UTILIZATIONS OF BY-PRODUCTS FROM ALCOHOLIC BEVERAGE PROCESSES FOR PRACTICAL DIETS FOR JUVENILE NILE TILAPIA (*OREOCHROMIS NILOTICUS*)

Kunthika Vechklang

A Thesis Submitted in Partial Fulfillment of the Requirements for the

Degree of Doctor of Philosophy of Biotechnology

Suranaree University of Technology

Academic Year 2010

การใช้ผลพลอยได้จากกระบวนการผลิตเครื่องดื่มมีแอลกอฮอล์เพื่อการผลิตอาหารสำเร็จรูปสำหรับลูกปลานิลวัยอ่อน (*Oreochromis niloticus*)

นางสาวกุณฑิกา เวชกลาง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาเทคโนโลยีชีวภาพ
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2553

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Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

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กุณฑิกา เวชกลาง : การใช้ผลพลอยได้จากกระบวนการผลิตเครื่องดื่มมีแอลกอฮอล์ในการผลิตอาหารสำเร็จรูปสำหรับลูกปลานิลวัยอ่อน (*Oreochromis niloticus*) (UTILIZATIONS OF BY-PRODUCTS FROM ALCOHOLIC BEVERAGE PROCESSES FOR PRACTICAL DIETS FOR JUVENILE NILE TILAPIA, *OREOCHROMIS NILOTICUS*) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.โชคชัย วนภู, 115 หน้า.

ผลพลอยได้จากกระบวนการผลิตเครื่องดื่มมีแอลกอฮอล์มีสารอาหารจำนวนมากที่ได้มาจากวัตถุดิบและจุลินทรีย์ ซึ่งมีคุณค่าทางโภชนาการ เหมาะที่จะนำมาใช้เป็นวัตถุดิบในการประกอบสูตรอาหารสัตว์ การศึกษาในครั้งนี้จึงได้ดำเนินการทดลองโดยแบ่งเป็นสองการทดลองเพื่อประเมินศักยภาพของการใช้กากสาโทเป็นแหล่งโปรตีนทางเลือกและผลของการเสริมกากยีสต์แห้งในอาหารที่ระดับต่างๆเป็นพรีไบโอติกสำหรับเลี้ยงลูกปลานิลวัยอ่อน

ในการทดลองที่หนึ่งได้ศึกษาระดับการใช้กากสาโทในสูตรอาหารสำหรับลูกปลานิลวัยอ่อน โดยมีสูตรอาหารในการทดลอง 8 สูตร ประกอบด้วย สูตรที่ 1 (กลุ่มควบคุม) ไม่ผสมกากสาโทในอาหาร สำหรับสูตรที่ 2 ถึง 7 ผสมกากสาโทที่ระดับ 7.5, 15.0, 22.5, 30.0, 37.5 และ 45.0% ตามลำดับ ส่วนสูตรที่ 8 ใช้อาหารสำเร็จรูปทางการค้า โดยกำหนดให้อาหารทุกสูตรที่ใช้ทดลองมีระดับโปรตีน 32% เท่ากัน นำอาหารสูตรต่างๆไปเลี้ยงปลาเป็นระยะเวลา 6 สัปดาห์ ทำการประเมินสมรรถนะการเจริญเติบโต ณ สัปดาห์ที่ 4 จากนั้นศึกษาค่าทางโลหิตวิทยา ค่าภูมิคุ้มกันบางชนิด และค่าเคมีในเลือด ณ สัปดาห์ที่ 3 และ 6 รวมทั้งศึกษาค่าจุลสัณฐานวิทยาของลำไส้ ในสัปดาห์ที่ 4 ผลการศึกษาพบว่า สมรรถนะการเจริญเติบโตของปลาที่ได้รับอาหารสูตรที่ 1 ถึง 4 ไม่มีความแตกต่างทางสถิติ (P>0.05) การศึกษาองค์ประกอบของเนื้อปลา ค่าไลโซไซม์ในซีรัม ค่าโลหิตวิทยา และค่ายูเรียในเลือด ของปลาทุกกลุ่มไม่แตกต่างกัน (P>0.05) ยกเว้นปริมาณเม็ดเลือดแดงอัดแน่น และค่ายูเรียในเลือด ณ สัปดาห์ที่ 3 และค่าฮีโมโกลบิน ในสัปดาห์ที่ 6 ของปลาในกลุ่มที่เลี้ยงด้วยอาหารสูตรที่ 6 และสูตรที่ 7 มีความแตกต่างจากกลุ่มควบคุม (P<0.05) การศึกษาระดับน้ำตาลในเลือด พบว่ามีการเพิ่มขึ้นตามการเพิ่มของระดับการเสริมกากสาโทในสูตรอาหาร ในขณะที่ค่าคอเลสเตอรอลในเลือดลดลงตามระดับกากสาโทที่เพิ่มขึ้นในสูตรอาหาร (P<0.05) การศึกษาจุลสัณฐานวิทยาของลำไส้พบว่าปลาในกลุ่มที่เลี้ยงด้วยอาหารสูตรที่ 7 มีค่าแตกต่างจากกลุ่มอื่นๆ (P<0.05) จากการศึกษาครั้งนี้พบว่าการใช้กากสาโทที่ระดับ 22.5% ในอาหารลูกปลานิลวัยอ่อนเป็นระดับที่มีความเหมาะสม เนื่องจากไม่ส่งผลเสียต่อสมรรถนะการเจริญเติบโต และสุขภาพของปลา

การทดลองที่สองได้ศึกษาผลของการเสริมผลิตภัณฑ์ทางการค้าจากยีสต์ที่ใช้ผลิตเบียร์ในอาหารสำหรับเลี้ยงลูกปลานิลวัยอ่อนสองชนิด ดังนี้ พรีไบโอติก GB (GroBiotic®-A) ซึ่งเป็นส่วนผสมของ autolyzed brewers yeast ผลิตภัณฑ์นม และ ผลพลอยได้จากกระบวนการหมัก และ BY (Brewtech®) ซึ่งเป็น autolyzed brewers yeast การทดลองครั้งนี้ใช้ BY และ GB มาเสริมในอาหารสูตรควบคุม (ที่กำหนดให้มีโปรตีน 32% ปริมาณไขมัน 6%) ที่ระดับ 1% และ 2% และนำไปเลี้ยงลูกปลานิลวัยอ่อนในตู้ เป็นระยะเวลา 12 สัปดาห์ เพื่อประเมินสมรรถนะการเจริญเติบโต การตอบสนองทางระบบภูมิคุ้มกัน และความต้านทานต่อเชื้อ *Streptococcus iniae* ของลูกปลานิลวัยอ่อน พบว่าสมรรถนะการเจริญเติบโตของปลาทุกกลุ่มการทดลองไม่แตกต่างกัน ($P>0.05$) และเมื่อทำการศึกษาค่าโปรตีนในซีรัม ค่าอิมมูโนกลอบูลินรวม ค่าไลโซไซม์ในซีรัม พบว่าไม่มีความแตกต่างอย่างมีนัยสำคัญทางสถิติ ($P>0.05$) ทั้งในสูตรอาหารที่เสริม BY และ GB อย่างไรก็ตามพบว่าค่าซีรัมฮีโมไลติกคอมพลีเม้น (serum hemolytic complement activity; SH50) ของปลาในกลุ่มที่มีการเสริม BY ที่ระดับ 1% มีค่าสูงกว่ากลุ่มควบคุมอย่างมีนัยสำคัญทางสถิติ ($P<0.05$) จากนั้นนำปลาที่เลี้ยงด้วยอาหารแต่ละกลุ่มทดลองมาฉีดเชื้อ *S. iniae* เพื่อทดสอบความต้านทานโรคต่อเชื้อ *S. iniae* ผลการศึกษาพบว่าค่าระบบภูมิคุ้มกันอะกลูติเนตติ้งต่อเชื้อ *S. iniae* ไม่แตกต่างกัน ($P>0.05$) และพบว่าปลาที่เลี้ยงด้วยอาหารที่เสริมด้วย BY หรือ GB ทั้ง 2 ระดับมีอัตราการตายสะสมต่ำกว่ากลุ่มควบคุม แต่อย่างไรก็ตามไม่พบความแตกต่างอย่างมีนัยสำคัญทางสถิติ ($P>0.05$)

โดยสรุปผลการศึกษานี้ได้แสดงว่ากากสาโทมีศักยภาพที่จะนำมาใช้เป็นวัตถุดิบแหล่งโปรตีนในสูตรอาหารปลานิลวัยอ่อน และผลิตภัณฑ์ GB และ BY ก็มีศักยภาพที่จะใช้เป็นสารเสริมพรีไบโอติกในอาหารปลานิลวัยอ่อน

สาขาวิชาเทคโนโลยีชีวภาพ ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2553 ลายมือชื่ออาจารย์ที่ปรึกษา _____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

KUNTHIKA VECHKLANG : UTILIZATIONS OF BY-PRODUCTS FROM ALCOHOLIC BEVERAGE PROCESSES FOR PRACTICAL DIETS FOR JUVENILE NILE TILAPIA (*OREOCHROMIS NILOTICUS*) ; THESIS ADVISOR : ASST. PROF. CHOKCHAI WANAPU, Ph.D., 115 PP.

RICE WINE RESIDUAL/NILE TILAPIA/BREWERS YEAST/GROWTH PERFORMANCE/IMMUNE STIMULATION

By-products from alcoholic beverage production processes which contain many nutritious substances derived from both raw materials and microorganisms have become available on a regular basis and are constant in their nutritious values for use as animal feed ingredients. This study was conducted with two experiments to evaluate the potential of utilizing the rice wine residual (RWS) as an alternative protein source and the effect of dietary levels of dried brewers yeast as prebiotic for Nile tilapia at juvenile stage.

The first experiment investigated the utilization level of RWS in a diet for juvenile Nile tilapia. The dietary treatments (each diet in triplicate groups) consisted of seven isonitrogenous (32% crude protein) that were formulated to include RWS at the levels of 0 (control diet), 7.5, 15.0, 22.5, 30.0, 37.5, and 45.0% dry diet (Diets 1-7, respectively), and a commercial diet (Diet 8). The effects of RWS in the diets were evaluated for growth (at week 4), fillet composition, hematological and blood chemical parameters (at weeks 3 and 6), and intestinal morphology (at week 6). The growth performances of fish in the groups on Diets 1-4 did not differ significantly. There were no marked variations in fillet composition, serum lysozyme,

hematological profiles and blood urea nitrogen (BUN) among treatments, except for hematocrit and BUN (week 3) and hemoglobin (week 6) of fish on Diets 6-7. Increasing the amount of RWS incorporation caused blood glucose to increase (P<0.05) and the cholesterol to decrease (P<0.05). Only intestinal morphometry of fish fed with Diet 7 differed significantly from that of the other treatments. Taken together, RWS (at 22.5 % dry diet) has the potential for use in juvenile Nile tilapia diet without negative effects.

The second experiment was conducted to evaluate the effect of feeding diets supplemented with two commercial products of brewers yeast: a prebiotic GB (GroBiotic®-A), a mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products; and BY (Brewtech®), partially autolyzed brewers yeast. A basal diet which was formulated to contain 32% crude protein and 6% lipid (control) was supplemented with 1% and 2% of BY or GB. Each diet was fed to Nile tilapia in quadruplicate aquaria for 12 weeks. Weight gain, feed intake, survival and whole body proximate composition of fish were not significantly affected by dietary treatments. Serum total protein, total immunoglobulin, and lysozyme activity were unaffected by dietary treatments. However, serum hemolytic complement activity (SH50) of fish fed with 1% BY was significantly higher than those of fish fed with the control diet (P<0.05). In order to evaluate the effect of dietary BY or GB on resistance of fish to *Streptococcus iniae*, fish from each treatment diet was injected with *S. iniae*. The result showed that agglutinating antibody titer to *S. iniae* was unaffected by dietary treatments. Furthermore, cumulative mortality at 20 days post-challenge with *S. iniae* of fish fed

with 1% and 2 % of BY or GB was lower than that of fish fed with control diet. However, there was not significant difference ($P>0.05$).

In conclusion, the present study demonstrated the potential benefit of utilizing the by-products from rice wine industry as an alternative protein source in fish feeds. In addition, both GB and BY showed potential prebiotic effects in fish.

