many problems associated with the use of antimicrobial agents in aquaculture. These included antibiotic resistant gene development in bacteria, residue of antimicrobial agents remained in fish products and the suppressing of the immune system of aquatic animals (CFS, 2006a, b, c; Sapkota et al., 2008; Sørum, 2006). Commonly used prebiotic fibres in fish diets include inulin, fructooligosaccharides (FOS), mannanoligosaccharides (MOS) and galactooligosaccharides (GOS) (Ringø et al., 2010). In this experiment, two types of prebiotic fibres, inulin and mannan-oligosaccharide (MOS), being 2 of the most extensively studied prebiotic fibres, were added to the food waste based diets to investigate their effects on fish growth.
Inulin is an oligosaccharide with fructose as the repeating unit. Inulin is commonly found in different plant species. Various studies have shown that inulin supplementation could have positive effects on fish growth and/or innate immune response. Ibrahem et al. (2010) reported that Nile tilapia treated with 5 g inulin per kg diet had significantly higher body weight gain, specific growth rate, condition factor, survival rate and nitroblue tetrazolium (NBT) value. The feed supplemented with fructooligosaccharide (FOS) in the form of inulin led to improved feed efficiency, with a feed conversion ratio of 1.38±0.003 compared with control 1.32±0.015 (Grisdale-Helland et al., 2008).

Mannan-oligosaccharide (MOS) is a glucomannoprotein complex derived from yeast cell wall (Saccharomyces cerevisiae) (Sohn et al., 2000). It has been reported that rainbow trout fed with MOS (2 g/kg) showed improved growth performance, survival as well as antibody titer and lysozyme activity (Staykov et al., 2007). He et al. (2003) noted improved survival, enhanced lysozyme and alternative complement pathway activities (ACH50) in hybrid tilapia fed with 0.6% MOS compared to fish fed the control diet, but the supplement had no effects on growth. With the noted beneficial effects of MOS on fish, inulin and MOS were incorporated into food waste based diets to study their effects on fish growth and non-specific immunity.

In this experiment, two feeding trials were conducted to rear grass carp and Nile tilapia with food waste based diets supplemented with inulin or MOS (2% or 0.2% w/w). It was hypothesized that the prebiotic fibres could improve growth performance of fish as well as their non-specific immunity. The objectives of this experiment were to study the effects of incorporating prebiotic fibres in food waste based diets on: (1) fish growth performance in
terms of relative weight gain (RWG), feed conversion ratio (FCR), protein efficiency ratio (PER), specific growth rate (SGR) and diet digestibility; and (2) selected innate immune parameters (serum total immunoglobin, serum bactericidal activity and anti-protease activity).

5.2 Materials and Methods

5.2.1 Fish, fish tanks and maintenance

Two separate feeding trials were conducted using grass carp (*Ctenopharyngodon idella*) and Nile tilapia (*Oreochromis niloticus*). One hundred and fifty of individuals of each species were obtained from local fish farms in both Hong Kong and mainland China, respectively. Fish were treated with 3% of salt solution for 60 s for eliminating parasites before randomly dividing into 12 200 L plastic fish tanks. Each tank was connected to an external filter and continuously aerated. Fish were acclimatized for at least 2 weeks before introducing into the experimental tanks (60 L). Jiefeng® 613 formulated feed (contained flour, wheat middling, fishmeal, rapeseed meal, bean pulp, bean oil, fish oil and vitamin-mineral supplement) was provided to fish twice per day approximately according to 2% body mass of fish. One-third of water was replaced with de-chlorinated tap water twice a week. The salinity of water was maintained at about 4 ppt, using raw salt. The light-dark cycle was set at 12L:12D, using florescent lamps as a primary lighting source. Three days prior to experiment commenced, eight fish were divided into each experimental tank (60L), with each diet groups with 3 replicates. At day 0, the weight of grass carp and Nile tilapia were 59.4±3.2 g and 56.2±5 g, respectively.

5.2.2 Experimental diets and diet preparation
Food Waste Diet B (FWB) was used for this feeding experiment. DietB composed of 10% fruits and vegetables, 25% meat products, 28% cereal, 8% bone meal, 10% fishmeal, 15% corn starch and 4% other food waste. Two types of prebiotic fibres: inulin and mannan-oligosaccharide from yeast were used. 2% (w/w) of fibre (2% of prebiotic fibre or 0.2% of prebiotic fibre with 1.8% of α-cellulose) were added into grounded FWB, along with 0.5% (w/w) chromium(III) oxide and 0.3% (w/w) vitamin-mineral premix. Ingredients were mixed with water and re-pelletized using a meat grinder. Re-pelletized feeds were placed in an oven at 60°C, until completely dried. Two control diets, ContA (Jiefeng® 613 feed pellets as basal diet with 2.3% α-cellulose, no VMP) and ContB (food waste based Diet B with 2% of α-cellulose, with 0.3% VMP), were also produced similarly to other experimental diets. Fish were fed twice per day manually at 2.5% body mass of fish. The detailed formulations of all experimental diets are listed in Table 5.1.

5.2.3 Growth performance of fish

Fish from the same fish tank were measured in bulk at day zero and then once every 2 weeks for 8 weeks (for calculating the amount of feed provided and fish weight gain). Prior to weighing, all fish were anesthetized with 100 mg/L MS222. Fish were weighed to nearest 0.1 g using a balance and then placed back to the aquarium. Growth performance of fish was calculated according to the following equations:

Relative weight gain, RWG (%) = \[\frac{\text{final weight (g)} - \text{initial weight (g)}}{\text{initial weight}}\] \times 100

Specific growth rate, SGR (%/day) = \[\frac{\ln \text{final weight (g)} - \ln \text{initial weight (g)}}{\text{feeding period (day)}}\] \times 100
**Table 5.1 Composition of experimental diets.**

<table>
<thead>
<tr>
<th>Ingredients (%w/w)</th>
<th>ContA*</th>
<th>ContB</th>
<th>Inu02</th>
<th>Inu2</th>
<th>MOS02</th>
<th>MOS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jiefeng® 613 formulated feed</td>
<td>97.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Food waste diet B</td>
<td>97.2</td>
<td>97.2</td>
<td>97.2</td>
<td>97.2</td>
<td>97.2</td>
<td>97.2</td>
</tr>
<tr>
<td>Inulin</td>
<td>0.2</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOS</td>
<td></td>
<td>0.2</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-cellulose</td>
<td>2.3</td>
<td>2</td>
<td>1.8</td>
<td></td>
<td>1.8</td>
<td></td>
</tr>
<tr>
<td>chromium(III) oxide</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Vitamin-mineral premix</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
</tbody>
</table>

*Jiefeng® 613 formulated feed is a complete diet (contains flour, wheat middling, fishmeal, rapeseed meal, bean pulp, bean oil, fish oil and vitamin-mineral supplement) vitamins and minerals added. ContA = Control diet A, commercial diet based; ContB = control diet B, food waste based.*
Feed conversion ratio FCR = feed provided (g) / [Final biomass – Initial biomass (g)]

Protein efficiency ratio, PER (%) = wet body weight gain (g)/protein intake

\[(\text{g}) \times 100\]

5.2.4 Fish immune parameters

Blood collection

Blood samples were also collected from fish right after weight measurements. Six blood samples were collected from fish from each treatment, from the caudal vein using syringes. The blood sample was then transferred to a 0.65ml centrifuge tube for clotting at 4°C overnight. Serum was separated after centrifugation at 3,000 g and aliquots of serum samples were stored at -18°C, until analyzed for total serum immunoglobin, serum bactericidal activity, and serum anti-protease activity.

Bactericidal activity of blood serum

Serum bactericidal activity against *Aeromonas hydrophila* was conducted following the procedures described by Abidov and Mirismailov (1979). An equal volume (100 µl) of serum and bacterial suspension (10⁴ cfu/ml) was mixed and incubated for 1 h at 25 °C. Blank control was also prepared by replacing serum with sterile saline. The mixture was then diluted with sterile phosphate buffered saline (PBS) at a ratio of 1:10. Diluted suspension (100 µl) was spread plated onto LB agar plates and incubated for 24 h at 28°C. The number of viable bacteria was determined by counting the colonies grown on LB agar plates.

Total protein and total immunoglobulin of fish serum

Total immunoglobulin in serum was estimated according to the method described by
Anderson and Siwicki (1995) and modified by Sharma et al. (2010). 0.1 ml of serum was placed into a plastic serum vial and mixed with 0.1ml of 12% polyethylene glycol in DI water. The mixture was incubated at room temperature (22°C) for 2 h under constant mixing. The sample was then centrifuged at 5,000 g for 10 min, and the supernatant was collected. Its protein concentration of supernatant was determined (Bradford, 1976). Protein reading from the supernatant was the amount of protein precipitated by absorption to polyethylene glycol. Total immunoglobulin was expressed as mg per ml.

**Anti-protease activity of serum**

Anti-protease activity of fish serum was measured according to the method described by Hjelmeland (1983) with modification. 10 µl of serum was incubated with 20 µg of trypsin dissolved in 100 µl of Tris-HCl (50 mM, pH 8.2). In serum blank, 100 µl of Tris-HCl was added to 10 µl of serum, instead of trypsin in Tris-HCl, and in the positive control, no serum was added to trypsin. All tubes were filled with 200 µl of Tris-HCl and incubated for 1 h in room temp (22°C). After incubation, 2 ml of 0.1 mM substrate BAPNA (Nα-Benzoyl-L-arginine 4-nitroanilide hydrochloride) that dissolved in Tris-HCl (containing 20 mM calcium chloride), was added to the samples and further incubated for 15 min. 500µl of 30% acetic acid was added to cease the reaction. The absorbance was measured at 410 nm by using an UV-Visible spectrophotometer (UV-1601, Shimadzu, Tokyo, Japan). The percentage of trypsin inhibition was calculated according to the following equation:

\[
\text{Trypsin inhibition (% per mg protein) = } \left(\frac{A1-A2}{A1}\right) \times 100/ M
\]

where, A1= control trypsin activity (without serum); A2 = activity of trypsin remained after addition of serum, M = total protein in serum (mg protein).
5.2.5 Apparent protein digestibility of diets

The method for determining protein digestibility of the diets was listed in Section 4.2.4.

5.2.6 Statistical analyses

Normality and homogeneity of data were tested prior to ANOVA. The growth parameters and immune response parameters of fish were compared using one-way ANOVA, and Duncan's multiple comparison test was used to compare means. Significant differences between experimental groups were expressed at the significance level of \( p < 0.05 \). All statistics were calculated using SPSS Statistics 19.0 (IBM, USA).

5.3 Results and Discussion

Table 5.2 shows the growth performance of grass carp fed with different experimental diets. For grass carp, groups fed with ContA, Inu02, Inu2 and MOS2 showed significant better growth performances \( (p<0.05) \) in terms of wet weight gain, relative weight gain (RWG), specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER) than groups fed with ContB (without prebiotic fibre supplementation of prebiotic fibres in the food waste diet) and MOS02. Inu02, Inu2 and MOS2 were effective in improving the food waste feeds for grass carp.

Table 5.3 shows the growth performance of Nile tilapia fed with different experimental diets. For tilapia, the groups fed with ContB, Inu02 and Inu2 had significantly better RWG, FCR, PER and SGR among all treatment groups \( (p<0.05) \), followed by the groups treated with
Table 5.2 Growth performance of grass carp fed with different experimental diets after 8 weeks.

<table>
<thead>
<tr>
<th></th>
<th>ContA</th>
<th>ContB</th>
<th>Inu02</th>
<th>Inu2</th>
<th>MOS02</th>
<th>MOS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative weight gain (%)</td>
<td>100.71 ± 7.83b</td>
<td>91.65 ± 1.92a</td>
<td>104.89 ± 9.31b</td>
<td>105.79 ± 7.05b</td>
<td>87.53 ± 6.30a</td>
<td>101.82 ± 1.06b</td>
</tr>
<tr>
<td>Feed conversion ratio</td>
<td>2.07 ± 0.18b</td>
<td>2.37 ± 0.09a</td>
<td>2.08 ± 0.12b</td>
<td>2.08 ± 0.01b</td>
<td>2.46 ± 0.14a</td>
<td>2.10 ± 0.09b</td>
</tr>
<tr>
<td>Protein efficiency ratio</td>
<td>6.01 ± 0.53b</td>
<td>5.40 ± 0.21a</td>
<td>6.18 ± 0.38b</td>
<td>5.95 ± 0.03b</td>
<td>5.22 ± 0.30a</td>
<td>6.09 ± 0.26b</td>
</tr>
<tr>
<td>Specific growth rate (%) per day</td>
<td>1.24 ± 0.07b</td>
<td>1.16 ± 0.02a</td>
<td>1.28 ± 0.08b</td>
<td>1.29 ± 0.06b</td>
<td>1.12 ± 0.06a</td>
<td>1.25 ± 0.01b</td>
</tr>
<tr>
<td>Protein digestibility (%)</td>
<td>86.58 ± 1.47a</td>
<td>90.99 ± 1.83b</td>
<td>86.86 ± 2.18a</td>
<td>91.76 ± 0.85b</td>
<td>92.74 ± 0.68b</td>
<td>92.02 ± 4.02b</td>
</tr>
</tbody>
</table>

Mean ±SD having the same letters in the same row are not significantly different. ContA = Control diet A, commercial diet based diet; ContB = control diet B, food waste based diet; Inu02 = 0.2% inulin and food waste based diet; Inu2 = 2% inulin and food waste based diet; MOS02 = 0.2% mannan-oligosaccharide and food waste based diet; MOS2 = 2% mannan-oligosaccharide and food waste based diet.
MOS02 and MOS2 and then ContA. The feeding trial using Nile tilapia showed that prebiotic fibre supplements had no positive effects on fish growth compared with those treated with ContB or other supplemented diets.

There were no previous studies focusing on the effects of inulin or MOS on the growth of grass carp. Prebiotic fibre supplements are not always beneficial to fish. For example, a negative correlation between weight gain, specific growth rate, protein efficiency ratio, energy retention, feed efficiency, protein retention and prebiotic fibre dosage (inulin, 1-3%) was noted in beluga (Huso huso) (Reza et al., 2009). However, turbot (Psetta maxima) larvae fed with inulin resulted in increased growth rate (Mahious et al., 2006), and this was the only published report concluding that inulin could enhance fish growth. In the present study, inulin and MOS supplementations (to food waste diets) resulted in positive effects on the growth performance for grass carp, indicating that both inulin (0.2% and 2%) and MOS (2% only) could effectively enhance growth of grass carp.

Samrongpan et al. (2008) observed that 0.4% or 0.6% of MOS increased the average daily growth (0.040±0.010 and 0.042±0.009 g/day, while 0% = 0.037±0.007) of Nile tilapia fry, while Ibrahim et al. (2010) also reported that 0.5% of inulin in the diet promoted body weight gain and specific growth rate of Nile tilapia. However, in the present study, no positive effects of the two prebiotic fibres (the dosage used of MOS or inulin, 0.2% and 2%) on growth performance of Nile tilapia were observed, indicating the dosages (0.2% or 2%) used in the present study had no positive effects on Nile tilapia. Surprisingly, for both grass carp and tilapia, the groups fed with 0.2% of MOS showed inferior growth performance, compared
Table 5.3 Growth performance of Nile tilapia fed with different experimental diets after 8 weeks.

<table>
<thead>
<tr>
<th></th>
<th>ContA</th>
<th>ContB</th>
<th>Inu02</th>
<th>Inu2</th>
<th>MOS02</th>
<th>MOS2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Relative weight gain (%)</strong></td>
<td>108.61 ± 5.23a</td>
<td>121.79 ± 12.72b</td>
<td>122.63 ± 6.73b</td>
<td>117.08 ± 6.86b</td>
<td>95.52 ± 28.81ab</td>
<td>107.25 ± 23.05ab</td>
</tr>
<tr>
<td><strong>Feed conversion ratio</strong></td>
<td>1.66 ± 0.04a</td>
<td>1.48 ± 0.07b</td>
<td>1.48 ± 0.06b</td>
<td>1.48 ± 0.10b</td>
<td>1.95 ± 0.65ab</td>
<td>1.69 ± 0.42ab</td>
</tr>
<tr>
<td><strong>Protein efficiency ratio</strong></td>
<td>1.31 ± 0.04a</td>
<td>1.42 ± 0.10b</td>
<td>1.43 ± 0.05b</td>
<td>1.38 ± 0.06b</td>
<td>1.18 ± 0.28ab</td>
<td>1.29 ± 0.20ab</td>
</tr>
<tr>
<td><strong>Specific growth rate (%) per day</strong></td>
<td>2.01 ± 0.05a</td>
<td>2.26 ± 0.11b</td>
<td>2.26 ± 0.09b</td>
<td>2.26 ± 0.16b</td>
<td>1.83 ± 0.51ab</td>
<td>2.05 ± 0.45ab</td>
</tr>
<tr>
<td><strong>Protein digestibility (%)</strong></td>
<td>64.78 ± 8.10</td>
<td>75.74 ± 2.72</td>
<td>77.88 ± 6.06</td>
<td>86.45 ± 11.94</td>
<td>86.16 ± 3.10</td>
<td>81.96 ± 5.57</td>
</tr>
</tbody>
</table>

Mean ±SD having the same letters in the same row are not significantly different. ContA = Control diet A, commercial diet based diet; ContB = control diet B, food waste based diet; Inu02 = 0.2% inulin and food waste based diet; Inu2 = 2% inulin and food waste based diet; MOS02 = 0.2% mannan-oligosaccharide and food waste based diet; MOS2 = 2% mannan-oligosaccharide and food waste based diet.
to groups fed with other diets (with or without supplements). Further investigations would be necessary to understand the causal effects.

Enhanced FCR values were noted in grass carp fed with inulin and MOS only (not in Nile tilapia). Kaleeswaran et al. (2011) showed that inclusion of *Cynodon dactylon* (a plant) mixed diet improved the feed conversion ratio of Indian major carp (*Catla catla*). When comparing the overall feed conversion ratio (FCR) of the 2 species, Nile tilapia always showed better (smaller) FCR values than grass carp, even they were given a similar feeding rate (2% body mass per day). FCR is a measure of the efficiency for an animal to convert feed into biomass and a low FCR value is a good indicator of a high quality of feed (USAID-HARVEST, 2011). Although the treatments added with dietary prebiotic did not improve the FCR value, Nile tilapia had about 20% higher relative weight gain than grass carp. In the natural environment, tilapia consumes plankton, aquatic macrophytes, invertebrates, larval fish, detritus, and decomposed organic matter, and it could also digest animal protein more efficiently than plant protein (Popma, 1999). It is possible that the omnivorous dietary preferences rendered tilapia more favorable to utilize the food waste based diets, which made of both plant and animal proteins.

Fig 5.1a shows the total serum immunoglobin of grass carp. There were no significant differences in serum immunoglobin concentrations among all treatment groups at week 2 (p>0.05). Immunoglobin concentrations in groups fed with 0.2% of MOS at week 4 and week 8, and 2% of inulin at week 6 were significantly higher than the control groups (p<0.05). On the other hand, there were no significant differences in serum immunoglobin concentrations
among all treatments for Nile tilapia (Fig 5.1b) (p>0.05). The total immunoglobulin of grass
carp fed with 2% inulin and 0.2% MOS was significantly higher in the present study, which
were comparable with the findings of Indian major carp (*Labeo rohita*) fed with diet
supplemented with *Withania somnifera* (Sharma et al., 2010), greasy groupers (*Epinephelus
tauvina*) fed with herbal diet with purified active principle of *Ocimum sanctum, Withania
somifera*, and *Myristica fragrans* (Sivaram et al., 2004), and Siberian sturgeon (*Acipenser
baeri*) with epin (a plant growth regulator epibrassinopid) (Kolman, 2001). Contrary to grass
carp, supplementation with both MOS and inulin had no significant effects on total serum
immunoglobulin of tilapia. This is in line with a previous report that hybrid striped bass
receiving vitamin C and E supplement in the diet had no effects on immunoglobulin (Sealey and
Gatlin, 2002). Natural antibody is produced in certain B cells in mammalian system (Casali
and Schettino, 1996), and it is involved in both viral and bacterial defense of rainbow trout
and goldfish (Gonzalez et al., 1989; Sinyakov et al., 2002). The increased total serum
antibody in the present study could be related to the stimulation of MOS and inulin.

Figure 5.2a shows the bactericidal activity of grass carp serum. At week 2 and week 4, there
were no significant differences in the activities among all treatment groups (p>0.05). At week
6, the activities of the groups fed with Inu02, Inu2 and MOS2 were significantly higher than
other groups (p<0.05). At week 8, the activities of groups fed with ContA, Inu02, Inu2 and
MOS2 were significantly higher than other groups (p<0.05). For tilapia, at week 2, week 4
and week 6, there were no significant differences among all treatment groups in bactericidal
activities (p>0.05). At week 8, groups fed with ContB, Inu2, MOS02 and MOS2 were
significantly higher than ContA and Inu02 (p<0.05). The bactericidal activities of both fish
Figure 5.1 Total serum immunoglobin of (a) grass carp and (b) Nile tilapia. Different superscripts (a,b,c) indicated significant difference (p<0.05) at the same sampling week (n=6). ContA = Control diet A, commercial diet based diet; ContB = control diet B, food waste based diet; Inu02 = 0.2% inulin and food waste based diet; Inu2 = 2% inulin and food waste based diet; MOS02 = 0.2% mannan-oligosaccharide and food waste based diet; MOS2 = 2% mannan-oligosaccharide and food waste based diet.
species were enhanced, and same results were observed in rohu (*Labeo rohita*) fed with the diets containing garlic (Sahu et al. 2006). Stronger bactericidal activities would indicate stronger innate immune responses in fish (Das et al., 2009).