School of Biotechnology

Academic Year 2010

Student's Signature _____

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Co-advisor's Signature _____

Co-advisor's Signature _____

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Kunthika Vechklang

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LIST OF ABBREVIATIONS

BUN	=	blood urea nitrogen
cm	=	centimeter
CFU	=	colony-forming units
CFU mL^{-1}	=	colony-forming units per milliliter
CFU $fish^{-1}$	=	colony-forming units per fish
DE	=	digestible energy
DM	=	dry matter
DNA	=	Deoxyribonucleic acid
ER	=	energy retention
FCR	=	feed convenience ratio
FE	=	feed efficiency
FER	=	feed efficiency ratio
fish $aquarium^{-1}$	=	number of fish per aquarium
FM	=	fish meal
x g	=	gravity force
g	=	gram
g dL^{-1}	=	gram per deciliter
h	=	hour
Hb	=	hemoglobin
H&E	=	Haematoxylin and Eosin
HSI	=	Hepatosomatic index

LIST OF ABBREVIATIONS (Continued)

Ht	=	haematocrit values
IP	=	Intraperitoneal
IU	=	International Unit
kcal	=	kilocalorie
L	=	liter
L L^{-1}	=	lenght per liter
L min^{-1}	=	liter per minute
µL	=	microliter
µm	=	micrometer
µg mL^{-1}	=	microgram per mililiter
mg	=	miligram
mg dL^{-1}	=	miligram per deciliter
mg kJ^{-1}	=	miligram per kilojoule
mg mL^{-1}	=	miligram per mililiter
mg kg^{-1}	=	miligram per kilogram
mg L^{-1}	=	miligram per liter
nm	=	nanomete
mM	=	milimole
µm	=	micromete
µg mL^{-1}	=	microgram per mililiter
µL	=	microliter
µL $well^{-1}$	=	microliter per well

LIST OF ABBREVIATIONS (Continued)

µs cm^{-1}	=	microsiemens per centimeter
mL	=	mililiter
mL L^{-1}	=	mililiter per liter
M	=	mola
min	=	minute
mm	=	millimeter
mol L^{-1}	=	mole per liter
nm	=	nanometer
MOS	=	mannan oligosaccharide
mt	=	metric tons
PER	=	protein efficiency ratio
ppm	=	part per million
PBS	=	phosphate buffer saline
PR	=	protein retention
RBC	=	red blood cells number
RNA	=	Ribonucleic acid
rpm	=	round per minute
RWS	=	rice wine residual
SB	=	soybean
S.E.M.	=	standard error of the mean
SH50	=	spontaneous haemolytic complement activity
SGR	=	specific growth rate

LIST OF ABBREVIATIONS (Continued)

SRBC	=	sheep red blood cells
TSB	=	tryptic soy broth
TB	=	thermophilic bacteria
YB	=	yeast biomass
WG	=	weight gain

CHAPTER I

INTRODUCTION

1.1 Significance of the study

Rapid expansion of fish culture in recent years is demanding the development of nutritious fish feeds, as well as better feed utilization, due to the fact that feed cost may increase the cost of fish production by 50–80%. In fish culture, there is an increasing emphasis towards developing cost-effective fish meal substitutes using by-products from animal such as shrimp meal, plant such as cottonseed meal and alcoholic beverage processes such as rice wine, sake, and beer for replacers and/or supplement for fish meal in fish diets. Furthermore, success in the use of alternative proteins in artificial diets would reduce the requirement for trash fish that is presently the main food source for culture of most fish species (Oseni, 2002; José et al., 2007; Se-Jin and Kyeong-Jun 2009; Li and Gatlin, 2003). The alcoholic beverages industry includes beer, wine and liquor production. There is extensive environmental guidance available for breweries. Moreover, by-products from alcoholic beverage processes found that there had contained many nutritious substances such as protein, carbohydrate and vitamins derived from both raw material and yeast.

Sato (Thai rice wine) is a traditional alcoholic beverage made from rice fermented by *Aspergillus oryzae* or *Rhizopus oryzae* for breaking starches in rice into sugars that can be fermented by yeast cells, and it is very popular in the rural parts of

Thailand (Techakriengkrai and Surakarnkul, 2007; Liu et al., 2007). Yeast is hence single cell protein in sato residual. Part of the sato residual is deposed of as waste, so finding ways to recycle are a matter of concern. Because sato residual or lees contain many nutritious substances such as protein, carbohydrate and vitamins derived from both rice and yeast (Manabe et al., 2004). On the other hand, brewing waste with enrich nutrient for living animals are also considered. Brewtech® dried brewers yeast (BY) and GroBiotic®-A (GB) are playing a greater role in the evolution of aquaculture diets. With excellent nutrient profiles and capacity to be mass produced economically, BY and GB have been added to aquaculture diets as supplement for fish meal. Some yeast strains have probiotic properties, such as *Saccharomyces cerevisiae* and *Debaryomyces hansenii*, boost larval survival either by colonizing the gut of fish larvae, thus triggering the early maturation of the pancreas, or via the immune stimulating glucans derived from the yeast cell wall. The cell wall of *S. cerevisiae* may account for up to 20–30% of the cell dry mass. It is mainly composed of β-glucans and mannoproteins. It also contains small amount of chitin and lipids. The glucan found in *S. cerevisiae* consists approximately 85% of β-(1, 3)-glucan and 3% of β-(1, 6)-glucan (Kim and Yun, 2006). However, many of these yeast supplements are deficient in sulfated amino acids, particularly methionine, which restricts their extensive use as the sole protein source (Lim et al., 2005). When *S. cerevisiae* is tested alone, growth and feed efficiency are improved in Israeli carp and Nile tilapia. In tilapia fed a control diet, survival and digestibility are reduced by increasing the population density, while this stress did not affect the groups treated with the yeast (Gatesoupe, 2007).

From the report of fisheries government Thailand (Fisheries, 2009) found that the yield of freshwater culture, during 1987 to 2006 Nile tilapia has high from 17.0 Tons to 498.3 Tons. Rapid expansion of fish culture in recent years is demanding the development of nutrient fish feeds, as well as better feed utilization, due to the fact that feed cost may increase the cost of fish production by 50–80%. Dietary protein ranging from 40% to 45% has been reported to obtain an optimum spawning performance of Nile tilapia. This dietary protein content is considerably less than the protein level of nearly 34%, which supports maximum growth (Cavalheiro et al., 2007). However, the major problem associated with intensive fish culture is the increased susceptibility of fish to infectious diseases, including streptococcal disease in tilapia caused by *Streptococcus iniae*. The problem of streptococcal disease is worldwide (Muzsquiz et al., 1999) and the annual loss to the aquaculture industry is estimated to be over $100 million (Shoemaker et al., 2001). Commercially, antibiotics have been supplemented in aqua feeds for treatment and prevention of bacterial disease of aquatic animals (Li and Gatlin, 2005). The use of antibiotics can lead to the emergence of antibiotic-resistant bacteria, and contamination in food products and the environment (FAO, 2002). Consequently, a wide variety of products ranging from polysaccharides, plant extracts and some nutrients have been added in fish diets as immunostimulants to stimulate immune system function, and/or their resistance to infectious diseases or serve as adjuvant to improve vaccine efficacy (Sakai, 1999; Gannam and Schrock, 2001). Fish meal is traditionally used as the main dietary protein source for tilapia. However, a reduction of fish meal production and its increasing demand has caused a substantial increase in fish meal prices. Therefore, numerous studies have been conducted to evaluate less expensive fish meal

replacements (Lara-Flores et al., 2007). The alternative is the use of proteins from animal or plant, such shrimp meal, blood meal or soybean meal and cottonseed meal. The applications of sato residual are source of protein replacement fish meal and BY and GB for supplementation in fish diets are challenge for research. This research was focused on the effect of sato residual for fish meal replacement, BY and GB for supplement on growth performance and immunological stimulation in practical diets of juvenile sex reversal Nile tilapia.

1.2 Research objectives

The purposes of this study were as follows:

1. To optimize and formulate fish diets from sato residual replacement fish meal, and Brewtech® dried brewers yeast and GroBiotic®-A for supplement.
2. To investigate the effects of formulated fish diets in juvenile sex reversal Nile tilapia (*Oreochromis niloticus*) on growth performance, immunostimulation and resistance to *Streptococcus iniae* challenge.

1.3 Research hypothesis

The practical fish diets could be produced from sato residual, BY and GB which unknown replace fish meal and supplement, respectively. These diets could be effect on growth performance, immunological stimulation and resistance to *S. iniae* challenge of fish for aquaculture use and also for industrial scale production.

1.4 Scope and limitation of the study

Sato residual was selected for protein source replacement fish meal formulates of fish diets production base on ability. Then, the selected formulates were cultivated on juvenile sex reversal Nile tilapia hapa. The formulation and production processes of fish diets were observed water quality, growth performance, survival and immune response of sex reversal Nile tilapia during cultivation. BY and GB were supplemented and fed on fish, then observed the growth performance and resistance to *S. iniae* challenge.

1.5 Expected results

From this study are expected the high potential of sato residual, BY and GB would be can obtained and would be applied for fish diets. Moreover, the information of proximate, feed formulation, growth performance, hematology, blood chemistry, histological, of the replacement fish meal with sato residual in practical fish diets which could be applied for fish diets industry. In addition, BY and GB would be supplement in fish diets had affect on growth performance, immune response and bacterial challenge on fish.

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CHAPTER II

LITERATURE REVIEWS

2.1 The general concept of sato production

Sato is an alcoholic beverage made from rice. It is unlike wine, which is made by fermentation of naturally sweet grapes and other fruits, Sato results from the fermentation of rice starch converted to sugars. This process is related to the production of beer; however, beer production employs a mashing process to convert starch to sugars whereas sato uses the different amylolytic process. Alcoholic beverages distilled from sato exclusive to East and Southeast Asian countries, with knowledge of the distillation process reaching China, India and parts of South Asia later through trade. Rice brew typically has higher alcohol content 9-25% than wine (10-15%), which in turn has higher alcohol content than beer (3-8%) (Wikimedia Foundation, Inc., www, 2009). In Thailand sato locally produced in large amounts every year, is traditional alcoholic beverages made from rice starch, and they are very popular in most parts of Thailand. Recently, the popularity of the traditional sato has enormously increased throughout the country, for both homemade and commercial production (Techakriengkrai and Surakarnkul, 2007). The principle of sato manufacturing consists of the saccharification of steamed rice starch by fungal enzymes under aerobic solid state fermentation and the mould mass is mixed with water and is allowed to undergo submerged alcoholic fermentation by yeasts (Jeyaram et al., 2008). The sato residual or lees come out after filtration.

2.1.1 Sato processing

Rice is polished to remove the protein, lipids and minerals, which are in excess in the bran and germ, and then washed, steeped in water and streamed. After being cooled to room temperature, the rice is inoculated with *Aspergillus oryzae* or *Rhizopus oryzae* starter for 5-6 days.

Sato making begins with the introduction of koji, with break down rice starch into glucose in process known as saccharification. This step is to produce a high concentration of polymer degrading fungal enzyme. Then, yeast is added and fermentation will be begun. At first step, sato can be characterized by solid state fermentation followed by a liquid fermentation.

Lastly, sato is filtered and pasteurized before packaging (Sirisantimethakom et al., 2008; Lertpinyochaithaworn, 2007). The overall of step is shown in Fig. 2.1. The sato residual is generated as a by product which is rich in nutrition.

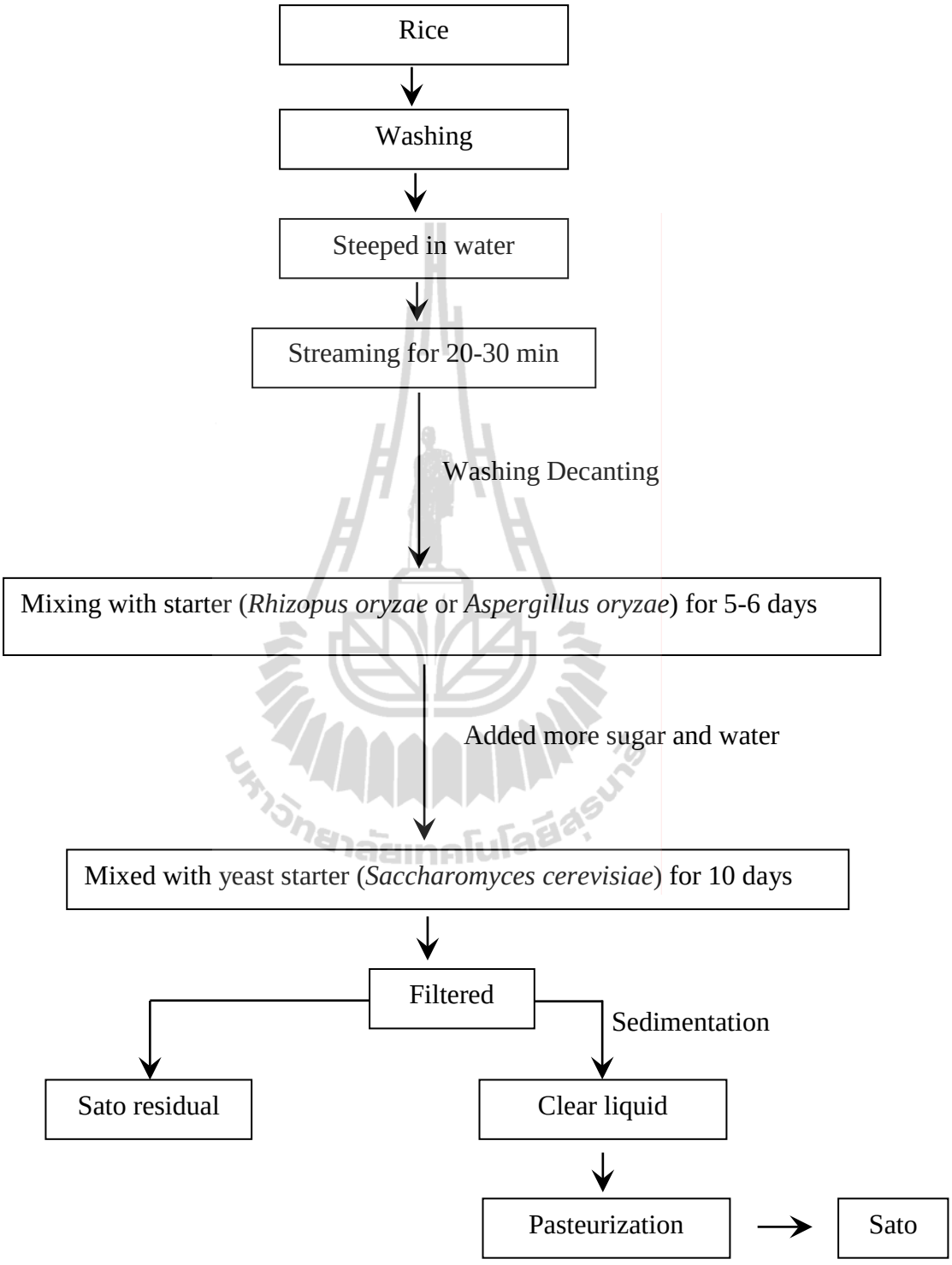

Figure 2.1 Flow diagram of sato production process.

2.2 Nutritional Yeast as Animal Nutrients

Yeast cells exhibit great diversity with respect to cell size, shape and colour. Even individual cells from a particular yeast strain of a single species can display morphological and colour heterogeneity. This is mainly due to alterations of physical and chemical conditions in the environment. Yeasts are abundant throughout the environment. They can be found on cereal grains, grain-by-products, silages, hays and are even present in the soil and water. The yeast ferments simple sugars into ethanol and carbon dioxide. Today, pure cultures of yeast are grown specifically for breweries, wineries, distilleries, bakeries and home use.

The nutritional yeast products consist of yeast biomass or pure, dead yeast cells which are fed for their nutrient value. They include primary dried yeast, brewers dried yeast, and whey yeast. Yeast cells have long contributed to the nutritional value of fermented foods, like breads and beers. In some societies, cloudy beers make a major contribution to daily nutritional needs. The cloudy sediment of yeast cells provides essential vitamins B, minerals and amino acids. And during the middle ages, infants were often fed the sediment from cloudy beer to keep them healthy and avoid nutritional deficiencies. Yeasts are a good source of protein or amino acids. Approximately 40% of the weight of dried yeast consists of protein. The quality of yeast protein is excellent for a vegetable protein and it is about equivalent in quality to soybean protein. Both are rich in lysine, and are excellent supplements to cereals, whose proteins are generally low in lysine (Table 2.1). As with other plant proteins, yeast protein is low in the sulfur amino acids, but supplementing dried yeast with 0.5% methionine can raise its protein quality up to that of casein. However, there is a limit to how much yeast can be fed, because about 20% of the crude protein nitrogen

in yeast is in the form of nucleic acids. Nucleic acids can cause problems if over fed, because excessive nucleic acid intake results in elevated uric acid levels in the blood. While the nutritional value of yeast was recognized early, the identification of the nutritional factors which cured certain nutritional diseases did not take place until the early 20th century. That is when the vitamins B are discovered. Several of these vitamins are first extracted and characterized from yeast, including biotin, niacin, pantothenic acid, and thiamin. Yeast has long been recognized as a rich source of natural vitamins B (Stone, www, 2009).

Table 2.1 Amino acids requirements of juvenile Nile tilapia diets.

Amino acid	**Protein in diets %**	**Requirement as percentage of dietary protein**	**Requirement as percentage of dry diets**
Arginine	28	4.20	1.18
Histidine	28	1.72	0.48
Isoleucine	28	3.11	0.87
Leucine	28	3.39	0.95
Lysine	28	5.12	1.43
Methionine	28	2.86	0.75
Phenylalanine	28	3.75	1.05
Threonine	28	3.75	1.05
Tryptophan	28	1.00	0.28
Valine	28	2.80	0.78

Source : NRC, 1993

When whole yeast cells are fed, like brewer's yeast or active dry yeast, their primary nutritional contribution comes from the proteins, peptides, vitamins, and

minerals contained within the cell the intracellular biochemical found in the yeast cell compare with other source (Table 2.2). Thus, for these nutrients to become available the yeast cell must be lysed or broken open so that the contents within the cell become available for digestion and absorption.

Nutritional studies to assess the nutrient requirements of a species are always carried out under standard, well defined, and benefit conditions. Because of nutritional, environmental, and husbandry factors in commercial aquaculture facilities however, the fish may be subject to a combination of stressors which can have short and long term effects. Use of micronutrients for example vitamins, trace minerals, probiotics, and immunostimulants, as dietary supplements may benefit animal health by improving the non-specific immune system of the animal by availability or utilization of nutrients through a variety of pathways (Staykov et al., 2007).

Table 2.2 Nutritional profile of yeast biomass (YB), thermophilic bacteria (TB), fish meal (FM) and soybean (SB) to be source of protein for fish diet (% dry matter basis)

Component	**Content (%)**			
	YB	**TB**	**FM**	**SB**
Dry matter	2-5	96.41	93.7	89.1
Crude Protein	50-52	51.77	59.37	53.5
Crude Lipids	4-7	5.41	9.78	1.4
Ash	nd	13.01	17.72	6.4

Source : Reed and Nagodawithana, 1991; Gonzales Jr., and Brown, 2007; Wang et al., 2006; Gómez-Requeni et al., 2004

Abdel-Tawwab et al. (2008) carried out to evaluate the use of commercial live bakers' yeast, *S. cerevisiae* as a growth and immunity promoter for Nile tilapia. From the results indicate that bakers' yeast supplement is promising as an alternative method to antibiotics for disease prevention in tilapia aquaculture, and the optimum level of live bakers' yeast is about 1.0 g per kg diet.

Guedes et al. (2008) studied on the effects of *S. cerevisiae* yeast on ruminal fermentation and fiber degradation of maize silages in cows found that *S. cerevisiae* posses the potential to reduce the risk of rumen acidosis in commercial cattle fed maize silage based diets. Li and Gatlin (2003) found that fish fed the diet with 2% brewers yeast are found to have significantly ($P < 0.01$) higher blood neutrophil oxidative radical and extracellular superoxide anion production of head kidney macrophages than control fish. However, no significant differences in intracellular superoxide anion and serum lysozyme were observed among the treatments.

White et al., (2002) used brewers dried yeast as a source of mannan oligosaccharides for wealing pigs found that it had minimal effects on growth, microbial populations, and intestinal health traits of early-weaned pigs, but certain serum immunological traits are enhanced by feeding yeast.

Oliva-Teles and Gonçalves (2001) found that using brewers yeast replacement fish meal in diets for sea bass juvenile can replace 50% of fish meal protein with no negative effects in fish performance. Moreover, the inclusion of up to 30% brewers yeast in the diet improved feed efficiency. There is no beneficial effect of supplementing the brewers yeast diets with methionine.

2.3 Susceptibility of fish to infectious diseases

Nowadays, one major problem associated with intensive fish culture is the increased susceptibility of fish to infectious diseases, including streptococcal disease in tilapia caused by *Streptococcus iniae*. The problem of streptococcal disease is worldwide (Muzsquiz et al., 1999) and the annual loss to the aquaculture industry is estimated to be over $100 million (Shoemaker et al., 2001). *Streptococcus iniae* has been identified as one of the principal etiologic agents of streptococcal disease in cultured hybrid striped bass (*Morone chrysops* x *Morone saxatilis). S.iniae* is a Gram-positive, aerobic, coccus-shaped bacterium that has been isolated from brain, eye, or kidneys of naturally infected fish. A potential source of *S. iniae* infection is water contaminated with *S. iniae* infected morbid and dead fish. Entry of *S. iniae* through the nares andror the eyes of cultured fish may be a likely route of infection from contaminated water, although the nares are not a commonly cultured site (Joyce et al., 2000; Chhorn et al., 2010).

Commercially, antibiotics have been supplemented in aqua feeds for treatment and prevention of bacterial disease of aquatic animals (Li, and Gatlin, 2005). The use of antibiotics can lead to the emergence of antibiotic-resistant bacteria, and contamination in food products and the environment (FAO, 2002). The use of antibiotics in animal production has been banned in EU countries and is increasingly under public scrutiny and criticism in most other countries. Consequently, a wide variety of products ranging from polysaccharides, plant extracts and some nutrients have been added in fish diets as immunostimulants to stimulate immune system function, and/or their resistance to infectious diseases or serve as adjuvant to improve vaccine efficacy (Sakai, 1999; Gannam and Schrock, 2001).

In aquaculture, traditional methods for treating infective pathogens include a limited number of government-approved antibiotics and chemotherapeutics. However, the disadvantages such as marginal effectiveness and high cost are obvious (Sealey and Gatlin, 2001). These treatments also may cause the accumulation of chemicals in the environment and fish, thus posing potential threats to consumers and the environment. An alternative strategy, besides vaccine development, is nutritional modulation of immune responses and disease resistance of aquaculture species. Research on the subject of nutritional modulation, especially evaluation of natural extracts or synthetic compounds, which may enhance the immune responses and disease resistance of hybrid striped bass, is still in its infancy. Yeast by-products from the brewing industry are natural diet additives that have been shown to positively influence non-specific immune responses as well as growth of some fish species (Oliva-Teles and Goncalves, 2001). In addition, doses and time of administration have been recognized to have important effects on immunostimulant function, and efficacy of oral administration of immunostimulants has been reported to decrease over time (Li and Gatlin, 2003).

Brewtech® dried brewers yeast, *S. cerevisiae*, is a natural product of the International Ingredient Corporation, St. Louis, MO, USA containing various immunostimulating compounds such as β-glucans, nucleic acids and mannans. It has been shown to positively influence non-specific immune responses as well as growth of various fish species and thus may serve as an excellent health promoter for fish culture (Li and Gatlin, 2003; 2004; 2005; Waszkiewicz-Robak and Karwowska, 2004). World-wide spent brewer's yeast is generally sold primarily as inexpensive animal feed after inactivation by heat. Proper nutrition has long been recognized as a

critical factor in promoting normal growth and sustaining health of fish. Brewer's yeast has been recognized to have potential as a substitute for live food in the production of certain fish and as a potential replacement for fishmeal (Oliva-Teles and Gonçalves, 2001). Brewer's yeast can replace 50% of fishmeal protein with no negative effects in fish performance. Moreover, the inclusion of up to 30% brewer's yeast in the diet improved feed efficiency. As a protein feedstuff, brewer's yeast has been included in commercial diet formulations for several fish species, including salmonids. The cell wall has been suggested to cause the reduced nitrogen digestibility commonly found in single cell protein sources (Ferreira et al., 2010).

2.3.1 Evaluation of the prebiotic GroBiotic®-A with fish

Evidence of the beneficial effects of probiotics gave rise to the concept of prebiotics, which are defined as nondigestible food ingredients which beneficially affect the host by selectively stimulating the growth of and/or activating the metabolism of one or a limited number of health promoting bacteria in the intestine tract, thus improving the host's intestinal balance. The dietary supplementation with a commercial prebiotic significantly enhanced growth and disease resistance of fish that achieved with brewers yeast (Delbert and Peng 2004).

The prebiotics have several advantages, but the main advantage of prebiotics over probiotics is that they are natural feed ingredients. Their incorporation in the diet does not require particular precautions and their authorization as feed additives may be more easily obtained, in spite of some concerns about their safety and efficacy. Prebiotic, unlike probiotic, is not an organism and has less influence in natural environment. Based on definition of Gibson and Roberfroid (1995), prebiotics are 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. The prebiotic in the diet has been reported to increase the uptake of glucose (Breves et al., 2001) and bioavailability of trace elements. Bongers and van den Heuvel (2003), explained this enhancing effect of prebiotics on mineral absorption, the osmotic effect with the exchange of protons and possible decrease in proteins such as calcium-binding protein which may increase the availability of trace elements in the small intestine, acidification of the colonic content due to fermentation and production of short chain fatty acids, formation of calcium and magnesium salts of these acids, and hypertrophy of the colon wall. Prebiotic may have the role of increasing growth rate, improve immune system as well as change the community of bacterial in gastrointestinal track. Many scientists have worked to optimize the dosage of supplementary prebiotic in feed to achieve better growth rate and survival. Reports about effect of prebiotic on growth parameters in fish are inconclusive. Supplementation of Beluga's (*Huso huso)* diet with 1, 2 and 3% inulin showed negative relationship between some performance indices including weight gain (WG), specific growth rate (SGR), protein efficiency ratio (PER), energy retention (ER), feed efficiency (FE), protein retention (PR) and supplementation level of inulin. Also growth parameters in fish fed inulin was lower than control group (Akrami et al., 2009). The reduction of some growth parameter in treatment groups may be due to affecting some other parameters in experimental place of work or the condition of fish itself and not by the versus effect of inulin, but conclude that at least the inulin had no positive effect in growth rate of *Huso huso* young fishes. In using commercial prebiotic GroBiotic®-A (International Ingredient Corporation, St. Louis, MO, USA), feed efficiency was significantly improved when using a 7- week diet was

supplemented with 1 – 2 % of this commercial food on hybrid striped bass, but the growth was not significant (Li and Gatlin, 2004). The growth of the fish my increase by using supplementary prebiotic in feed. In a 3-week trial, Refstie et al. (2006) found that Atlantic salmon fed with a fish meal based diet supplemented with 7.5 % inulin had increased relative mass of the gastrointestinal tract, but the absorptive capacity of the fish was not affected. Considerations in supplementing prebiotics in fish diets have been arisen to some extent.

The type of prebiotic such as mannanoligosaccharides, lactose, as well as oligofructose and inulin to supplement specific animal characteristics (species, age, and stage of production) and type of diet are important considerations. In addition, practical formulations and economic considerations should be carefully considered. The use of different chemotherapies is advisable to avoid bacterial infection of fish. The use of GroBiotic®-A have shown improved survival rate of hybrid stripped bass challenged with live *Streptococcus marinum* and *Mycobacterium marinum* (Li and Galin, 2005). In trials with rainbow trout (Staykov et al., 2007), common carp (Staykov et al., 2005) and Jian carp (Zhou and Li, 2004), the non-specific immune system was positively affected when the diet was supplemented with a mannan oligosaccharide (MOS) (Staykov et al., 2007). Torrecillas et al. (2007) reported dietary incorporation of MOS at 0.4% activated sea bass' immune system and increased its resistance to a bacterial infection directly inoculated in the gut, one of the main sites of infection in fish. However, prebiotics have been reported to have numerous beneficial effects in fish such as increased disease resistance and improved nutrient availability.

The potential using of a specialized prebiotic preparation in aquaculture, laboratory evaluated the commercial product GroBiotic®-A (International Ingredient Crop., St. Louis, MO, USA) in three separate feeding trials with hybrid striped bass. This product is mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products containing 35.2% crude protein, 1.7% crude lipid, and ~53% simple and complex carbohydrates including oligosaccharides. The dairy ingredient components and dried fermentation products has been shown to enhance the growth performance, feed efficiency and survival of hybrid striped bass to *S. iniae* and *Mycobacterium marinum* (Li and Gatlin, 2004; 2005).

From reviewing the literature, the advantages of using yeast fermented sato residual, brewers yeast and GroBiotic®-A, found that these can a few report on used for animal, especially fish diet. Therefore the replacement fish meal with the level of sato residuals and supplement with the level of brewers yeast and GroBiotic®-A 1% and 2% in practical fish diets for juvenile Nile tilapia on growth performance, immune response and bacterial challenge were used in this study.