Figure 5.3a shows the anti-protease activity of grass carp serum. At week 2, the groups fed with ContB, Inu2 and MOS showed significantly higher anti-protease activities. The groups fed with MOS2 and ContA showed significantly higher activities (p<0.05) than other treatment groups at week 4 and week 6, respectively. No significant differences of the activities among all groups were noted at week 8. The group fed with Inu02 had a significantly lower (p<0.05) anti-protease activity than other treatment groups during week 2 (Fig 5.3b). The group fed with Inu2 at week 4 and week 8 showed significantly higher anti-protease activities than other treatment groups (p<0.05). The anti-protease activity of tilapia fed with inulin (2%) was significantly enhanced in the present study, comparable with the findings of Indian major carp (*Catla catla*) fed with diet containing *Achyranthes aspera* (0.5%) (Rao and Chakrabarti, 2004) and *Cynodon dactylon* (5%) (Kaleeswaran et al., 2011)

Non-specific immune system, or innate immune system, is thought to be more important than the adaptive immune system in fish defence mechanism against pathogens, as there are several limitations of the adaptive immune system, including poikilothermic nature, limited repertoire of antibodies and the slow proliferation, maturation and memory of their white cells (Whyte, 2007). Non-specific immune response of different species of fish were different, according to different treatments of dietary prebiotic fibres. Staykov et al. (2007) noted that rainbow trout fed with MOS supplemented diet (0.2% w/w) had improved antibody titer and
Figure 5.2 Serum bactericidal activity of (a) grass carp and (b) Nile tilapia. Different superscripts (a,b,c) indicated significant difference (p<0.05) at the same sampling week (n=6). ContA = Control diet A, commercial diet based diet; ContB = control diet B, food waste based diet; Inu02 = 0.2% inulin and food waste based diet; Inu2 = 2% inulin and food waste based diet; MOS02 = 0.2% mannan-oligosaccharide and food waste based diet; MOS2 = 2% mannan-oligosaccharide and food waste based diet.
Figure 5.3 Anti-protease activity of (a) grass carp and (b) tilapia serum. Different superscripts (a,b,c) indicated significant difference (p<0.05) at the same sampling week (n=6). ContA = Control diet A, commercial diet based diet; ContB = control diet B, food waste based diet; Inu02 = 0.2% inulin and food waste based diet; Inu2 = 2% inulin and food waste based diet; MOS02 = 0.2% mannan-oligosaccharide and food waste based diet; MOS2 = 2% mannan-oligosaccharide and food waste based diet.
lysozyme activity, while bactericidal activity was not affected. Torrecillas et al. (2007) showed that European sea bass (*Dicentrarchus labrax*) fed with MOS supplemented diet (4%) had significantly higher head kidney macrophage phagocytic activity. Incorporation of dietary supplements may exert no effects or even inhibit non-specific immune responses. It has been revealed that Channel catfish (*Ictalurus punctatus*) fed with MOS supplemented did not affect growth performance or immune function (Welker et al., 2007).

The mode of action of MOS on immunity is related to the interaction between the mannose molecules on MOS and the corresponding receptors. Macrophages and dendritic cells possess mannose receptors (MR) which recognize a range of carbohydrates present on the surface and cell walls of micro-organisms (Apostolopoulos and McKenzie, 2001). It has been assumed that MOS can exert a direct effect on the immune cells through its mannose molecule (Che, 2010). The interaction between mannose on MOS and immune cells may induce intracellular signaling for stimulating the production of proinflammatory cytokines and may possess beneficial features as feed additives to fish and shellfish (Ringø et al. 2010). It has been revealed that Atlantic cod possesses receptors comparable to mammal MRs (Sørensen et al., 2001), while C-type lectin in shrimp possesses MR features (Zhao et al., 2009). It could be possible that both grass carp and Nile tilapia also possess suitable receptors on their immune cells, based on the observations that the non-specific immune parameters of grass carp (anti-protease activity, bactericidal activity and total serum antibody) and Nile tilapia (anti-protease activity and bactericidal activity) were improved.

Wang and Wang (1997) attempted the first study on the effects of prebiotic fibres on pathogen resistance of grass carp by intra-peritoneal injection. It was concluded that inulin had no
significant effects on the immune responses, and there was no further investigation regarding to the use of any prebiotic fibres in grass carp. It has been suggested that insoluble inulin (γ-inulin) may possess adjuvant activity, as it could activate the alternative complement pathway (Silva et al. 2004). The alternative complement pathway stimulated by foreign bacteria is an important defence system of fish (Holland and Lambris, 2002). On the other hand, a number of studies reported that the long-chained inulin stimulates the human immune system by binding to specific lectin-like receptors on leucocytes and inducing macrophage proliferation (Causey et al., 1998; Meyer, 2008; Seifert and Watzl, 2007). It was hypothesized that gilthead seabream did not possess the receptors for inulin binding to white cells, rendering inulin an unsuitable choice of immuno-stimulant for gilthead seabream (Cerezuela et al., 2008). With the observations that both fish species had enhanced non-specific immune parameters, it may be possible both fish species possess the suitable receptors for inulin. In the present study, no significant adverse effects on growth performances (FCR, PER, RWG and SGR) were observed in grass carp. Diets supplemented with inulin significantly increased serum total immunoglobin as well as serum bactericidal activity of grass carp, while anti-protease activities were not significantly affected by inulin. These indicated that both 0.2% and 2% are suitable dosage for rearing grass carp. Nile tilapia fed with inulin supplemented diets showed improved bactericidal activities (both 2% and 0.2%) only. The hypothesis was accepted as the prebiotic fibres could enhance the growth and non-specific immunity of fish.

5.4 Conclusions
The growth performance (as reflected by RWG, SGR, PER and FCR) of grass carp and Nile tilapia, responded differently to the fibre-supplemented diets. Inulin (0.2% or 2%) and MOS (2%) significantly improved RWG, SGR, PER and FCR of grass carp, while 0.2% of MOS had no effects on the tested growth indices. On the other hand, inulin supplements had no effects on the growth indices of tilapia while MOS supplements resulted in inferior growth when comparing to the group fed with ContB (food waste based diets). Grass carp fed with inulin supplemented food waste diet showed improvement in both growth performance and non-specific immunity, while Nile tilapia fed with various prebiotic fibre supplemented diets did not show any significant improvements in growth performance. This indicated that the dosages (0.2% and 2%) of prebiotic fibres used in the present study were more suitable for feeding grass carp than Nile tilapia. In terms of non-specific immunity, addition of prebiotic fibre supplementations seemed to be more beneficial for grass carp than Nile tilapia, as all 3 tested parameters (total serum immunoglobin, bactericidal activity and anti-protease activity) were found enhanced in grass carp, while the prebiotic fibre supplementations had no effects on total serum antibody of Nile tilapia. In general, 2% of inulin supplementation to food waste diets was more preferable than other experimental diets (0.2% inulin, 0.2% MOS and 2% MOS), as it could support growth of grass carp as well as the non-specific immune systems of both grass carp and Nile tilapia. Incorporation of 2% inulin would be a more suitable supplement to enhance fish health as well as fish growth.
Chapter 6 Upgrading food waste based feeds with Chinese herbs: effects on feed digestibility, growth and non-specific immunity of fish

6.1 Introduction

Antimicrobial agents such as antibiotics and antifungals have been used for years to control the outbreak of diseases in animal production facilities. It has been estimated that over 80% of antimicrobials applied in aquaculture are discharged into the environment while remained active (Armstrong et al., 2005; Le and Munekage, 2004; Sørum, 2006). The discharged antimicrobial agents would remain in the aquatic environment for a long period of time and could lead to the development of antibiotic resistant genes by exerting selective pressures to bacteria (Ding and He, 2010; Marshall and Levy, 2011; Petersen et al., 2002).

Chinese herbal medicines have been used for years by the Chinese society as immuno-stimulants as well as for treating various diseases. Chinese herb (such as huangqi) possesses antimicrobial activities as well as immuno-stimulating properties to prevent or combat against fish disease (Anderson, 1992; Ardó et al., 2008; Secombes, 1994; Yuan et al., 2008). Therefore, they could serve as possible alternatives to replace antibiotics or antimicrobial agents (Galina et al. 2009). Citarasu (2010) concluded that herbal based medicines possess positive effects on fish growth, disease resistance, stimulating appetite and anti-stress.

Two Chinese herbs, Huangqi (*Astragalus membranaceus*) and Goji (*Lycium barbarum*), were
used in this experiment to study their effects on fish growth as potential supplements incorporated into food waste based feeds. Huangqi is the root of *Astragalus membranaceus* and has been used as an immuno-booster for nearly 2000 years (Galina et al., 2009). Ardó et al. (2008) reported that inclusion of 0.1% of *Astragalus membranaceus* in the diet of Nile tilapia significantly enhanced phagocytic and respiratory burst activities of blood phagocytic cells and plasma lysozyme. However, there is no report available regarding the herb on stimulating fish growth.

Goji or wolfberry, is the dried fruit of *Lycium barbarum* and it is widely used as a functional food as well as a medicine in Chinese society. Medicinal values of goji included but not limited to treat and prevent diseases such as insomnia, liver dysfunction, diabetes, visual degeneration and cancer (Chen et al., 2009). *Lycium barbarum* polysaccharides (LBP) were found to enhance innate immune response by activating marcophages in both mouse and murine (Chen et al., 2009), and induce T lymphocytes proliferation in mouse (He et al., 2005; Chen et al., 2008). Unlike Huangqi, there has been no study regarding its use on the fish production nor on fish immunity.

Non-specific immune system, or innate immune system, plays an more important role than the adaptive immune system in fish defence mechanism against pathogens, due to limitations of the adaptive immune system, their poikilothermic nature, limited repertoire of antibodies, and the slow proliferation, maturation and memory of their lymphocytes (Whyte, 2007). Commonly used immunostimulants include glucan, herbs, prebiotic fibres and probiotic bacteria (Choi et al., 2013; Ibrahem et al., 2010; Uribe et al., 2011). Treatments with
immunostimulants aim primarily at enhancing non-specific immunity, which could serve as
general protective measures (Magnadóttir, 2010). Thus, a number of studies focused on the
effects of immunostimulants on non-specific immune responses of fish (Ardó et al., 2008;
Sharma et al., 2010; Yin et al., 2006; Zakęś et al., 2008).

In order to further improve the food waste based feeds, crude extracts (using water as solvent)
of huangqi and goji were added into the food waste based feeds for rearing grass carp and
tilapia. Two control feeds, commercial available diet (Jiefeng® 613) and food waste based
feed only (without addition of Chinese herb) were used. The synergistic effects of combining
food waste based feeds and Chinese herbal medicine on growth and non-specific immunity of
grass carp and tilapia were studied. It was hypothesized that the Chinese herbs could improve
growth performance of fish as well as their non-specific immunity. The major objectives of
this experiment were to study the effects of Chinese herbs addition on (1) fish growth
performance in terms of relative weight gain (RWG), feed conversion ratio (FCR), protein
efficiency ratio (PER), specific growth rate (SGR) and diet digestibility; and (2) selected
innate immune parameters (serum total immunoglobin, serum bactericidal activity and
anti-protease activity) of fish.

6.2 Materials and Methods

6.2.1 Fish, fish tanks and maintenance

Two separate feeding trials were conducted using grass carp (Ctenopharyngodon idella) and
Nile tilapia (Oreochromis niloticus). One hundred and fifty of individuals of each species
were obtained from local fish farms in both Hong Kong and mainland China, respectively.
Fish were treated with 3% of salt solution for 60 s for eliminating parasites before dividing into 12 200 L plastic fish tanks. Each tank was connected to an external filter and continuously aerated. Fish were acclimatized for at least 2 weeks before introducing into the experimental tanks (60 L). Jiefeng® 613 formulated feed (contained flour, wheat middling, fishmeal, rapeseed meal, bean pulp, bean oil, fish oil and vitamin-mineral supplement) was provided to fish twice per day approximately according to 2% body mass of fish. One-third of water was replaced with de-chlorinated tap water twice a week. The salinity of water was maintained at about 4 ppt, using raw salt. The light-dark cycle was set at 12L:12D, using florescent lamps as a primary lighting source. Three days prior to experiment commenced, eight fish were weighted and divided into each experimental tank (60L), with each diet groups with 3 replicates. At day 0, the weight of grass carp and Nile tilapia were 56.4±3.2 g and 59.7±5 g, respectively.

### 6.2.2 Experimental diets and diet preparation

Food waste feed formulation B feed pellets (DietB) were used for this feeding experiment. DietB composed of 10% fruits and vegetables, 25% meat products, 28% cereal, 8% bone meal, 10% fishmeal, 15% corn starch and 4% other food waste. The crude-hot water extract of Chinese herbs and their residue were added into fish feed. Two types of Chinese herbs: 2% (w/w) of huangqi or goji, 2% of herb or 0.2% of herb with 1.8% of α-cellulose were used. The crude extract of the herb was prepared by boiling for 30 min with 400 mL of deionized water and the aqueous extracts were filtered through Whatmen No.1 filter paper. The herb residues were boiled with another 400 mL of deionized water two more time. The crude extract and the
herb residue were then added into grounded DietB, with 1.8% (w/w) cellulose (for 0.2% herbs only, no cellulose added for formulation containing 2% herb) along with 0.5% (w/w) chromium(III) oxide and 0.3% (w/w) vitamin-mineral premix. Ingredients were mixed with water and re-pelletized using a meat grinder. Re-pelletized feeds were dried in an oven at 60°C, until completely dried. Two control diets, ContA (Jiefeng® 613 feed pellets as basal diet with 2.3% α-cellulose, no VMP) and ContB (food waste based Diet B with 2% of α-cellulose and 0.3% VMP), were also produced similarly to other experimental diets. Fish were fed twice per day manually at 2.5% body mass of fish. The detailed formulations of all experimental diets are listed in Table 6.1.

6.2.3 Growth performance of fish

Fish from the same fish tank were measured in bulk at day zero and then once every 2 weeks for 8 weeks (for calculating the amount of feed provided and fish weight gain). Prior to weighing, all fish were anesthetized with 100 mg/L MS222. Fish were weighed to nearest 0.1 g using a balance and then placed back to the aquarium. Growth performance of fish was calculated according to the following equations:

Relative weight gain, RWG (%) = \[
\frac{\text{final weight (g)} - \text{initial weight (g)}}{\text{initial weight}} \times 100
\]

Specific growth rate, SGR (%/day) = \[
\frac{\ln \text{final weight (g)} - \ln \text{initial weight (g)}}{\text{feeding period (day)}} \times 100
\]

Feed conversion ratio FCR = \[
\frac{\text{feed provided (g)}}{\text{Final biomass} - \text{Initial biomass (g)}}
\]

Protein efficiency ratio, PER (%) = \[
\frac{\text{wet body weight gain (g)}}{\text{protein intake (g)}} \times 100
\]
Table 6.1 Detailed compositions of experimental diets.

<table>
<thead>
<tr>
<th>Ingredients (%w/w)</th>
<th>Experimental diets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jiefeng® 613 formulated feed#</td>
<td>97.2</td>
</tr>
<tr>
<td>Food waste diet B</td>
<td>97.2 97.2 97.2 97.2 97.2 97.2</td>
</tr>
<tr>
<td>Inulin</td>
<td>0.2 2</td>
</tr>
<tr>
<td>MOS</td>
<td>0.2 2</td>
</tr>
<tr>
<td>α-cellulose</td>
<td>2.3 2 1.8 1.8</td>
</tr>
<tr>
<td>chromium(III) oxide</td>
<td>0.5 0.5 0.5 0.5 0.5 0.5</td>
</tr>
<tr>
<td>Vitamin-mineral premix</td>
<td>0.3 0.3 0.3 0.3 0.3 0.3</td>
</tr>
</tbody>
</table>

#Jiefeng® 613 formulated feed is a complete diet (contains flour, wheat middling, fishmeal, rapeseed meal, bean pulp, bean oil, fish oil and vitamin-mineral supplement) vitamins and minerals added. ContA = Control diet A, commercial diet based; ContB = control diet B, food waste based; AM = Huangqi, Astragalus membranaceus; WB = Goji, Lycium barbarum.
6.2.4 Fish immune parameters

The details of blood collection and determination of non-specific immune parameters were listed in Section 6.2.4.

6.2.5 Apparent protein digestibility of diets

The method for determining protein digestibility of the diets was listed in Section 4.2.4.

6.2.6 Statistical analyses

Normality and homogeneity of data were tested prior to ANOVA. The growth parameters and immune response parameters of fish were compared using one-way ANOVA, and Duncan's multiple comparison test was used to compare means. Significant differences between experimental groups were expressed at the significance level of \( p < 0.05 \). All statistics were calculated using SPSS Statistics 19.0 (IBM, USA).

6.3 Results and Discussion

Table 6.2 shows the growth performance of grass carp fed with 6 experimental diets (ContA, ContB, AM02, LB02, WB2). As 2 aquaria (replicates) of grass carp fed with 2% AM were found dead after feeding for 2 weeks and 4 weeks respectively, the treatment feeding with 2% AM was stopped. The group fed with LB02 (0.2% Goji) showed a significantly better growth performance (\( p < 0.05 \)) than other groups, in terms of relative weight gain (RWG), specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER). The growth performance of other groups fed with different experimental diets: including ConA, ContB, AM02 and WB2, was not significantly different (\( p > 0.05 \)) based on various growth
<table>
<thead>
<tr>
<th>Grass carp</th>
<th>Diets</th>
<th>ContA</th>
<th>ContB</th>
<th>AM02</th>
<th>AM2#</th>
<th>LB02</th>
<th>LB2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative weight gain (%)</td>
<td></td>
<td>107.8 ± 4.91b</td>
<td>103.3 ± 3.6b</td>
<td>107.4 ± 5.3b</td>
<td>29.6 ± 1.1a</td>
<td>119.3 ± 1.9c</td>
<td>108.4 ± 5.2b</td>
</tr>
<tr>
<td>Feed conversion ratio</td>
<td>1.46 ± 0.07ab</td>
<td>1.58 ± 0.13b</td>
<td>1.48 ± 0.05b</td>
<td>2.23 ± 0.06a</td>
<td>1.33 ± 0.01c</td>
<td>1.48 ± 0.09b</td>
<td></td>
</tr>
<tr>
<td>Protein efficiency ratio</td>
<td>2.31 ± 0.09b</td>
<td>2.14 ± 0.17b</td>
<td>2.27 ± 0.06b</td>
<td>1.57 ± 0.04a</td>
<td>2.53 ± 0.03c</td>
<td>2.29 ± 0.11b</td>
<td></td>
</tr>
<tr>
<td>Specific growth rate (% per day)</td>
<td>1.31 ± 0.04b</td>
<td>1.27 ± 0.03b</td>
<td>1.30 ± 0.04b</td>
<td>1.05 ± 0.04a</td>
<td>1.40 ± 0.02c</td>
<td>1.31 ± 0.04b</td>
<td></td>
</tr>
<tr>
<td>Protein digestibility (%)</td>
<td>76.1 ± 1.9a</td>
<td>85.1 ± 0.4c</td>
<td>86.4 ± 0.2c</td>
<td>61.0 ± 5.2a</td>
<td>84.5 ± 2.5c</td>
<td>84.3 ± 4.2c</td>
<td></td>
</tr>
</tbody>
</table>

Mean ±SD having different letters in the same row are significantly different (p<0.05). #Growth performance of AM2 group was not recorded after 4 weeks only as most of the fish died 1 month after the commence of the feeding trial. ContA = Control diet A, commercial diet; ContB = food waste based diet with 2% cellulose; AM02 = 0.2% Huangqi and food waste based diet; AM2# = 2% Huangqi and food waste based diet; LB02 = 0.2% Goji and food waste based diet; LB2 = 2% Goji and food waste based diet.
performance parameters. The group fed with AM2 showed a significantly lower weight gain, FCR, SGR, PER and protein digestibility (p<0.05).

Table 6.3 shows the growth parameters of Nile tilapia fed with different experimental diets. Similar to the results of grass carp, the group fed with LB02 (2% goji) had a significantly better growth performance than other groups, in terms of wet weight gain, % weight gain, SGR, FCR and PER (p<0.05). The growth performance of other groups fed with different experimental diets: including ContA, ContB, AM02 and AM0 2 did not show any significant difference in all the growth performance parameters (p>0.05), while the group fed with LB2 showed the most inferior growth performance among all groups.