2.4 Nile tilapia

Nile tilapia is the common name for nearly a hundred species of cichlid fishes from the tilapia Cichlid tribe. They inhabit a variety of fresh and brackish water habitats from shallow streams and ponds through to rivers, lakes, and estuaries (Wikipedia Foundation, Inc., www, 2009). Most tilapias are omnivorous, feeding on algae, aquatic plants, small invertebrates, detritus material and the associated bacterial films (Fitzsimmons, www. 2009). They have historically been of major importance in artisanal fishing in Africa and the Levant, and are of increasing importance

in aquaculture around the world. It is first introduced into Thailand in 1965 when the Emperor of Japan gave a few fish to H.M. King of Thailand. The fish is bred at Chitralada Palace and hence the name Chitralada strain is born (Wikipedia Foundation, Inc., www, 2009). The tilapias are one of the most commonly cultured fish on the Earth. Their ability to utilize a wide range of feed ingredients, tolerance of poor environmental conditions, ease of reproduction, and fast growth rates make Nile tilapia a good candidate (Gonzales and Brown, 2006). In general, the male of Nile tilapia is popular culture because it is fast growth rates, resistant disease and high survival rate. Nowadays, sex reversal Nile tilapia farm is popular culture. There are a number of ways to control reproduction in mixed sex population. One of these is the culture of all male tilapia. Sex reversal by oral administration of feed incorporated with methyl testosterone is probably the most effective and practical method for the production of all male tilapia (Cagauan et al., www. 2009).

Kingdom: Animalia

Phylum: Chrodata

Class: Actinopterygii

Order: Perciformes

Family: Cichildae

Genus: *Oreochrmis*

Species: *O. niloticus*

Tilapia is the generic name of a group of Cichlids endemic in Africa. A group consists of three aqua culturally important genera *Oreochromis, Sarotherodon* and *Tilapia* (Fig. 2.2).

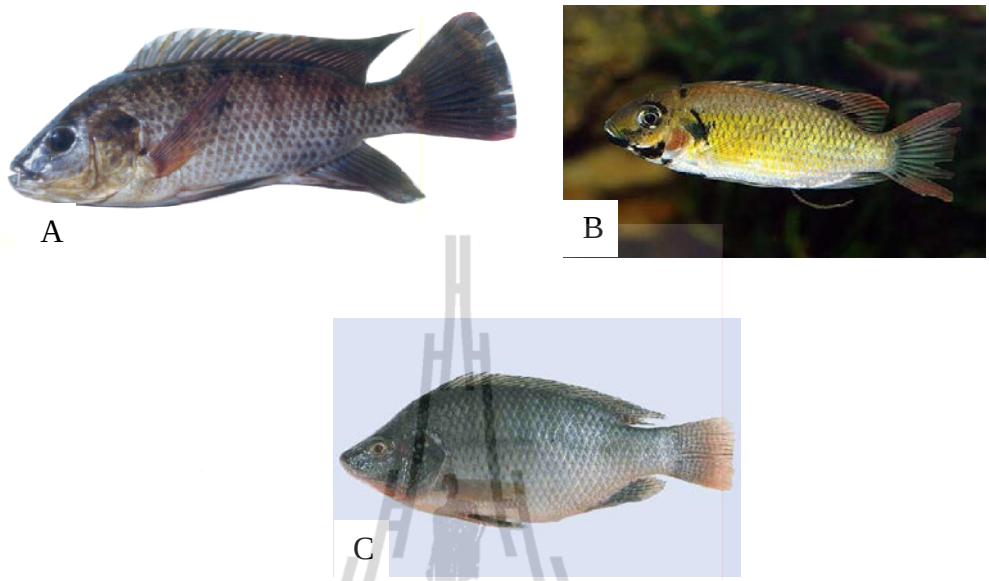

Figure 2.2 The morphological characteristics of cichlids fishes; A) *Oreochromis,* B) *Sarotherodon* and C) *Tilapia*.

Source: Seaburst, www. 2009

Several characteristics distinguish these three genera, but possibly the most critical relates to reproductive behavior. All tilapia species are nest builders, which fertilized eggs are guarded in the nest by a brood parent. Species of both *Sarotherodon* and *Oreochromis* are mouth brooders; eggs are fertilized in the nest but parents immediately pick up the eggs in their mouths and hold them through incubation and for several days after hatching. In *Oreochromis* species only females practice mouth brooding, while in *Sarotherodon* species either the male or both male and female are mouth brooders. During the last half century fish farmers throughout the tropical and semi-tropical world have begun farming tilapia. The scientific name of the Nile tilapia is *Oreochromis niloticus*. Tilapia are shaped much like sunfish or crappie but can be easily identified by an interrupted lateral line characteristic of the

Cichlid family of fishes. They are laterally compressed and deep-bodied with long dorsal fins. The forward portion of the dorsal fin is heavily spine. Spines are also found in the pelvis and anal fins. The front portion of the dorsal fin is spiny and the rear is soft rayed. Spines are also found in the pelvic and anal fins. The external anatomy of tilapia is given in Fig. 2.3. There are usually wide vertical bars down the sides of fry, fingerlings, and sometimes adults (Popma1 and Masser 1999; Nandlal and Pickering, 2004).

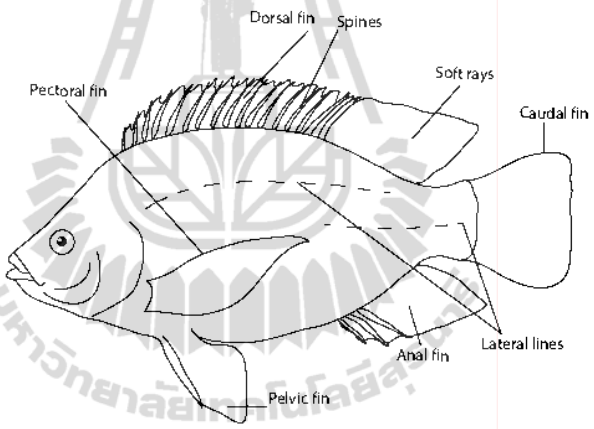

Figure 2.3 External anatomy of tilapia. (Nandlal, and Pickering, 2004)

Under good growth conditions, start at 1 g of fish body is cultured in nursery ponds until fish weight 20 to 40 g for 5 to 8 weeks and then restocks into grow out pond. Mono sex grows out pond under good temperature regimes; males generally reach a weight of 200 g in 3 to 4 months, 400 g in 5 to 6 months, and 700 g in 8 to 9 months. To produce 400 to 500 g fish, common practice is to stock 6,000 to 8,000 males per acre in static water ponds with aeration or 20,000 to 28,000 males per acre where 20 percent daily water exchange is economically practical. After 6 months of

feeding with good quality diets, such ponds can produce 2,000 to 3,000 kg per acre and 18,000 to 9,000 kg per acre, respectively. If grow out cycles are longer than 5 to 6 months there is a risk that offspring from reproduction of the few females, that were unintentionally included in the all male culture will have time to reach sexual maturity and overpopulate the pond (Popma1 and Masser, 1999). Currently, the aquaculture of Nile tilapia has expanded throughout the country, and become the No. 1 freshwater fish produce in Thailand with a volume of 1.39 million mt (metric tons) in 2009. In addition, world aquaculture production of Nile tilapia has drastically increased from 2.15 million mt in 2007 to 2.54 million mt in 2010 (FAO, 2010). The global aquaculture production of tilapia has drastically increased from 124 thousand mt in 1997 to 2.5 million mt in 2010 (FAO, 2010).

2.5 Feed ingredient for Nile tilapia

Dietary ingredients must be highly digestible, be available on a consistent basis, be easily handled in the manufacturing process, be able to withstand the rigors of the manufacturing process, and be economical. A brief summary of the commonly available feed ingredients that used in Nile tilapia feeds.

1. Protein source; These commonly used in Nile tilapia feeds include fish meal, meat meal, bone meal, blood meal, and poultry by-product meals. Animal proteins are generally considered to be of a higher nutritional value than plant protein because of their balanced indispensable amino acids. The plant protein sources used in Nile tilapia feeds are oilseed meals, such as soybean meal and cottonseed meal. Replacement of fish meal with plant proteins presents problems, as the quality and concentration of proteins from plant sources is generally inferior to fish meal and the

palatability of most plant proteins relative to fish meal is low. However, the cost and availability of plant proteins is superior to fish meal and this cost advantage may allow processing of crops to improve their nutritive value in finfish (Drew, 2007).

2. Energy sources; Energy feedstuffs are those that contain less than 20% crude protein. These include grain and grain by-products, and animal fats or vegetable oils.

3. Vitamin and mineral supplements; Nile tilapia feeds are supplemented with a vitamin premix that provides vitamins in quantities necessary to meet dietary requirements and compensate for losses due to feed processing (Li et al., 2006).

2.5.1 Nutrient requirements for Nile tilapia

Fish diet contains nutrients and energy sources essential for fish growth, reproduction, and health. Deficiencies of these substances can reduce growth rates or lead to diseases, and in some cases, excesses can cause a reduction in growth rate. Dietary requirements can be established for energy, amino acids, protein, lipids, minerals, and vitamins (NRC, 1993). In intensive systems, tilapias have the advantage that they can be fed a prepared diet that includes a high percentage of plant proteins. Carnivorous fish require fish meal or other animal proteins in their diets, which in general are more expensive than plant proteins. Nutritional studies which substitute plant proteins supplemented with specific amino acid supplements may lower costs, but still not to the level that can be achieved with tilapia diets. Complete diets are used in systems that cannot provide any dependable nutrition. Tilapia exhibits the best growth rate when it is fed a balanced diet that provides a proper mix of protein, carbohydrates, lipids, vitamins, mineral, and fiber. The nutritional requirements are slightly different for each species and more importantly vary with life stage. Fry and

fingerling fish require a diet higher in protein, lipids, vitamins and minerals but lower in carbohydrates as they are developing muscle, internal organs and bone with rapid growth. Sub-adult fish need more calories from fat and carbohydrates for basal metabolism and a smaller percentage of protein for growth (Table 2.3). The absolute amount the fish is eating will still be increasing as the fish is much larger. Adult fish need even lee protein, however the amino acids that makeup that protein needs to be available in certain ratios (Table 2.1). Feed formulators will adjust protein sources to fit the desired pattern of amino acids through the growth cycle. Brood fish may require elevated protein and fat levels to increase reproductive efficiency (NRC, 1993; Fitzsimmons, www. 2009).

Table 2.3 Typical protein requirements of juvenile Nile tilapia.

Weight of whole body	Protein
First feeding fry	45 - 50 %
0.02 - 2.0 g	40 %
2.0 - 35 g	35 %
35 g - harvest	30 - 32 %

Source: Fitzsimmons, www. 2009

For several indispensable amino acids, intake and weight gain are apparently linearly related and this relationship is presumed to hold for all indispensable amino acids. On this basis amino acid requirements of fish are expressed as a percentage of dietary protein as well as on a dry matter basis (NRC, 1983). In general, the lipid requirements for fish under 2 g represent 10% of the diet. This decreases to 6-8% from 2 g to harvest. The lipids should contain both omega 3

and omega 6 fatty acids. Each fatty acid should represent 1% of the diet, although some reports suggest that fish grow better with a higher proportion of omega 6 to omega 3. The fiber component is usually the reciprocal of the lipid content. That is starting at 6-8% in small fish up to 35 g and increasing to 10% above 35 g. Carbohydrates usually represent lee than 25% of the diet for fish under a gram and increases to 25 - 30% for fish greater than a gram up to harvest (Fitzsimmons, www. 2009).

2.6 Replacement of fish meal in fish diets

Nile tilapia is the third largest group of farmed finfish species. Fish feeding represents over 50% of operating costs in intensive aquaculture, with protein being the most expensive dietary source. The development of commercial aqua feeds has been traditionally based on fish meal (FM) as the main protein source due to its high protein content and balanced essential amino acid profile. FM is also an excellent source of essential fatty acids, digestible energy, minerals and vitamins. Therefore, it is no surprise that FM is the most expensive protein source in animal and aquaculture feeds (El-Sayed, 1999). Most published research on the use of low cost plant and industrial waste proteins, as a substitute of fish meal, in fish diets i.e. faba beans (Azaza et al., 2009), X' pelon seed (*Vigna unguiculata*) (Lara-Flores et al., 2007), soybean meal (Wang et al., 2006), gilthed sea bream (Gómez-Requeni et al., 2004), brewers yeast (Oliva-Teles, and Gonçalves, 2001), cottonseed meal (Yue, and Zhou, 2008) in diets for Nile tilapia.

Hernández et al., 2010 suggest that the high-quality of poultry by-product meal-pet food grade and porcine meal used could completely replace FM protein in practical diets for fingerling Nile tilapia without affecting growth performance.

El-Sayed, 1998 found that the soybean meal, meat and bone meal, bone meal, and poultry by-product meal can totally replace FM in practical Nile tilapia diets under the experimental conditions employed.

2.7 Immunostimulants on fish

2.7.1 Fish and immune system

Fish is a heterogeneous group of organisms that include the agnathans, condryctians and teleosteans. As in all vertebrates, fish have cellular and humoral immune responses and a central organ that's the main function is involved in immune defence. Fish and mammals show some similarities and some differences regarding immune function. Taking into account differences due to body compartments and cell organization, most of the generative and secondary lymphoid organs present in mammals are also found in fish, except for the lymphatic nodules and the bone marrow. Instead, the head kidney, aglomerular, assumes hemopoietic functions, and unlike higher vertebrates is the principal immune organ responsible for phagocytosis, antigen processing and formation of IgM and immune memory through melanomacrophagic centres. The kidney in fish is a disperse organ with a Y shape that is placed along the body axis (Fig. 2.4). The lower part is a long structure situated parallel to the vertebral column, most of which works as a renal system. The active immune part, the head kidney or pronephros, is formed by two Y arms, which penetrate underneath the gills. In fish, this structure has a unique feature: the head

kidney is also an important endocrine organ, homologous to mammalian adrenal glands, releasing corticosteroids and other hormones. In addition, it is a well innervated organ. Thus, the head kidney is an important organ with key regulatory functions and the central organ for immune-endocrine interactions and even neuroimmunoendocrine connections. The thymus, another lymphoid organ situated near the opercular cavity in teleosts, produces T Lymphocytes involved in allograft rejection, stimulation of phagocytosis and antibody production by B cells. The involution of thymus in fish is more dependent on hormonal cycles and seasonal variations than on the age. Blood filtration and erythrocytic destruction is performed by the melanomacrophagic centres, formed by accumulation of macrophages associated to elipsoid capillaries. These centers may retain antigens as immune complexes for long periods.

Fish immune cells show the same main features than that of other vertebrates, and lymphoid and myeloid cell families have been determined. The lymphoid system is a relatively recent evolutionary development since most animals prior to vertebrates rely on non-lymphoid cells or serum molecules. The existing functional analysis together with the reactivity of monoclonal antibodies suggests the presence of helper and cytotoxic T lymphocytes and subpopulations of B cells. The monocyte/macrophage cell lineage is the most studied in fish although no exact cell specific markers are available. Therefore, the term macrophage is often used as a loose definition for phagocytic cells independent of the differentiation status and the anatomical location. The majority of cytokines identified to date and of data concerning cytokine regulation has been obtained in macrophage cell cultures (Tort et al., 2003).

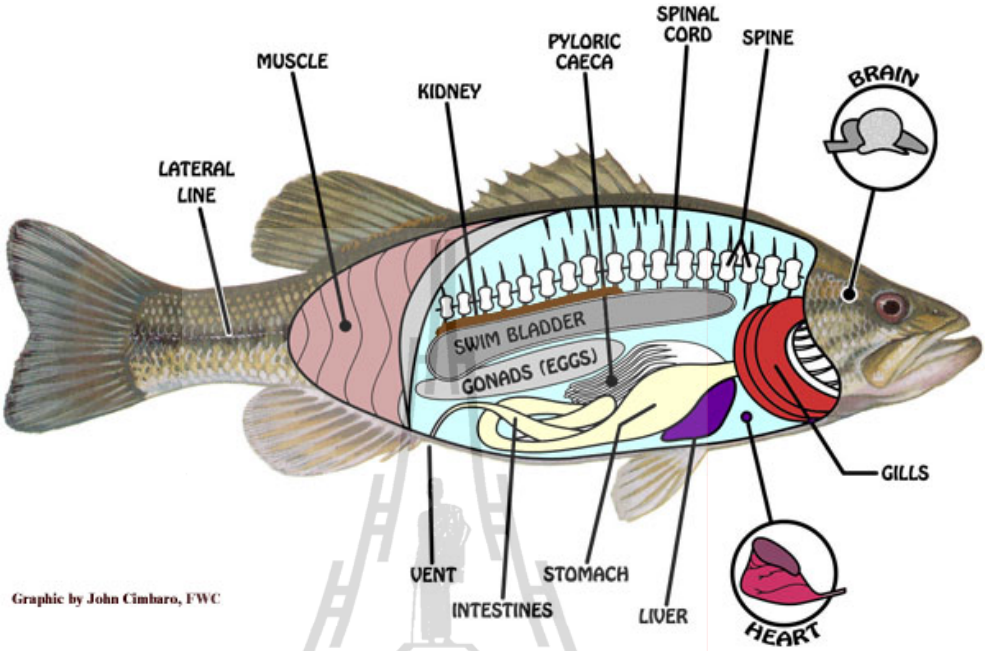

Figure 2.4 Internal anatomy structure of fish.

Source: www. kentuckylake.com/fishing/anatomy.shtml (2011)

Lysozyme is one of the most studied innate responses in fish. Lysozyme can act on the peptidoglycan layer of bacterial cell walls resulting in the lysis of the bacteria. Lysozyme has been found in mucus and ova, and serum lysozyme, probably coming from peritoneal macrophages and blood neutrophils, has been used as an indicator of non-specific immune response. The lysozyme response has been found to be variable in its potency depending on the species and the tissue location. It appears that the lysozyme response in fish may be induced very rapidly and not only related to bacterial presence but also to other alarm situations such as after stress. Thus lysozyme in fish would be involved in the overall alarm response, acting as an acute-phase protein (Tort et al., 2003).

Immunostimulants have been used as feed additives for many years in aquaculture (Dalmo and Bøgwald, 2008). A number of immunostimulants has molecular possessing repeating units of a certain moiety such as fatty acid chains in bacterial lipopolysaccharides (LPS) and certain lipoproteins, and (deoxy)riboses in DNA/RNA, particularly of glucose in β-Glucan. The β-Glucan has been common knowledge in the scientific community that β-glucan is the most known powerful immune stimulant and a very powerful antagonist to both benign and malignant tumors; it lowers cholesterol and triglyceride level, normalizes blood sugar level, heals and rejuvenates the skin and has various other benefits.

2.7.2 β-Glucan sources and structure

The β-Glucans are naturally occurring polysaccharides. These glucose polymers are produced by a variety of plants, such as oat, barley, and seaweed. β-Glucans are the constituents of the cell wall of certain pathogenic bacteria (*Pneumocystis carinii, Cryptococcus neoformans, Aspergillus fumigatus, Histoplasma capsulatum, Candida albicans*) and fungi (*S. cerevisiae*) (Cain et al., 2003; Kim and Yun, 2006; Lee et al., 2001). The main components of the fungal cell wall are polysaccharides and glycoproteins. Yeast β-glucan is a particulate carbohydrate that consists of glucose and mannose and is a major constituent of the cell membrane. Of the major fish diet producers include yeast β-glucan in their fish feed mixture (Dalmo and Bøgwald, 2008). Yeast (*S. cerevisiae)* cell wall consists of three layers: an inner layer of insoluble β-glucan (30-35%), middle layer of soluble β-glucan (20-22%), external layer of glycoprotein (30%). The β-Glucan has been purified from brewers and backer's yeast is the common name for the strains of yeast commonly used as a leavening, agent in baking, bread and bakery products. It is almost always of the

species *S. cerevisiae* which is the same species commonly used in alcoholic fermentation, where it converts the fermentable sugars present in the dough into carbon dioxide and ethanol. There are from oats and barley bran (Wikipedia, www. 2011; Shu et al., 2006; Rodŕiguez et al., 2009). The β-Glucans derived from different sources have some differences in their structure. It is a heterogeneous group of glucose polymers, consisting of a backbone of β-(1,3)-linked β-D-glucopyranosyl units with β-(1,6)-linked side chains of varying length and distribution. Oat and barley β-glucans are primarily linear with large regions of β-(1,4) linkages separating shorter stretches of β-(1,3) structures. Mushrooms β-glucans have short β-(1,6)-linked branches coming off of the β-(1,3) backbone. Yeast β-glucans have β-(1,6) branches that are further elaborated with additional β-(1,3) regions (Fig. 2.5). These structural differences can have large implications for the activity of the β-glucan. For example, differences in the length of the polysaccharide chain, extent of branching, and the length of those branches can result in the difference between material extractable by hot water, as mushroom β-glucans, and insoluble cell wall component, as yeast β-glucan, and in different molecular weight. In general, *in vitro* studies have suggested that large molecular weight or particular β-glucans can directly activate leukocytes, stimulating their phagocytic, cytotoxic, and antimicrobial activities, including the production of reactive oxygen and nitrogen intermediates. Intermediate or low molecular weight β-glucans possess biological activity *in vivo*, but their cellular effects are lee clear. Very short β-glucans are generally considered inactive. Yeast and mushrooms β-glucan are easily purified, there are a lot of experiments performed in Japan, China, and Korea are mostly investigated (Akramienė et al., 2007).

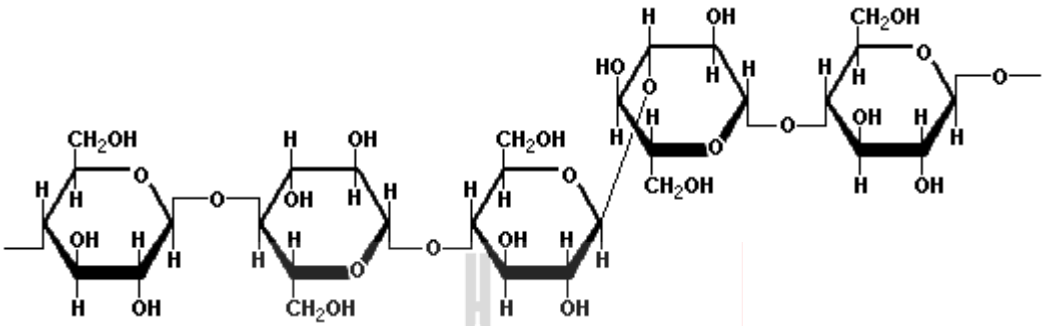

Figure 2.5 Typical 1, 3 and 1, 6 β-glucan structure (MW ≥1000 kDa)

Source: Foodnetworksolution, www 2011

2.7.3 β-Glucan immunostimulating activity in fish

Immunostimulants are natural and synthetic compounds that counteract the immunosuppressive state of fish by promoting the non-specific immune response, antibody production and up-regulation of inflammatory response. Natural immunostimulants are biocompatible, biodegradable and safe for the environment and human health. The use of natural immunostimulants in aquaculture can improve the immune response of fish. Therefore, the health of fish and enhancement of immunity are of primary concern and worthy of more attention. β-glucan are widespread in plants, algae, bacteria, yeast and mushrooms. β-glucan from different sources are different in their structure and immunomodulatory potencies. Oral administration of these compounds could potentiate the general immune response. The use of the whole yeast (*S. cerevisiae*) in fish feeds could be successful as a protein source substituting the expensive fish-meal-protein. The dietary effects of the whole yeast on the immune response of seabream and hybrid tilapia were investigated. Enhancement of specific and non-specific immune response after the administration of β-glucan isolated from yeast was documented inyellowcroaker, Asian catfish, carp and zebrafish. β-glucan

has been found to enhance the resistance against *Aeromonas hydrophila* in Asian catfish, carp and zebrafish. On the other hand, β-glucan has a little prospect in preventing columnaris disease in rainbow trout (El-Boshy et al., 2010; Bagni et al., 2005; Sahoo et al., 2005; Kumari and Sahoo, 2006).

Whittington et al. (2005) found that at the levels of commercial diet supplemented with 0, 50, 100 and 200 mg β-glucan had no effect on the growth performance of immunized and non-immunized juvenile Nile tilapia. Specific antibody responses are not affected by dietary β-glucan. Serum lysozyme activity significantly decreased in fish fed the 200 mg β-glucan diet. Immunization with *Streptococcus iniae* ARS-98-60 vaccine alone resulted in the enhancement of specific antibodies and protection of Nile tilapia against *S. iniae* infection. β-Glucan is not beneficial in improving immune responses and resistance of tilapia against *S. iniae* infection.

Ai et al. (2007) investigate the effects of dietary β-1,3 glucan on the innate immune response and protection against *Vibrio harveyi* infection in large yellow croaker, *Pseudosciaena crocea*. A basal diet is supplemented with 0% (control), 0.09% (low) and 0.18% (high) β-1,3 glucan to formulate three experimental diets. The results of 8 weeks feeding trial showed that low glucan supplementation (0.09%) significantly enhanced fish growth, whereas high supplementation (0.18%) did not. The serum lysozyme activity is significantly increased with the increase of dietary glucan ($P < 0.05$), and fish fed the diet with high glucan had significantly higher lysozyme activity compared with low glucan. There are no significant differences in alternative complement pathway activity between fish fed diets with and without supplementation of glucan. The phagocytosis percentage and respiratory burst activity in fish fed the diet with 0.09% glucan are significantly higher than those in fish fed with

the control diet ($P < 0.05$), but both immunological parameters significantly decreased in fish fed the diet with high supplementation compared with low supplementation and no significant difference is observed between the control and high supplementation groups. The challenge experiment showed that fish fed the diet with low glucan had significantly lower cumulative mortality compared with the control and high glucan groups ($P < 0.05$), but no significant differences observed between the control and high supplementation groups. These results suggested that low glucan could enhance growth and innate immunity of large yellow croaker with an 8 week oral administration, but higher supplementation did not influence growth, or further improve immunity of large yellow croaker.

Bonaldo et al. (2007) found that the European Sea Bass can be immunomodulated with oral administration of β-glucan. Optimal doses and administration times have been established when β-glucans are fed alone.

Jhowang and Nhooma (2009) found that at the levels of the dietary β-glucan with 0, 250, 500 and 1,000 ppm had no effect on the growth performance and significantly feed efficiency ratio ($P>0.05$). The β-glucan of 1,000 ppm has effect on non-specific immune responses increase efficiencies of phagocytes, lysozyme and stimulate function of complement.

From reviewing the literature, yeast fermented sato residual, brewers yeast and GroBiotic®-A have effect immune response on animal. Therefore the replacement fish meal with the level of sato residuals in practical fish diets for juvenile Nile tilapia on growth performance and immunological stimulation. In addition, the supplement with the level of brewers yeast and GroBiotic®-A of 1% and 2% in practical fish diets for juvenile Nile tilapia on growth, immune response and bacterial challenge were used in this study.

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CHAPTER III

THE POTENTIAL FOR RICE WINE RESIDUAL AS AN ALTERNATIVE PROTEIN SOURCE IN PRACTICAL DIET FOR NILE TILAPIA (*OREOCHROMIS NILOTICUS*) AT THE JUVENILE STAGE

3.1 Abstract

This study investigated the utilization level of rice wine residual (RWS) in a diet for juvenile Nile tilapia. The effects of RWS in diets were evaluated for growth (at week 4), fillet composition, hematological and blood chemical parameters (at weeks 3 and 6), and intestinal morphology (at week 6). The dietary treatments (each diet in triplicate groups) consisted of seven isonitrogenous (32% crude protein) servings that were formulated to include RWS at levels of 0 (control diet), 7.5, 15.0, 22.5, 30.0, 37.5, and 45.0 % (Diets 1-7, respectively) and a commercial diet (Diet 8). The growth performances of fish in the groups on Diets 1-4 did not differ significantly. There were no marked variations in fillet composition, serum lysozyme, hematological profiles and blood urea nitrogen (BUN) among treatment diets, except for hematocrit and BUN (week 3) and hemoglobin (week 6) of fish on Diets 6-7. While the blood glucose increased as the amount of RWS incorporation increased (P<0.05), the cholesterol decreased (P<0.05). Only intestinal morphometry of Diets 7

differed significantly from any of the other treatments. Taken together, RWS (at 22.5%) has the potential for use in juvenile Nile tilapia diet without negative effects.