There are no published reports available on the growth promoting effects of LB on fish. Zhang and Chen (2006) noted that inclusion of a glycoconjugate from *Lycium barbarum* reduced food conversion rate and the subcutaneous fat of mice. Zhang et al. (2002) also reported that *Lycium barbarum* polysaccharides (LBP-4) can enhance the feed conversion rate of weanling mice. The present results suggested that goji could also enhance growth performance of fish. However, diets supplemented with huangqi had no positive effects on fish growth (in both grass carp and tilapia). It has been reported that herbal medicines could act as appetite stimulant (Citarasu, 2010). Rohu (*Labeo rohita*), an omnivorous fish, fed with Livol (a herbal growth promoter) incorporated diet stimulated digestive enzyme activity and enhanced food consumption (Maheshappa, 1993). Moreover, Venketramalingam et al. (2007) revealed that giant tiger prawn (*Penaeus monodon*) fed with brine shrimp enriched with ginger (*Zingiber officinalis*) showed a significant improvement
Table 6.3 Growth performance of Nile tilapia fed with different experimental diets after 8 weeks.

<table>
<thead>
<tr>
<th>Growth performance</th>
<th>ContA</th>
<th>ContB</th>
<th>AM02</th>
<th>AM2#</th>
<th>LB02</th>
<th>LB2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative weight gain (%)</td>
<td>98.9 ± 17.5ab</td>
<td>107.6 ± 31.3b</td>
<td>118.9 ± 4.2b</td>
<td>121.2 ± 2.9b</td>
<td>150.2 ± 11.3c</td>
<td>74.0 ± 17.4a</td>
</tr>
<tr>
<td>Feed conversion ratio</td>
<td>1.88 ± 0.26b</td>
<td>1.73 ± 0.41bc</td>
<td>1.60 ± 0.06bc</td>
<td>1.64 ± 0.08bc</td>
<td>1.31 ± 0.13c</td>
<td>2.44 ± 0.46a</td>
</tr>
<tr>
<td>Protein efficiency ratio</td>
<td>1.85 ± 0.23ab</td>
<td>2.07 ± 0.47b</td>
<td>2.09 ± 0.07b</td>
<td>2.10 ± 0.06b</td>
<td>2.62 ± 0.22c</td>
<td>1.44 ± 0.27a</td>
</tr>
<tr>
<td>Specific growth rate (％ per day)</td>
<td>1.23 ± 0.15ab</td>
<td>1.30 ± 0.25b</td>
<td>1.41 ± 0.05b</td>
<td>1.43 ± 0.04b</td>
<td>1.64 ± 0.08c</td>
<td>0.98 ± 0.17a</td>
</tr>
<tr>
<td>Protein digestibility (%)</td>
<td>82.6 ± 4.0b</td>
<td>78.4 ± 1.8a</td>
<td>81.7 ± 1.7b</td>
<td>87.9 ± 1.3c</td>
<td>90.4 ± 1.7c</td>
<td>73.5 ± 5.7a</td>
</tr>
</tbody>
</table>

Mean ±SD having same letters in the same row are not significantly different. ContA = Control diet A, commercial diet; ContB = food waste based diet with 2% cellulose; AM02 = 0.2% Huangqi and food waste based diet; AM2# = 2% Huangqi and food waste based diet; LB02 = 0.2% Goji and food waste based diet; LB2 = 2% Goji and food waste based diet.
of digestive enzyme activities (amylase, lipase and protease), better FCR, feed consumption and conversion, and production efficiencies. Other than stimulating the digestive enzyme activities, consumption of herbal medicines could also result in altered microbial communities. It has been noted that 1% incorporation of herbs formulation containing Scutellaria baicalensis, Astragalus membranaceus, Poria cocos, Houttuynia cordata and Isatis indigotica could increase the quantity of microflora in the intestine of common carp (Liu et al., 2004). In the present study, goji (0.2%) seemed to possess similar effects which stimulate digestive enzymes activities and/or yield microbial communities that could enhance digestion. Huangqi did not achieve such effects, as huangqi supplemented diets had no positive effects on growth of both fish species. Nevertheless, the addition of 0.2% of goji in the diet significantly enhanced the protein quality of the food waste diets which resulted in a significantly lower FCR than other groups (both species), as a lower FCR value indicates a higher quality of feed (USAID-HARVEST, 2011). Further studies focusing on the stimulatory effects of herbs would be necessary to understand how the herbs interact with the digestive system of fish.

For tilapia, there was no significant difference (p>0.05) noted among all groups in total serum immunoglobulin (Tlg) at week 2 and week 6, respectively (Fig. 6.1). The Tlg levels of groups fed with AM02, AM2 and LB02 were significantly higher than ContA at week 4 (p<0.05). At week 8, Tlg levels of AM02 and LB02 were significantly higher than ContA. For grass carp, the serum Tlg levels of groups fed with goji supplemented diets were significantly higher than other groups (p<0.05) (Fig 6.1). At week 6 and week 8, there was no significant difference in Tlg levels among all groups, respectively (p>0.05).
Figure 6.1 Total serum immunoglobin in (a) grass carp and (b) Nile tilapia fed with various experimental diets. Values are mean ± SD. Different superscripts (a,b,c) indicate significant differences (p<0.05) at the same sampling week (n=6). #Growth performance of AM2 group was not recorded after 4 weeks only as most of the fish died 1 month after the commence of the feeding trial. ContA = Control diet A, commercial diet; ContB = food waste based diet with 2% cellulose; AM02 = 0.2% Huangqi and food waste based diet; AM2 = 2% Huangqi and food waste based diet; LB02 = 0.2% Goji and food waste based diet; LB2 = 2% Goji and food waste based diet.
For tilapia, the serum bactericidal activities of LB02 (0.2% goji) were significantly higher than ContA at week 4 and week 6 (p<0.05), respectively (Fig. 6.2). The activities of the group fed with LB2 (2% goji) were also higher than ContA at week 6 (p<0.05). At week 8, all groups (except for LB02) showed significantly lower (p<0.05) bactericidal activities than ContA. At week 2 and week 4, grass carp fed with ContB, AM02, AM2, LB02 and LB2 showed significantly higher bactericidal activities than ContA (p<0.05), accordingly (Fig. 6.3). At week 6, the activities of the groups fed with ContB and AM02 were significantly higher than other diet groups (p<0.05).

For grass carp, the anti-protease activities of grass carp fed with ContB and LB2 were significantly lower (p<0.05) than groups fed with ContA, AM02, AM2 and LB02 at week 2. At week 4, activities of ContA, ContB and LB02 were significantly higher than AM02, AM2 and LB2 (Fig. 6.3). The activities of the group fed with LB02 at week 6 and the groups fed with AM02, LB02 and LB2 at week 8 were significantly higher than ContA (p<0.05). On the other hand, at week 2 and week 4, serum anti-protease of tilapia fed with various experimental diets were all significantly higher than ContA (p<0.05). The activities of groups fed with ContB and LB2 at week 6 and ContB, AM02, AM2 and LB2 at week 8 were significantly higher than ContA (p<0.05).

Without the supplement of huangqi or goji, ContB showed no significant difference in growth performance when comparing to ContA (p>0.05), which was in line with the results of the previous experiments related to both tilapia and grass carp (Chapter 4). However, when taking
Figure 6.2 Serum bactericidal activity of (a) grass carp and (b) Nile tilapia fed with various experimental diets. Values are mean ± SD. Different superscripts (a,b,c) indicate significant differences (p<0.05) at the same sampling week (n=6). #Growth performance of AM2 group was not recorded after 4 weeks only as most of the fish died 1 month after the commence of the feeding trial. ContA = Control diet A, commercial diet; ContB = food waste based diet with 2% cellulose; AM02 = 0.2% Huangqi and food waste based diet; AM2 = 2% Huangqi and food waste based diet; LB02 = 0.2% Goji and food waste based diet; LB2 = 2% Goji and food waste based diet.
Figure 6.3 Serum anti-protease activity of (a) grass carp and (b) Nile tilapia fed with various experimental diets. Values are mean ± SD. Different superscripts (a,b,c) indicate significant differences (p<0.05) at the same sampling week (n=6). #Growth performance of AM2 group was not recorded after 4 weeks only as most of the fish died 1 month after the commence of the feeding trial. ContA = Control diet A, commercial diet; ContB = food waste based diet with 2% cellulose; AM02 = 0.2% Huangqi and food waste based diet; AM2 = 2% Huangqi and food waste based diet; LB02 = 0.2% Goji and food waste based diet; LB2 = 2% Goji and food waste based diet.
non-specific immune response into account, fish fed with ContB responded differently on immune responses. Anti-protease activities of grass carp fed with ContB were lower than fish fed with ContA at week 2 and week 4 (p<0.05), while the activities of Nile tilapia fed with ContB were always significantly higher than fish fed with ContA (p<0.05). No significant differences were noted in total serum immunoglobulin fed with ContA or ContB for both tilapia and grass carp. These results suggested that the formulation of food waste based diet (25% meat and 28% cereals with vitamin-mineral premix addition) was comparable to the commercial diet used in the present study (Jiefeng® 613, contains flour, wheat middling, fishmeal, rapeseed meal, bean pulp, bean oil, fish oil and vitamin-mineral supplement).

Chinese herbs were found to be effective in improving the immunity of various fish. For example, Yin et al. (2006) noted that the inclusion of 0.5% and 1% of *Scutellaria radix* in the diet of Nile tilapia inhibited phagocytosis and respiratory burst activity. Choi et al. (2013) reported that grass carp juvenile fed with diets containing 2% mixture of *Radix scutellaria*, *Rhizoma coptidis*, *Herba andrographis*, and *Radix sophorae flavescentis* at a ratio of 1:1:2:3 resulted in a significantly lower mortality after *Aeromonas hydrophila* (a common fish pathogen causing enteritis) challenge. The dosage given to fish is an important factor for optimizing productivity and immunity of fish. In previous studies conducted by various authors, the effective dosage of Chinese herbs to improve non-specific immunity ranged from 0.05% to 2% (w/w) herb (Zakęś et al., 2008; Choi et al., 2013). In the present study, it was also observed that 0.2% huangqi supplemented diet improved non-specific immune response (bactericidal activity and total immunoglobulin for Nile tilapia). Ardó et al. (2008) revealed Nile tilapia fed with 0.1% *Astragalus membranaceus* extract showed improved phagocytic
and respiratory burst activities of blood phagocytic cells. Yuan et al. (2008) found that injection of Astragalus polysaccharide (major active component of Astragalus root) enhanced the level of IL-1β mRNA in the head kidney of common carp. Astragalus extract has a positive effect on carp immune system as an immuno-stimulant (Cao et al., 2008;; Yin et al., 2004).

The mechanism of the immuno-modulation effects of Astragalus membranaceous has been studied by Shao et al. (2004). They showed Astragalus polysaccharides (APS) could activate both B cells and macrophages of mouse, while APS activates B cells and macrophages via binding to membrane immunoglobin and TLR4, respectively. Lee and Jeon (2005) also noted that APS could stimulated mouse macrophage to express iNOS gene (for nitric oxide synthase for producing NO as a defense mechanism), via activating NF-κB (a protein complex that control transcription of DNA). Huangqi could probably interact with immune cells in grass carp and Nile tilapia similarly to the mammalian system, although macrophage activities were not tested in the present study. Moreover, although the fish specific immunity is considered to be less important in fish immune system (Whyte, 2007), some B cells are responsible to produce natural antibodies, which are involved in immune molecules. It has been reported that natural antibodies of rainbow trout and goldfish are involved in both viral and bacterial defense (Gonzalez et al., 1989; Sinyakov et al., 2002). The stimulation from huangqi could result in the increase in total serum immunoglobin in the present study.

This is the first study incorporating goji into the diet of fish. Results from both feeding trials of grass carp and tilapia indicating that goji is a suitable Chinese herb for culturing both fish
species. 0.2% of goji would be a suitable dose for both species, as it was the only experimental diet that significantly enhanced all tested non-specific immune response (anti-protease activity, total serum immunoglobulin and serum bactericidal activity). A study summarized the immuno-modulation effects of goji, which included the increase in white blood cell count, interleukins production and immune cells proliferation (Amagase and Farnsworth, 2011).

The possible molecular mechanisms of the immuno-modulating effect of *Lycium barbarum* have been previously described. Chen et al. (2009) revealed that *Lycium barbarum* polysaccharide (LBP) could enhance the innate immunity by activating macrophages. It could activate transcription factors NF-κB and AP-1 (using RAW264.7 macrophage cells), induce the expression of TNF-α, IL-1β, IL-12p40 mRNA expression, and enhance the production of TNF-α dose-dependently. Furthermore, LBP could significantly increase macrophage endocytic and phagocytic capacities *in vivo*. The mechanism may be through activation of transcription factors NF-κB and AP-1 to induce TNF-α production and up-regulation of major histocompatibility complex (MHC) class II costimulatory molecules. It has been suggested that LBP fraction 4 primarily acts on macrophages rather than B or T cells (Zhang et al., 2011). Stronger bactericidal activities observed in the present study could be associated with enhanced fish macrophage activity, as macrophages are involved in the complement activation process (Hartung and Hadding, 1983). Although B cells are not target of goji LBP, activated macrophages could produce more signaling molecules such as cytokines and chemokines (Magnadóttir, 2006), which would further activate B cells indirectly, leading to the increase of total immunoglobulin production.
Depending on dosage, Chinese herb supplementation could be harmful to fish. An attempt to incorporate *Astragalus membranaceus* into fish diets noted that 0.1% of AM could improve the non-specific immunity of Nile tilapia (Ardó et al., 2008). This was similar to the present results that 0.2% of huangqi in food waste diet could improve bactericidal activity and total immunoglobin (for Nile tilapia), although no effects on growth performance were observed. However, in this experiment, 2% of AM (huangqi) incorporated into the diet resulted in catastrophic effects on grass carp, which were stressed and all fish refused to consume the fish feed pellets. The digestibility of AM2 diet for grass carp was measured using survived grass carp and the apparent digestibility coefficient of AM2 was significantly lower than other treatment groups (p<0.05), which could explain why the fish had inferior growth FCR and SGR. Moreover, Nile tilapia that consumed 2% of goji also showed inferior growth performance, when compared to other groups (p<0.05). Although there are no previous studies regarding on the adverse effects of Chinese herbs on fish, the present results probably indicated that overdose of Chinese herbs could be harmful to the treated fish.

One possible mechanism is that the high dose (≥2%) of herbs would inhibit bacterial community in the fish gut. An *in vitro* study showed that goji extract is capable to inhibit 17 bacteria, including *Bacillus sp*. (Jin et al., 1995), which is one of the important fish gut bacteria helping digestion (Ray et al. 2012). Balachandar et al. (2011) reported that the extract of huangqi inhibited diarrheal bacterial pathogens such as *Shigella* and *Campylobacter*. It is possible that the diets supplemented with 2% herbs could be harmful to some bacteria that participated in food digestion. Bacteria in fish gut are important for food digestion. For
example, *Aeromonas hydrophila* is a common bacterium found in fish gut (Ray et al., 2012), which is capable to produce extracellular enzymes such as proteases and chitinase (Pemberton et al., 1997; Sugita et al., 1999), although the bacterium is also considered to be an opportunistic fish pathogen (Leung and Stevenson, 1988). *Astragalus membranaceus* was found effective against infection of *A. hydrophila* (Ardó et al., 2008). Similar to mammals, the community of microorganisms in fish gut is important for the health of the host (Rawls et al., 2004; Ringø et al., 2003). The bacteria present in gut of grass carp and tilapia fed with a high dose (≥2%) of herbs could be probably inhibited (growth) or altered (bacterial community) and thus resulted in impaired diet digestibility. The hypothesis was accepted as the Chinese herbs could enhance the growth and non-specific immunity of fish. Further studies would be necessary in order to understand the changes in bacterial community in response to the diets supplemented with Chinese herbs as well as the purified active ingredients present in the Chinese herbs.

### 6.4 Conclusions

Huangqi and goji were incorporated into food waste based diets to study the effects on growth performance and non-specific immune response. Inclusion of 0.2% of goji (*Lycium barbarum*) significantly improved the relative weight gain, feed conversion ratio, specific growth rate, protein efficiency ratio and digestibility of diets for both grass carp and tilapia, indicating that 0.2% of goji could act as a growth promoting agent. Moreover, 0.2% of goji supplement also resulted in significantly higher total immunoglobulin bactericidal activity and anti-protease activity than ContB for both grass carp and Nile tilapia. 0.2% of huangqi also elevated the bactericidal activities and anti-protease activities for both fish species, although huangqi had
no effects on fish growth performance. High amounts of herbs (2%) incorporated into the food waste diets could be harmful to fish. Both fish species fed with food waste diets with 2% of huangqi and 2% goji, respectively, resulted in significantly impaired weight gain, feed conversion ratio, specific growth rate, diet digestibility and high mortality. Goji appears to be a better option for incorporating into the food waste based diets and 0.2% of goji would be a suitable dose for both fish species in terms of better weight gain feed conversion ratio, specific growth rate, diet digestibility and all 3 tested non-specific immune parameters. Thus, 0.2% of goji would be a more suitable supplement for both fish species to enhance growth as well as fish health.
Chapter 7 General discussion and major conclusions

7.1 Introduction

Currently, fish products consumed in Hong Kong largely relied on the import from other regions, especially from Mainland China. In 2012, pond fish culture produced about 2,300 tonnes of fish, which equals to only 3% of local freshwater fish consumption (HK Yearbook, 2013). The results from different experiments in the present study demonstrated the feasibilities of applying food waste supplemented with different dietary supplements, including prebiotic fibres and Chinese herbs, for culturing fish. Using food waste to culture fish could ensure safe and quality fish products which also contributed to easing part of the waste disposal pressure to the landfill sites.

Grass carp and Nile tilapia are herbivore and omnivore respectively that adapted to feed pellets. Their relatively lower protein requirements and higher tolerances to carbohydrates render them suitable species for utilizing food waste (Cai et al., 2005; NRC, 1993; Wang et al., 1985). Results from Chapter 2 revealed that food waste based diets (FWA and B) are more nutritional suitable for feeding grass carp and tilapia than common fish feeds such as Napier grass, breads, noodles and soybean dreg. The effects of food waste based diets on growth of grass carp, bighead and mud carp were evaluated in a field trial (Chapter 3), and the results suggesting that FWB is more suitable for feeding grass carp as better growth was observed. The effects of incorporating different supplements (vitamin mineral premix, prebiotic fibres and Chinese herbs) on fish growth and immunity were evaluated in Chapters 4, 5 and 6.
The objectives of this chapter were to summarize and discuss: (1) the nutritional qualities of food waste based feeds and the growth performance of fish fed with food waste based feeds; (2) the effects of incorporating dietary supplements into food waste based diets on fish growth performance; (3) the effects of incorporating dietary supplements into food waste based diets on fish non-specific immunity; and (4) the overall conclusions and recommendations of the whole study.

7.2 General Discussion
7.2.1 Nutritional qualities of food waste based feeds and the growth performance of fish fed with food waste based feeds

Protein is the most expensive ingredient in fish feed (Shiau and Lan, 1996). Making use of food waste could provide less expensive protein source for producing fish feeds. According to Centre for Food Safety (CFS, 2010), cereals and grains contributed to 43% of solid food consumed in Hong Kong. Being one of the major food groups, it would be reasonable to have a high amount of cereals and grains present in food waste generated in Hong Kong. The higher tolerance to carbohydrates (in cereals and grains) of grass carp (herbivorous fish) (38%) and Nile tilapia (omnivorous fish) (30-70%) make them suitable candidates for utilizing food waste based diets (FAO, 2014b; Li et al., 2014).

The nutritional profiles of food waste based diets were compared with common fish feeds, including fish feed pellets, breads, rice bran, instant noodles and soybean dreg, using the requirements of grass carp and Nile tilapia as references (Chapter 2). Protein requirements of grass carp and Nile tilapia are both about 22-30% and 28-30%, respectively (Cai et al., 2005;
Wang et al., 1985). The requirements of lysine, crude lipid, phosphorous of grass carp and Nile tilapia were 5.44% and 5.12%, 4% and 5-15% (Du et al., 2004; Ng and Chong, 2004), and 1.419-1.577% and 0.9%, respectively. Fish does not require carbohydrate (NRC, 1993), and in general the maximum inclusion rate of carbohydrate in fish diets for herbivores and omnivores should be 40% (Ding, 1991; Lim, 1991; Satoh, 1991). With too high amount of carbohydrate included in diet, carnivores would experience hyperglycaemia and the increase in liver size and glycogen content (Brauge et al., 1994; Hauler, 1995). Herbivorous and omnivorous fish react to carbohydrates similarly to carnivorous fish when they fed with high carbohydrate diets (>40%), although the duration of the elevation of blood glucose is shorter (Wilson, 1994).

Phosphorous is essential to fish for proper bone mineralization (Lim et al., 2001) and phosphorous could also reduce mortality in channel catfish when challenged with *Edwardsiella ictaluri*, which is a bacteria causing enteric septicaemia of catfish (Eya and Lovell, 1998). Lipid and carbohydrate are important energy sources for fish. Inclusion of dietary lipid or carbohydrate in fish feed could promote growth and food utilization, as protein in fish is spared and more energy is derived from other energy source (carbohydrates or lipids) (De Silva et al., 2001; Skalli et al., 2004).