3.2 Introduction

The global aquaculture production of tilapia has drastically increased from 124 thousand mt (metric tons) in 1997 to 2.5 million mt in 2010 (FAO 2010). This trend suggests that there will be even greater increases in the future. Among the cichlid species, it is the Nile tilapia (*Oreochromis niloticus*) that has dominated global tilapia culture. The tilapia market has expanded from a subsistence level to meet the protein needs of the middle class because of the year-round supply, delicious flavor, and reasonable price of that fish. To maintain the tilapia as a global staple protein source during a period of limitations in the world supply of energy, a reduction in the production costs of tilapia is necessary. This presents a challenge to research. A great deal of consideration is generally given to reducing feed costs. Although the Nile tilapia is an omnivorous grazer, protein-rich feed generally has been used for intensive culture systems, especially in the juvenile stage. In tropical areas where most commercial tilapia culture is undertaken, the intensive production (in floating cages) of Nile tilapia takes 4 months from the juvenile stage (35-40 g) to reach marketable sizes (800 g) (S. Plymee, Suranaree University of Technology Farm, Nakhon Ratchasima, Thailand personal communication). During the production process, the culture of juvenile fish needs feed of a high quality to raise healthy fish with a high growth rate. Traditionally, fish meal (FM) has provided a major part of the protein source of formulated feeds because of its suitable protein quality. Since the recent scarcity and uncertain consistency of supply of FM, its replacement by

alternative protein sources that are of high quality, but less expensive, has been investigated. The limitations on the world's food supply provide additional motivation (Nayor et al. 2000; New and Wijkström, 2002). Therefore, numerous studies have been undertaken to examine the effects of replacing FM by another source of protein such as plant-based protein or animal by-products in diets that can be fed to tilapia (Martinez-Palacios et al. 1988; Richter, Siddhuraju and Becker, 2003; Cavalheiro et al., 2007; Nguyen and Davis, 2009).

Rice wine is a traditional alcoholic beverage that is widely produced in most Asian countries, including China (choujiu), Korea (cheong), Japan (sake), India (santi), and Thailand (sato). Although, the name of the rice wine and the rice material differ by country, the fermentation processes are similar. Unlike wine, which is generally made by the fermentation of fruit sugar, rice wine undergoes a process of multiple, parallel fermentation. Rice starch is first converted to sugar by the amylolytic process of fungi. Simultaneously, sugar is converted to alcohol by the fermentation of yeast cells. After separating the liquid, the rice wine residual (RWS) is disposed of as waste. In fact, the RWS contains many nutritious substances derived from both rice and microorganisms, which can be especially utilized as a protein source. Recently, many attempts have been made to use RWS for animal feed (see review Sugiura et al., 2009). Several factors restrict the recycling of food waste for animal feed. For example, variations in the amount, quality and nutritional values may prevent the food waste from being used as a raw material in animal feeds. Because of the development of fermentation technology in Asia, rice wine-brewing industries have been qualitatively improved. Consequently, the food waste of rice wine

production has become available on a regular basis and is constant in its nutritious value (Agrifood consulting International, 2005; Asia BioBusiness, 2006).

This study seeks to evaluate the potential for utilizing the RWS as an alternative protein source for Nile tilapia at the juvenile stage. The effects on growth performance and fillet chemical composition have been investigated. Additionally, in determine whether there are negative effects on fish that are fed with the RWS diet, we evaluated hematological values, serum lysozyme activity and some blood chemistry parameters. Further, the intestinal morphology was also investigated.

3.3 Materials and Methods

3.3.1 Proximate composition and amino acid composition of the rice wine residual

The RWS was obtained from the Samrithmankong factory in Nakhon Ratchasima, Thailand. The proximate analyses of RWS were performed according to the standard methods of AOAC (1990) for dry matter, protein, total lipid, fiber and ash. Moisture content was determined by drying samples in an oven at 105^{o}C until constant weight was reached. Protein was determined by Kjeldahl Method. Lipid content of samples was determined by petroleum ether extraction using a Soxtec System (2050 Soxtec^{TM}; Auto Fat Extraction System, Foss Tecator, Höganäs, Sweden). Fiber content of sample was determined by using a Systems Fibertec (2010 Fibertec ^{TM}; Auto Fiber Analysis System, Foss Tecator, Höganäs, Sweden). Samples used for ash were incinerated in a muffle furnace at 600^{o}C 3 h for measurement of ash contents. Proximate composition of experimental diets was determined in triplicate

using the same procedures. The amino acid composition was determined by AOAC (2000) using Gas Chromatography/Mass Spectrophotometry.

3.3.2 Feed formulation and pellet preparation

All test ingredients, except RWS, were obtained from animal feedstuff companies. Before formulating the feed, all feed ingredients were analyzed to determine the percentages of moisture, protein, lipid and ash according to AOAC (1990). Seven diets were formulated to incorporate 0, 7.5, 15.0, 22.5, 30.0, 37.5 or 45.0 % (designated as Diets 1-7, respectively) (Table 3.1). Corn meal and cassava chips were incorporated so that all diets would contain up to 25.0 % flour, which is needed to produce floating pellets. In addition, all diets were formulated to have a protein-to-energy ratio of not < 18 mg kJ^{-1}, which is required for the normal growth of juvenile Nile tilapia (Kaushik et al., 1995). All experimental diets were produced using a grinder, mixer, and extruder (Paktongchai Pasusat, Nakhon Ratchasima, Thailand). The dry ingredients were ground by a hammer grinder and mixed by a ribbon screw mixer (22 rpm). The floating experimental diets were produced using a single screw extruder (an extruding temperature of 120-160ºC). In addition, as a control for growth performance, a floating commercial diet was used as Diet 8. All diets were analyzed to determine their proximate composition and stored at room temperature until they were used.

Table 3.1 Ingredients composition (%) of eight experimental diets

Ingredient (%)	Diet							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**Commercial**
Rice wine residual	0	7.5	15.0	22.5	30.0	37.5	45.0	-
Fish meal	30.0	25.0	20.0	15.0	10.0	5.0	0	-
Soybean meal	27.0	27.0	28.5	30.0	30.0	31.0	32.0	-
Rice bran	15.0	15.0	14.5	14.0	14.0	15.0	12.0	-
Corn meal	14.5	14.0	10.0	9.0	6.0	4.5	4.5	-
Cassava chips	12.0	10.0	10.3	7.5	8.0	5.0	4.0	-
Premix[1]	1.5	1.5	1.5	1.5	1.5	1.5	1.5	-
Soybean oil	0	0	0.2	0.5	0.5	0.5	1.0	-

[1]Vitamin and trace mineral mix provided the following (IU kg^{-1} or g kg^{-1} diet): biotin, 0.25 g; folic acid, 0.003 g; inositol, 0.25 mg; niacin, 0.0215 g; pantothenic acid, 0.03 g; vitamin A, 5,000 IU; vitamin B1, 0.0025 g; vitamin B2, 0.0012 g; vitamin B6, 0.0075 g; vitamin B12 0.00005 mg; vitamin C, 1 g; vitamin D3, 1,000 IU**;** vitamin E, 100 IU; vitamin K, 0.008 g; copper, 0.02 g; iron, 0.2 g; selenium, 0.3 mg; zinc, 0.32 g.

3.3.3 Experimental fish, design, fish culture and fish performance evaluation

Several generations of Nile tilapia, *O. niloticus*, were reared at the Suranaree University of Technology Farm (SUT Farm; Nakhon Ratchasima, Thailand). As male Nile tilapia grows approximately twice as fast as female, most commercial tilapia has been grown from sex - reversed tilapia fry using 17α-methyltestosterone (17-MT) (FAO 2010). The experimental Nile tilapia that were used in this study were male Nile tilapia that had been produced by feeding the swim-up fry with the 50 mg kg^{-1} 17- MT-treated feed for 4 weeks and then with a diet that consisted of 35.0 %[1] crude protein until the experiment began.

The experimental design was completely randomized with eight treatment diets, each of which was replicated three times with 55 fish to test the validity of the conclusions. Twenty - four hapas (2 x 2.5 x 2 m^3) were maintained in the SUT Farm reservoir. Prior to the start of the experiment, fish (38-43 g) were randomly distributed in the experimental hapas. The fish were fed Diet 1 for adaptation to experimental environment for 2 week. During the experiment, the fish were hand-fed twice daily for 6 weeks. Diets were fed ad libitum. Any dead fish were recorded and removed daily. The growth performance and feed utilization were evaluated at the end of week 4.

3.3.4 Fish sampling and Blood collection

To determine whether the RWS diet caused any detrimental effect on health status, hematological and blood chemical parameters were evaluated. At the end of weeks 3 and 6, the fish were not fed for 24 h prior to blood sampling. Four representative fish from each diet replication were selected and anaesthetized with 2-phenoxyethanol (0.35 mL L^{-1}). Blood sample was collected by a hypodermic syringe from the caudal vein. The collected blood sample was divided into three sets. One set was added to the tube that contained K_2EDTA as an anticoagulant. The second blood set was added to the tube that contained sodium fluoride (NaF) as an anticoagulant. The third blood set was left to clot at 4° C for at least 3 h and centrifuged at 1980 x g for 5 min at room temperature. The serum collected was stored at -80 ºC for further analysis. After bleeding, the fillet was cut and frozen for proximate analysis according to AOAC (1990) with slight modification such as sample weight and equipments. In addition, the liver was dissected and the hepatosomatic index was determined.

3.3.5 Blood analysis

3.3.5.1 Hematological assays

Immediately after blood sampling, the K_2EDTA-blood was used to examine hematological indices. The haematocrit values (Ht) were measured in duplicate by placing fresh blood in glass capillary tubes and centrifuging for 5 min in a microcentrifuge. The hemoglobin (Hb) content was determined by the use of an Advia®60 hematology system (Bayer Healthcare, Tarrytown, NY, USA). The red blood cells (RBCs) were counted in duplicate for each sample under a light microscope using a Neubauer haemocytometer after dilution with phosphate-buffered saline (Rodak et al., 2007).

3.3.5.2 Blood chemistry analysis

The blood chemistry, including glucose, blood urea nitrogen (BUN) and cholesterol, was analyzed using the Bayer Express Plus Clinical Chemistry Analyzer (Bayer). The blood glucose was assayed with the supernatant obtained from the NaF-blood. The BUN and cholesterol were examined using the plasma that was obtained from the K_2EDTA-blood.

3.3.5.3 Lysozyme activity assay

Lysozyme activity in the serum was determined by turbidimetric assay in which the activity of lysozyme is determined from a standard curve that indicates the level of lysis of Gram-positive bacterium *Micrococcus lysodeikticus* by known concentrations of a lysozyme standard, as described in Kumari and Sahoo (2006), with slight modification. Briefly, the standard used was hen egg white lysozyme (Sigma, St Louis, MO, USA). Twenty–five microliters of standard hen lysozyme that ranged from 0 $\mu g\ mL^{-1}$ to 14 $\mu g\ mL^{-1}$ (in 0.1 M phosphate citrate buffer, pH 5.8) or serum samples were

placed into a 96-well plate in duplicate. One hundred and seventy-five microliters of a *M. lysodeikticus* suspension (75 mg mL^{-1} prepared in the same buffer) were then added to each well. After rapid mixing, the decrease in absorbance at 450 nm was recorded at 15-min intervals. Lysozyme activities were converted to lysozyme concentration using hen egg white lysozyme as the standard.

3.3.6 Histological analysis

To determine whether the RWS diets had any detrimental effects on digestive tissue, intestinal morphometry was undertaken. At the end of the experimental period (6 weeks), tree fish per treatment was used. The fish were anesthetized, and then portions of duodenal and jejunum intestine were removed and preserved in 10% phosphate-buffered formalin with a pH of 7.2. After dehydration, the tissue was embedded in a paraffin box, cut into slices of 5 μm thickness and mounted on glass slides. After deparaffination, the slides were stained by Haematoxylin and Eosin (H&E). The height and epithelial thickness of the villus were measured on the stained sections under a microscope and an ocular micrometer. The five, longest, intact villi in each intestinal position were selected for measurement in duplicate cross-sections for each sample. In addition, the globet cells along the selected intact villi were counted.

3.3.7 Water quality analysis

Water samples were collected weekly from each hapa at a depth of 15 cm for 8 weeks (2 weeks of acclimation period and 6 weeks of experimental period). The water quality analysis, including dissolved oxygen, turbidity, suspended solids, total dissolved solids, chemical oxygen demand and biochemical oxygen demand, was measured according to the methods described in APHA, AWWA and WEF (1995).

3.3.8 Data analysis

The statistical model utilized was $y_{ij} = \mu + \tau_I + \varepsilon_{ij}$, where y_{ij} was the response, μ was the general means, τ_I was diet effects, and ε_{ij} was the random error. Regression analysis and goodness of fit (R^2) were determined. Since the independent variables for isonitrogenous and isoenergetic values in the experimental diets were assumed to be fixed, regression analyses of the response (Y) and level of RWS incorporation parameters (x) were conducted. In addition, all data were analyzed by one-way analysis of variance (ANOVA) using SPSS for Windows (Release 10) (SPSS Inc., Chicago, IL, USA). When significant differences were found among the groups, Tukey's procedure was used to rank the groups. Throughout the experiment, effects and differences were declared to be significant when their values were less than 0.05 ($P < 0.05$).

3.4 Results

The proximate and amino acid composition of RWS are presented in Table 3.2 The RWS contained a high level of essential amino acids that were required in the Nile tilapia. The proximate composition of fish diets are presented in Table 3.3.

The growth performances of all Nile tilapia that were fed experimental feeds were determined at the end of 4 weeks (Table 3.4). The Nile tilapia that were fed diets containing RWS up to 22.5 % (Diet 2-4) showed a similar growth response to those that were fed Diet 1. Significant non-linear relationship was observed between RWS and weight gain (Fig. 3.1). In this study, the feed conversion ratio (FCR) and the protein efficiency ratio (PER) appeared to be similar in all treatment diets.