Except for the commercial feed pellets (Jiefeng® 613), the common fish feeds tested: breads, rice bran, noodles and soybean dreg, were nutritional insufficient for grass carp and Nile tilapia. Lysine is always the first limiting amino acid in all the tested fish feeds for grass carp, while lysine, methionine, cystine, and threonine could be limiting to tilapia, with lysine being
the most frequently limiting EAA (Chapter 2, Tables 2.2 and 2.3). Except for soybean dreg and Jiefeng® 613, all the feed items had a crude protein content than lower than 30%. Except for Napier grass and Jiefeng® 613 all of the diets contains more than 40% carbohydrate. Therefore, Jiefeng® 613 would be the most suitable fish feed among all common fish feeds for both grass carp and Nile tilapia.

Contrary to the common fish feeds tested (except Jiefeng® 613), FWA and FWB contained sufficient amounts of essential amino acids (Chapter 2, Tables 2.1, 2.2 and 2.3), sufficient amounts of essential amino acids (especially lysine), crude protein, and total phosphorous for both grass carp and Nile tilapia, although carbohydrate of FWA was slightly higher than 40% and crude lipid of FWA would be too low for tilapia (minimum requirement = 5%, but preferably 10-15%) (Ng and Chong, 2004), while FWB would be too high for grass carp (requirement = 4%) (Du et al., 2004). These results indicated food waste could be used to produce fish diets with nutrients comparable to the commercial fish feed pellets (Jiefeng® 613).

In the field feeding trial, the effects of the 3 fish feed pellets (Jiefeng® 613, FWA and FWB) were evaluated on the polyculture of three carp species (grass carp, bighead and mud carp) (Chapter 3). The higher lipid content (13.3%) and lower carbohydrate content (24.2%) of FWB gave rise to significantly better growth (in terms of length gain, wet weight gain, productivity, feed conversion ratio, specific growth rate and protein efficiency ratio) of grass carp. Inclusion of more cereals in FWA also resulted in a higher phosphorous content in the diet (FWA = 2770 μg/g feed, while control= 967 μg/g feed and FWB= 1942 μg/g feed), which
in turn resulted in a significantly higher plankton density ($p<0.05$) (Fig. 3.3). With more food, bighead carp grew better in polyculture ponds fed with FWA. Mud carp grew equally well in ponds fed with the 3 experimental diets, probably indicating that the different diets did not exert significant effects on its bottom feeding habit (mainly feeding on benthic organisms and organic matter deposited at the pond bottom) (FAO, 2014d).

In the laboratory feeding trial (Chapter 4), fish (both grass carp and tilapia) fed with FWB without supplement of vitamin-mineral premix (VMP) grew equally well as the group fed with control diet. The results deviated from those of the field trial. The differences between the two feeding trials could be influenced by the natural food sources available in the fish ponds. Grass carp fed with Diet B in the ponds could also consume plankton and the submerged part of macrophytes, to replenish vitamins and/or minerals, which could enhance their growth. The groups fed with other diets (Control and FWA) did not respond similarly to the group fed with FWB in the field trial, indicating that Control and FWA were less suitable for grass carp. Adding VMP into FWB significantly enhanced RWG, FCR, PER and SGR of both grass carp and Nile tilapia (Chapter 4).

When feeding with the same diet and at the same feeding rate, Nile tilapia always showed better growth than grass carp, which could be related to the differences in their digestive tracts. Grass carp lacks a stomach and its gut is only 1.6 to 2 times long of its standard length (from mouth and end of tail) (Hoa and Thi 1973). It has been reported that grass carp could only digest half of the plant materials ingested daily (Fedorenko and Fraser, 1978). On the other hand, Nile tilapia possesses a stomach with a pH value as low as 1.4 (Moriarty, 1973), and its
longer intestine (at least 6 times longer than its standard length) provides an abundant surface area for digestion and absorption of nutrients (Opuszynski and Shireman, 1995). It is expected that other cyprinidae, such as common carp (Cyprinus carpio) and edible goldfish (Carassius auratus) could respond similarly to grass carp when fed with food waste based diets.

7.2.2 Effects of incorporating dietary supplements into food waste based diets (FWB) on fish growth (Chapters 5 and 6)

In order to improve the growth of fish feeding with food waste based diets, two prebiotic fibres (inulin and mannan-oligosacharide) and two Chinese herbs were incorporated into the diets to feed grass carp. The growth performances of fish fed with various feed supplements are summarized in Table 7.1. Grass carp fed with 0.2% and 2% inulin, 2% MOS and 0.2% goji diets and Nile tilapia fed with 0.2% goji diets (all %w/w) showed significantly better growth performances (p<0.05), in terms of relative weight gain (RWG), feed conversion ratio (FCR), specific growth rate (SGR) and protein efficiency ratio (PER), than ContB (FWB supplemented with VMP). No published report is available on the appetite stimulating effects of prebiotic fibres on fish. It is likely the growth enhancing effects of prebiotic fibres supplements observed in the present study were solely based on their abilities in promoting the growth of beneficial bacteria in fish gut, as prebiotic fibres is believed to be the most promising mean to manipulate composition and activity of gut microbiota (Bauer et al. 2006; Gibson and Roberfroid 1995; Williams et al. 2001). It has been reported that inclusion of prebiotic fibres in fish diets could improve digestibility of nutrients (Burr et al., 2008), possibly through the microbial breakdown of indigestible carbohydrates (Turnbaugh et al., 2006), or the microorganisms in fish gut produce extracellular enzymes that are either
Table 7.1 Summary of feeding trials on growth of fish fed with food waste diets supplemented with vitamin-mineral premix, prebiotic fibres and Chinese herbs

<table>
<thead>
<tr>
<th>Fish feed</th>
<th>Feed supplements</th>
<th>Feeding duration</th>
<th>Fish species</th>
<th>Major findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chapter 3</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Control (Jiefeng® 613 formulated feed), FWA and FWB</td>
<td>No supplements</td>
<td>6 months</td>
<td>Grass carp, bighead and mud carp</td>
<td>-Grass carp fed with FWB showed the best growth performance, followed by Control and then FWA; bighead fed with FWA showed the best growth performance, followed by FWB=control; Mud carp grew equally well in all treatments.</td>
</tr>
<tr>
<td>Chapter 4</td>
<td>vitamin mineral</td>
<td>4 weeks</td>
<td>Grass carp, Nile tilapia</td>
<td></td>
</tr>
<tr>
<td>Control (without supplement) and FWB (with or without supplement)</td>
<td>premix (0.3%)</td>
<td></td>
<td></td>
<td>-VMP supplemented FWB significantly improved the growth performance of both fish species.</td>
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<tr>
<td></td>
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<td></td>
<td>-VMP significantly improved diet digestibility of FWB for grass carp, but not for Nile tilapia</td>
</tr>
<tr>
<td>Chapter 5</td>
<td>Inulin, MOS</td>
<td>4 weeks</td>
<td>Grass carp, Nile tilapia</td>
<td></td>
</tr>
<tr>
<td>Control (without supplement), FWB (VMP supplemented)</td>
<td>(0.2% and 2%)</td>
<td></td>
<td></td>
<td>-0.2% and 2% inulin and 2% MOS significantly enhanced growth of grass carp.</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-None of the prebiotic fibres enhanced the growth performance of Nile tilapia</td>
</tr>
<tr>
<td>Chapter 6</td>
<td>Huangqi and goji</td>
<td>4 weeks</td>
<td>Grass carp, Nile tilapia</td>
<td></td>
</tr>
<tr>
<td>Control (without supplement), FWB (VMP supplemented)</td>
<td>(0.2% and 2%)</td>
<td></td>
<td></td>
<td>-Growth of both species was the best when fed with FWB supplemented with 0.2% goji</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-2% of goji and huangqi resulted in impaired growth of Nile tilapia and grass carp, respectively</td>
</tr>
</tbody>
</table>

120
lacking or insufficient in the host (Burr et al., 2008), which would enhance digestion and
growth.

On the contrary, including herbal medicines in diets could act as appetite stimulant of fish by
stimulating gut secretions or by having a direct bactericidal effect on gut (Citarasu, 2010).
Enhanced digestive enzyme activities have been noted in rohu (Labeo rohita) fed with diets
incorporated with Livol (a herbal growth promoter) (Maheshappa, 1993) and giant tiger
prawn (Penaeus monodon) with ginger (Zingiber officinalis) (Venketramalingam et al., 2007),
respectively. On the other hand, incorporation of herbs formulation in feeds (1% w/w)
containing Scutellaria baicalensis, Astragalus membranaceus, Poria cocos, Houttuynia
cordata and Isatis indigotica could enhance the quantity of microflora in the intestine of
common carp (Liu et al., 2004). It has been reported that Lycium barbarum polysaccharides
(LBP-4) could enhance the feed conversion rate of mice (Zhang et al., 2002), though no such
information is available on fish. Goji could also possess the appetite stimulating properties.
There is no information available on the growth stimulating effects of huangqi. The present
study also indicated that huangqi did not exert stimulatory effects on the growth of both grass
carp and Nile tilapia.

However, fish fed with Chinese herbs could result in impaired growth. Grass carp and Nile
tilapia fed with the diet incorporated with 2% huangqi and 2% goji (w/w), respectively,
resulted in significantly impaired RWG, FCR, PER and SGR. Both huangqi and goji possess
antibacterial activities (Ardó et al., 2008; Jin et al., 1995). The bacteria present in fish gut that
aid digestions, such as Bacillus sp. (Ray et al. 2012) and Aeromonas hydrophila (Pemberton
et al., 1997; Sugita et al., 1999), would probably be inhibited or altered under high doses (≥2%) of herbs.

7.2.3 Effects of incorporating dietary supplements into food waste based diets on fish non-specific immunity

Non-specific immunity is an important defense mechanism against pathogens for fish. Thus, it has been the major research focus to strengthen the non-specific immunity to fight against pathogens (Choi et al., 2013; Christybapita et al., 2007; He et al., 2003; Jian and Wu, 2003; Sitjà-Bobadilla et al., 2005; Yin et al., 2006). In Chapters 5 and 6, prebiotic fibres and Chinese herbs, respectively, were incorporated into food waste based diets to feed grass carp and Nile tilapia. The effects of the supplements on non-specific immune responses are listed in Table 7.2.

The 4 dietary supplements used in the present study (inulin, MOS, huangqi and goji) are able to stimulate the immune system via different means. Inulin and MOS act on immune cells via interacting with the receptors on immune cells. Inulin possess adjuvant activity which could activate the alternative complement pathway (Silva et al. 2004). It could also bind to lectin-like receptors on leucocytes and induce macrophage proliferation (Causey et al., 1998; Meyer, 2008; Seifert and Watzl, 2007). MOS could interact with immune cells directly with the mannan molecules present on MOS (Che, 2010) and fish would require receptors comparable to mammal MRs for activation (Sørensen et al., 2001). It has been suggested that Astragalus polysaccharides (APS) could activate both mammalian B cells and macrophages
<table>
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<tr>
<th>Fish feed</th>
<th>Feed supplements</th>
<th>Feeding duration</th>
<th>Fish species</th>
<th>Major findings</th>
</tr>
</thead>
</table>
| Chapter 5 Control, FWB | Inulin, mannan-oligosaccharide (0.2% and 2%) | 8 weeks           | Grass carp, Nile tilapia | -Both inulin and MOS had no significant effects on serum immunoglobin of Nile tilapia, while grass carp fed with 2% inulin and 0.2% MOS enhanced serum immunoglobin.  
-Grass carp fed with 0.2% and 2% inulin and 2% MOS and Nile tilapia fed with 2% inulin significantly improved serum bactericidal activities  
-Grass carp and Nile tilapia fed with 2% MOS and 2% inulin showed significantly higher anti-protease activities  
-0.2% goji enhanced serum anti-protease activity of grass carp, while 0.2% and 2% goji and 2% huangqi significantly enhanced the activity of Nile tilapia.  
-0.2% goji significantly enhanced the bactericidal activity, serum total immunoglobulin and anti-protease activity of both grass carp and Nile tilapia |
| Chapter 6 Control, FWB | Huangqi and goji (0.2% and 2%) | 8 weeks           | Grass carp, Nile tilapia | -Grass carp fed with 0.2% and 2% goji and Nile tilapia fed with 0.2% goji significantly enhanced total serum immunoglobulin.  
-0.2% huangqi, 0.2% and 2% goji for grass carp, and 0.2% and 2% goji for Nile tilapia gave rise to significantly increased the bactericidal activity.  
-0.2% goji enhanced serum anti-protease activity of grass carp, while 0.2% and 2% goji and 2% huangqi significantly enhanced the activity of Nile tilapia.  
-0.2% goji significantly enhanced the bactericidal activity, serum total immunoglobulin and anti-protease activity of both grass carp and Nile tilapia |
via binding to membrane Ig and TLR4, respectively (Shao et al., 2004). Lee and Jeon (2005) also reported that APS could stimulate mouse macrophage via activating NF-κB. The immuno-modulation effects of goji includes the increase in white blood cell count, interleukins production and proliferation of immune cells (Amagase and Farnsworth, 2011). Chen et al. (2009) revealed that Lycium barbarum polysaccharide (LBP) could enhance the innate immunity by activating macrophages via activating transcription factors NF-κB. Goji, huangqi, inulin and MOS could activate macrophages in mammalian models. Although macrophages activities were not investigated in the present study, the enhanced non-specific immune responses noted in the present study could be the consequences of the enhanced macrophages activities, as macrophages could produce a wide range of signaling molecules for triggering different immune responses (Cavaillon, 1994; Kopydlowski et al., 1999).

Macrophages are found to be involved in the complement activation process (Hartung and Hadding, 1983). A stronger serum bactericidal activity (through complement activity) indicates stronger non-specific immunity, which could enhance the resistance of fish against pathogens (Das et al., 2009; Wang et al., 2005). In the present study, bactericidal activity of grass carp fed with 0.2% and 2% inulin, 2% MOS, 0.2% huangqi, 0.2% and 2% goji and tilapia fed with 2% inulin, 0.2% and 2% goji were significantly improved. On the other hand, activated macrophages could produce more signaling molecules such as cytokines and chemokines (Magnadóttir, 2006), which would further activate B cells indirectly. Increases in total serum immunoglobin could be related to enhanced natural antibody producing B cells and natural antibody in fish is involved in both viral and bacterial defense (Gonzalez et al., 1989; Sinyakov et al., 2002). In the present study, grass carp fed with 2% inulin, 0.2% MOS,
0.2% and 2% goji, and tilapia fed with the diet containing 0.2% goji showed a significantly higher total serum immunoglobin than groups fed with the two control diets (Jiefeng® 613 and FWB without any supplements).

### 7.3 Major Conclusions

Based on the results obtained from the present study, the following conclusions could be drawn:

1. Both food waste based feed feeds (FWA and FWB) contained sufficient essential amino acids (amino acid scores >100), crude protein (about 30%) and phosphorous (>0.9%), while both diets had carbohydrate contents lower than 40% (Chapter 2). These results suggested that using food wastes as major ingredients could produce high quality fish feeds for culturing low trophic level fish such as grass carp and Nile tilapia, which have lower nutritional requirements.

2. Results from the field feeding trial revealed that both FWA and FWB favoured the growth of different fish species (grass carp, bighead and mud carp) in polyculture ponds. FWA that possessing a higher P content favoured the growth of plankton, leading to a better growth of bighead carp (a filter feeder), while FWB favoured the growth of grass carp (a herbivore), probably due to the relatively lower amount of carbohydrate (24.2%) and CHO:L ratio (1.83) than Control and FWA.

3. In the laboratory feeding trial (Chapter 4), grass carp and Nile tilapia fed with ContB (FWB without vitamin-mineral premix supplementation) and control diet (Jiefeng® 613) showed similar growth performances (RWG, FCR, PER, and SGR). Vitamin-mineral premix (VMP) is an essential ingredient for food waste based fish feed pellets, as both fish species fed with
FWB supplemented with 0.3% (w/w) VMP incorporation showed significantly improved RWG, FCR, PER, and SGR. Protein digestibility of FWB was also improved in grass carp fed with VMP supplemented FWB (but not in Nile tilapia).

4. For grass carp, among all the studied feed supplements (0.2% or 2% inulin, MOS, huangqi or goji), 0.2% of goji, 0.2% and 2% inulin, and 2% MOS (w/w) enhanced both fish growth (RWG, FCR, PER and SGR) and the tested non-specific immune parameters (total serum immunoglobin, bactericidal activity and anti-protease activity). For Nile tilapia, among all of the studied feed supplements (0.2% or 2% inulin, MOS, huangqi or goji), 0.2% was the only supplement enhanced both growth and tested non-specific immune responses.

5. Inclusion of high dosages of Chinese herbs (≥2%) in the diets could be harmful to fish. Grass carp fed with the diet incorporated with 2% huangqi showed a high motility rate (>90%), while Nile tilapia fed with 2% of goji showed the most inferior growth among all treatment group.

6. Based on the results obtained from different experiments of both grass carp and Nile tilapia, the best formulation would be food waste B, supplemented with 0.3% vitamin-mineral premix and 0.2% goji, based on superior growth performance (RWG, FCR, PER and SGR) and the tested non-specific immune responses (total serum immunoglobin, bactericidal activity and anti-protease activity).

7.4 Limitations of Study

The food waste diets used in this study were formulated according to hypothetical ratios of food wastes, and the production requires intensive sorting at sources into different categories of wastes (i.e. fruit and vegetables, meat products, cereals and bone meal). In actual situations,
the compositions of food wastes would be more complex and highly fluctuated, rendering the usage of food wastes as fish feeds more difficult.

Due to the availabilities of fish fry, the fish species used in the present study were only limited to grass carp (both laboratory trial and field trial), tilapia (laboratory trial), mud carp (field trial) and bighead (field trial). The effects of food waste based feeds and their incorporation with Chinese herbs and prebiotic fibres on other polyculture compatible species should also be studied.

7.5 Future Work

As the majority of fish farmers employed polyculture in their fish ponds, it would be necessary to study the effects of food waste based diets on the growth of other common species farmed in polyculture ponds such as grey mullet (*Mugil cephalus*), edible goldfish (*Carassius auratus*), common carp (*Cyprius Carpio*) and silver carp (*Hypophthalmichthys molitrix*). The optimal dosages of different supplementations have to be carefully tested to ensure at least no adverse effects would exert on all the cultured fish species in the polyculture ponds, as different fish would respond differently to the supplements. Further studies should focus on the use of food wastes in culturing carnivorous (both freshwater and marine) fish, such as jade perch (*Scortum barcoo*), yellow finned seabream (*Acanthopagrus latus*) and giant grouper (*Epinephelus lanceolatus*). This is an important sector of local fish farming industry, as the retail prices of these species are generally higher than freshwater fish. However, carnivorous fish would require more protein in their diets for energy production, as they have much lower carbohydrase activities than omnivorous and herbivorous...
Fig. 7.1 Schematic diagram to show the potential applications of food waste based diets and their upgrades on fish.
fish for the effectively digestion if carbohydrates (Stone, 2003).

Fish gut is where food digestion and absorption occurs. Other than the enzymes secreted by fish itself, gut associated microorganisms play an important role in fish nutrition by secreting various extracellular enzymes. For example, *Aeromonas hydrophila* is capable to produce protease, amylase, lipase, cellulase and chitinase for digesting protein, carbohydrate, lipid, cellulose and chitin contained in the diets (Hamid et al., 1979; Jiang et al., 2011, Sugita et al., 1997; Trust et al., 1979). Similar to mammals, the community of microorganisms in fish gut is important for the health of the host (Rawls et al., 2004; Ringø et al., 2003). Gut is also a potential site for invasion of pathogens. Molecular technique is becoming more frequently used for investigating the gut microbial diversity of fish, as traditional plate culturing technique could only culture less than 1% of total bacteria (Ferguson et al., 1984) and that most members of the microbiota in the GI tract cannot be cultured when removed from the gut (Moya et al., 2008; Suau et al., 1999).

The use of culture-independent methods such as screening the 16S rRNA gene of bacteria could be more reliable to estimate microbial diversity in the gut of fish (Wu et al., 2010). A preliminary study using denaturing gradient gel electrophoresis (DGGE) revealed that grass carp fed with various diets (Jiefeng® 613, FWA and FWB) resulted in significant differences in the Dice coefficient of the gut bacteria (Mo et al., 2013). Dice coefficient is calculated using the following formula: $Dsc = \frac{2j}{a+b}$, where $a =$ number of DGGE bands in lane 1, $b =$ number of DGGE bands in lane 2, while $j =$ number of common DGGE bands in lane 1 and lane 2, and $Dsc = 1$ indicates identical profiles (Dice, 1945). The Dice coefficients between
grass carp fed with FWB and Control, FWA and before the experiment were 0.526, 0.818 and
0.538 to indicating that the gut bacterial diversity in fish fed with food waste based diets
(FWA and FWB) were more similar, but not the Control. Further studies on modifications in
bacterial communities in fish gut fed with food waste diets incorporated with prebiotic fibres
or Chinese herbs would be necessary in order to understand their effects on bacterial
communities.
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