Additionally, the condition factor (K) of Nile tilapia did not vary significantly among the groups. Through the end of the experimental period (6 weeks), the survival rate was high in all treatment diets and ranged from 97% to 99 %.

Table 3.2 Chemical composition and amino acid profile of rice wine residual (RWS) (% on dry matter basis)

Components	**% in dry matter**	**Amino acids**	**% in dry matter**
Crude protein	38.12	Alanine	1.52
Ether extract	5.67	Arginine	0.41
(lipid)	6.42	Aspartic acid	1.58
Crude fiber	1.36	Cystine	1.20
Ash		Glutamic acid	4.77
		Glycine	0.83
		Histidine	1.32
		Hydroxylysine	0.005
		Hydroxyproline	0.005
		Isoleucine	3.38
		Leucine	6.85
		Lysine	3.25
		Methionine	1.20
		Phenylalanine	8.20
		Proline	1.81
		Serine	0.53
		Threonine	0.44
		Tryptophan	0.55
		Tyrosine	6.16
		Valine	2.31

Table 3.3 The proximate composition (%) of eight experimental diets

Ingredient (%)	Diet							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**Commercial**
Rice wine residual	0	7.5	15.0	22.5	30.0	37.5	45.0	-
Fish meal	30.0	25.0	20.0	15.0	10.0	5.0	0	-
Soybean meal	27.0	27.0	28.5	30.0	30.0	31.0	32.0	-
Rice bran	15.0	15.0	14.5	14.0	14.0	15.0	12.0	-
Corn meal	14.5	14.0	10.0	9.0	6.0	4.5	4.5	-
Cassava chips	12.0	10.0	10.3	7.5	8.0	5.0	4.0	-
Premix[1]	1.5	1.5	1.5	1.5	1.5	1.5	1.5	-
Soybean oil	0	0	0.2	0.5	0.5	0.5	1.0	-
Proximate composition (% dry weight)								
Dry matter	90.0	90.0	90.0	90.0	90.0	90.0	90.0	90.0
Crude protein	32.1	32.0	32.1	32.4	32.1	32.3	32.2	32.2
Crude lipid	6.1	6.0	6.1	6.1	6.1	6.1	6.0	4.7
Crude ash	11.0	10.8	10.2	8.8	7.1	6.5	5.5	7.8
Crude fiber	3.5	3.3	3.1	4.3	5.0	5.6	5.8	5.8
Cholesterol	23.6	17.9	16.3	15.6	15.5	7.6	6.9	ND[2]
Reducing sugar	1.05	1.22	1.36	1.36	1.36	2.10	2.16	ND[2]
Glucose	0.24	0.23	0.20	0.20	0.2	0.17	0.15	ND[2]
Gross energy ($MJ\ kg^{-1}$)	17.2	17.3	17.3	17.7	17.5	17.7	17.8	22.8
Protein: energy ratio ($g\ MJ^{-1}$)	18.7	18.5	18.6	18.3	18.3	18.2	18.1	14.1

[1] Table 3.1

[2] not determined.

Table 3.4 Growth performance of Nile tilapia fed experimental diets for 4 weeks (mean ± SD, n = 3)[1]

Diet	Initial Body Weight (g)	Final Body Weight (g)	Weight Gain[2] (%)	SGR[3] (% day^{-1})	FCR[4]	PER[5]	K[6]
1	40.0 ± 2.0	117.2 ± 6.4^{a}	193.4 ± 18.6^{a}	3.84 ± 0.23^{a}	0.83 ± 0.07	3.78 ± 0.30	1.94 ± 0.06
2	40.0 ± 0.5	113.5 ± 1.9^{a}	191.2 ± 7.3^{a}	3.82 ± 0.09^{a}	0.89 ± 0.09	3.55 ± 0.37	1.98 ± 0.09
3	39.5 ± 1.5	114.0 ± 1.7^{a}	188.6 ± 7.2^{a}	3.78 ± 0.09^{a}	0.89 ± 0.02	3.52 ± 0.06	1.94 ± 0.04
4	39.9 ± 1.6	115.0 ± 1.0^{a}	188.4 ± 10.7^{a}	3.78 ± 0.13^{a}	0.85 ± 0.06	3.67 ± 0.27	1.93 ± 0.04
5	40.1 ± 0.6	108.6 ± 1.6^{ab}	170.6 ± 1.1^{ab}	3.55 ± 0.01^{ab}	1.03 ± 0.08	3.04 ± 0.22	1.95 ± 0.02
6	40.6 ± 1.8	90.9 ± 5.2^{c}	124.1 ± 7.2^{c}	2.88 ± 0.12^{cd}	1.00 ± 0.04	3.14 ± 0.14	1.95 ± 0.08
7	38.6 ± 1.7	84.2 ± 3.5^{c}	118.7 ± 18.4^{c}	2.79 ± 0.30^{d}	0.93 ± 0.18	3.43 ± 0.61	1.96 ± 0.04
8	40.9 ± 2.0	102.8 ± 0.4^{b}	151.9 ± 12.2^{bc}	3.30 ± 0.17^{bc}	1.01 ± 0.09	3.13 ± 0.29	1.99 ± 0.04

[1] Means with different superscript in each column differed significantly from each other ($P<0.05$).

[2]Weight gain (%) = 100 x (final mean body weight – initial mean body weight) x initial mean body weight^{-1}.

[3]Specific growth rate (SGR) = 100 x [(*ln* final body weight - *ln* initial body weight) x experimental days^{-1}].

[4]Feed conversion ratio (FCR) = dry feed fed x wet weight gain^{-1}.

[5]Protein efficiency ratio (PER) = wet weight gain x total protein intake g^{-1}.

[6]Condition factor (K) = 100 x body weight (g) x L (cm)$^{-3}$.

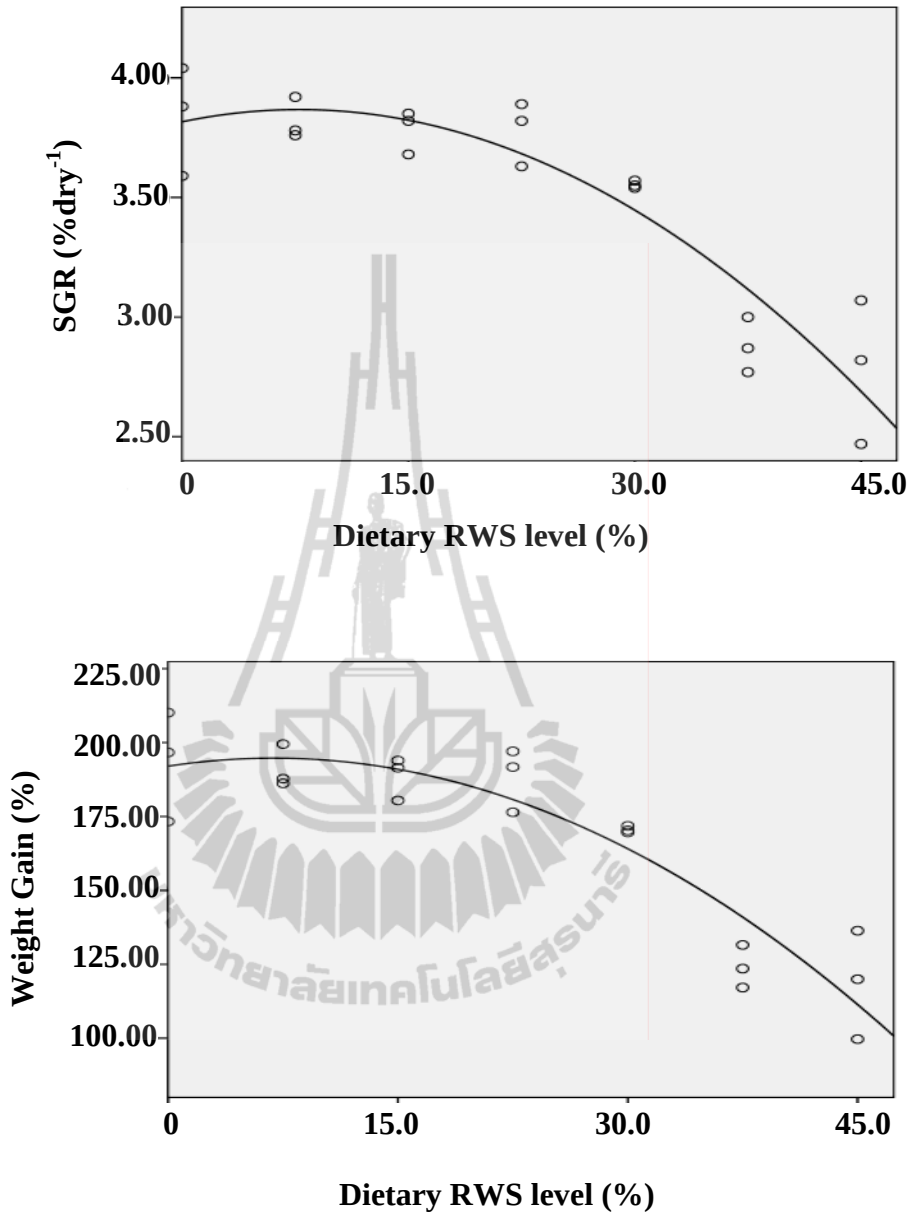

Figure 3.1 Regression plot of growth response and rice wine residual. Specific growth rate (SGR) (a) and weight gain (b) as a function of incorporation level of RWS. The non-linear relationship was observed between RWS and SGR ($y = -8.494E\text{-}6x^2 + 0.001x + 3.816$, $R^2 = 0.845$) or weight gain ($y = 6.863E\text{-}7x^3 - 0.001x^2 + 0.156x + 190.282$, $R^2 = 0.846$). These plots provide illustration of the maximum growth that is predicted. For Nile tilapia fed RWS.

At the ends of weeks 3 and 6, the hepatosomatic index and proximately chemical composition of fillets were determined (Table 3.5). The results showed that the hepatosomatic index of Nile tilapia did not vary significantly among the groups. No significant difference was observed in the fillet chemical compositions by experimental group.

The hematological parameters of Nile tilapia that were fed experimental diets appear in Table 3.6. The red blood cell number (RBC) did not vary significantly by treatment diet. The hematocrit of fish on Diets 6-7 was significantly lower than that of fish on Diet 1 (at week 3). By the end of week 6, hematocrit did not differ by treatment diet. At week 3, hemoglobin tended to decrease as the incorporation level of RWS increased, although they were not significantly different. Significantly lower hemoglobin contents were observed in Nile tilapia that were fed Diets 6-7 (at week 6) (P<0.05). A significant linear relationship (y = -0.02x + 101.75, R^2 = 0.608) was observed between RWS and hemoglobin content (Appendix A).

To evaluate the effect of RWS on humoral non-specific immunity, the serum lysozyme activity was determined. The lysozyme activity of the Nile tilapia appears to be similar, regardless of which experimental diet they were fed (Table 3.7). Several blood chemical aspects, such as BUN, glucose and cholesterol, were assessed. At week 3, increasing the RWS in the diet significantly lowered the BUN (P<0.05, Table 3.7). However, by the end of week 6, the BUN of fish that were fed all diets appeared to be similar. Moreover, the blood glucose rose with an increase in RWS in tested diets, and a significant increment was found at the second analyzed time (P < 0.05, Table 3.7). A significant non-linear relationship (y = −2.310E-5x^2 + 0.02x + 6.022, R^2 = 0.909) was observed between RWS and blood glucose (Appendix A).

Figure 3.2 shows that blood cholesterol decreases with an increase in RWS content in experimental diets ($P < 0.05$). In addition, the quadratic relationships were evaluated between RWS and blood cholesterol. The blood cholesterol of Nile tilapia with commercial diet (Diet 8) was also lower than that of fish that was fed Diet 1 ($P < 0.05$).

Table 3.5 Proximate composition of fillet (%) and hepatosomatic index of Nile tilapia fed on experimental diets (mean ± SD, n = 3)[1]

Diet	3 weeks					6 weeks				
	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	HSI[2]	Moisture (%)	Protein (%)	Fat (%)	Ash (%)	HSI[2]
1	77.47 ± 4.2	19.54 ± 0.6	1.47 ± 0.4	1.31 ± 0.7	1.81 ± 0.33	77.12 ± 11.8	20.31 ± 1.1	1.06 ± 0.2	1.46 ± 0.2	1.78±0.33
2	77.20 ± 3.4	19.99 ± 0.9	1.38 ± 0.3	1.32 ± 0.2	1.75 ± 0.10	76.88 ± 1.5	20.59 ± 1.3	1.05 ± 0.2	1.42 ± 0.2	1.76±0.20
3	76.39 ± 7.9	20.27 ± 0.7	1.78 ± 0.6	1.30 ± 0.3	1.79 ± 0.13	77.47 ± 4.1	19.61 ± 0.6	1.39 ± 0.4	1.41 ± 0.2	1.64±0.19
4	77.27 ± 12.1	19.79 ± 0. 6	1.48 ± 0.4	1.28 ± 0.3	1.91 ± 0.17	77.10 ± 7.5	19.87 ± 0.2	1.07 ± 0.2	1.39 ± 0.2	1.81±0.06
5	76.53 ± 18.9	19.56 ± 0.8	1.49 ± 0.4	1.27 ± 0.6	1.57 ± 0.14	76.65 ± 9.7	21.02 ± 0.4	1.00 ± 0.1	1.40 ± 0.2	1.85±0.08
6	76.96 ± 4.4	20.33 ± 0.8	1.43 ± 0.3	1.21 ± 0.6	1.69 ± 0.29	77.17 ± 15.6	20.25 ± 0.8	0.93 ± 0.1	1.37 ± 0.2	1.73±0.06
7	77.24 ± 17.4	202.3 ± 0.7	1.10 ± 0.4	1.23 ± 0.4	1.33 ± 0.42	77.09 ± 17.9	20.42 ± 0.4	1.09 ± 0.1	1.46 ± 0.1	1.74±0.06
8	76.81 ± 18.7	203.8 ± 1.5	1.54 ± 0.4	1.24 ± 0.5	1.85 ± 0.20	76.39 ± 11.0	20.81 ± 0.2	1.31 ± 0.8	1.49 ± 0.1	1.93±0.13

[1]No significant differences (P>0.05) were observed among treatment means.

[2]HSI, Hepatosomatic index.

Table 3.6 Hematology values of Nile tilapia fed experimental diets (mean ± SD, n = 3)[1]

Diet	3 weeks			6 weeks		
	RBC[2] (10^6 cells μL^{-1})	Hemoglobin (g dL^{-1})	Hematocrit (%)	RBC[2] (10^6 cells μL^{-1})	Hemoglobin (g dL^{-1})	Hematocrit (%)
1	3.04 ± 0.43	10.66 ± 0.49	40.36 ± 2.75^{a}	3.38 ± 0.31	10.26 ± 0.28^{a}	38.13 ± 1.23
2	3.09 ± 0.54	10.36 ± 0.30	40.70 ± 2.00^{a}	3.43 ± 0.15	9.68 ± 0.16^{ab}	37.50 ± 1.38
3	2.96 ± 0.43	9.73 ± 0.39	36.77 ± 1.28^{ab}	3.37 ± 0.24	9.76 ± 0.42^{ab}	38.40 ± 1.11
4	3.11 ± 0.63	9.99 ± 0.41	39.23 ± 1.82^{ab}	3.28 ± 0.50	9.34 ± 0.58^{ab}	34.27 ± 3.42
5	2.94 ± 0.46	9.48 ± 0.62	34.71 ± 1.36^{ab}	3.16 ± 0.50	9.54 ± 0.40^{ab}	36.52 ± 2.04
6	2.95 ± 0.26	9.63 ± 0.27	35.46 ± 1.88^{b}	3.16 ± 0.46	9.15 ± 0.34^{b}	34.23 ± 1.25
7	2.96 ± 0.64	9.62 ± 0.20	34.98 ± 2.45^{b}	3.23 ± 0.56	9.18 ± 0.26^{b}	33.52 ± 1.31
8	3.11 ± 0.41	9.70 ± 0.83	35.62 ± 0.78^{ab}	3.14 ± 0.53	9.53 ± 0.05^{ab}	35.52 ± 2.21

[1]Means with different superscript in each column differed significantly from each other (P<0.05).

[2]RBC, red blood cells number.

Table 3.7 Lysozyme activity, glucose and BUN of Nile tilapia that were fed experimental diets (mean ± SD, n = 3)[1]

Diet	Lysozyme activity (μg mL^{-1})		Glucose (mg dL^{-1})		BUN[1] (mg dL^{-1})	
	3 weeks	6 weeks	3 weeks	6 weeks	3 weeks	6 weeks
1	15.28 ± 2.71	14.15 ± 2.45	109.75 ± 18.42	119.92 ± 26.56^{b}	5.33 ± 1.59^{a}	1.78 ± 1.03
2	16.51 ± 2.27	14.47 ± 1.86	127.42 ± 20.64	158.58 ± 45.50^{ab}	4.27 ± 0.62^{ab}	2.42 ± 0.81
3	13.34 ± 1.78	14.12 ± 2.52	145.58 ± 15.04	176.58 ± 17.90^{ab}	4.19 ± 0.63^{ab}	2.38 ± 0.58
4	14.86 ± 1.82	13.49 ± 2.40	138.92 ± 15.69	168.08 ± 11.88^{ab}	4.63 ± 0.05^{ab}	2.58 ± 0.28
5	14.09 ± 1.22	12.69 ± 4.09	149.42 ± 16.61	171.83 ± 4.94^{ab}	3.58 ± 0.13^{ab}	2.13 ± 0.38
6	13.86 ± 3.79	14.26 ± 2.37	143.25 ± 3.54	190.58 ± 3.79^{a}	3.19 ± 0.20^{b}	2.49 ± 0.52
7	12.82 ± 1.74	13.96 ± 1.33	154.58 ± 17.79	184.33 ± 9.27^{ab}	2.96 ± 0.20^{b}	2.14 ± 0.30
8	15.42 ± 1.37	16.10 ± 0.87	150.83 ± 18.65	176.58 ± 28.44^{ab}	3.07 ± 0.40^{b}	1.98 ± 0.72

BUN, blood urea nitrogen.

[1]Means with different superscript in each column differed significantly from each other (P<0.05) (means ± SD)

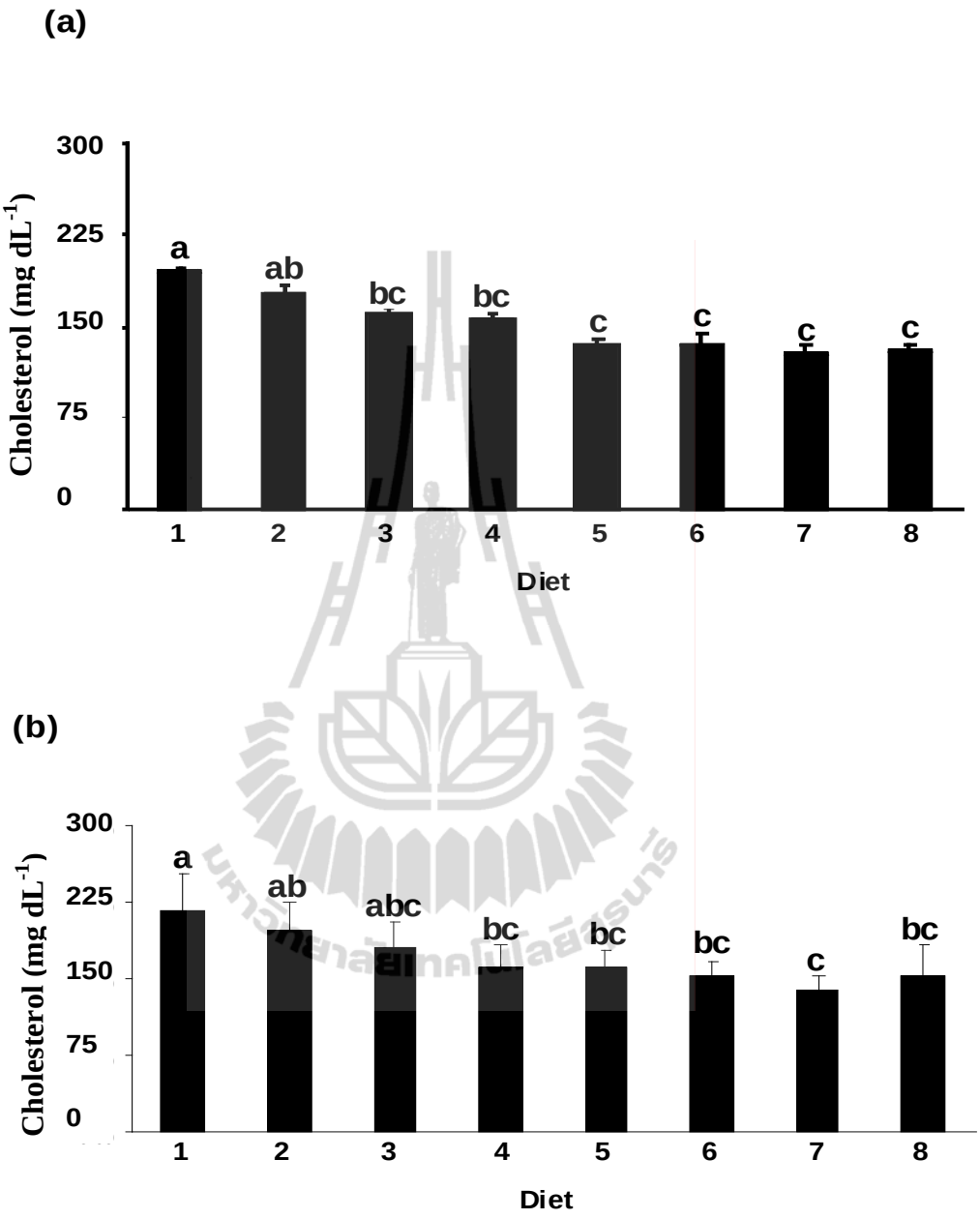

Figure 3.2 Cholesterol level in blood of Nile tilapia with different levels of rice wine residual diets at 3 weeks (a) and 6 weeks (b). Different superscript letters in the bar graph indicate significantly different $P<0.05$. The quadratic relationship was predicted between RWS and cholesterol level at week 3 ($y = 5.680E\text{-}6x^2 - 0.007x + 5.19$, $R^2 = 0.665$) and week 6 ($y = 6.763E\text{-}6x^2 - 0.008x + 6.221$, $R^2 = 0.879$).

To investigate the effect of RWS on the morphology of digestive tracts, we analyzed the intestinal histomorphometry of fish in the various experimental groups (Table 3.8, Fig. 3.3 and Fig. 3.4). The lowest villus height of duodenum was observed in fish that were fed Diet 7, although no significant difference was detected (P>0.05). The duodenal epithelium thickness appeared to be similar in all treatment diets. The number of goblet cells in the duodenum part declined significantly in fish that were fed Diet7 (P < 0.05). A significant non-linear relationship ($y = -9.218E\text{-}7x^3 - 0.052x + 39.679$, $R^2 = 0.763$) was observed between RWS and goblet cell number. In the jejunum part, the lowest significant villus height was found in fish that were fed Diet 7 (P < 0.05). A significant non-linear relationship ($y = -1.292E\text{-}5x^3 + 0.007x^2 - 1.039x + 521.821$, $R^2 = 0.904$) was found between RWS and villus height. Moreover, although there was no significant difference, the lowest epithelium thickness was detected in fish that were fed Diet 7. The globet cell number was the lowest in fish that were fed Diet 7 (P<0.05). Again, A significant non-linear relationship ($y = -1.679E\text{-}6x^3 + 0.001x^2 - 0.175x + 42.964$, $R^2 = 0.821$) was determined between RWS and goblet cell number (Appendix A).

The water quality analysis in the hapas, the conductivity and pH were measured using a conductivity meter and a pH meter, respectively. During the experimental period, the water quality parameters were within the acceptable ranges, i.e., pH of 8.0-8.6, suspended solids of 7-24 mg L^{-1}, Total dissolved solids of 135-218 mg L^{-1}, dissolved oxygen of 5.3-9.1 mg L^{-1}, conductivity of 173-222 µs cm^{-1}, chemical oxygen demand of 31-41 mg L^{-1}, biological oxygen demand of 4.2-10.2 mg L^{-1}, turbidity of 10.05-14.15 Nephelometric Turbidity Units, and total plate count of $2.15 \times 10^2 – 9.25 \times 10^2$ CFU mL^{-1}.

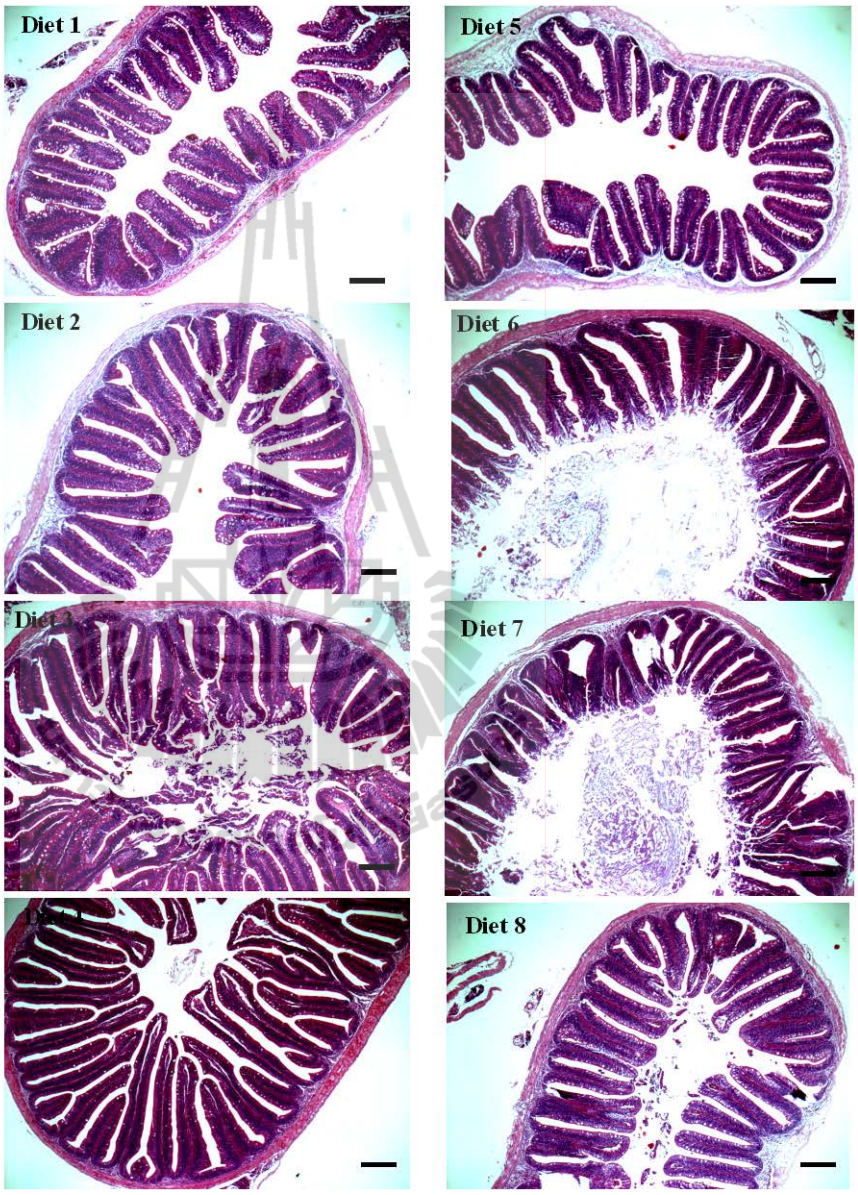

Figure 3.3 Representative hematoxylin-eosin-stained section of duodenum with different levels of RWS in practical fish diets for juvenile sex reversal Nile tilapia. The bar represents 100 µm.

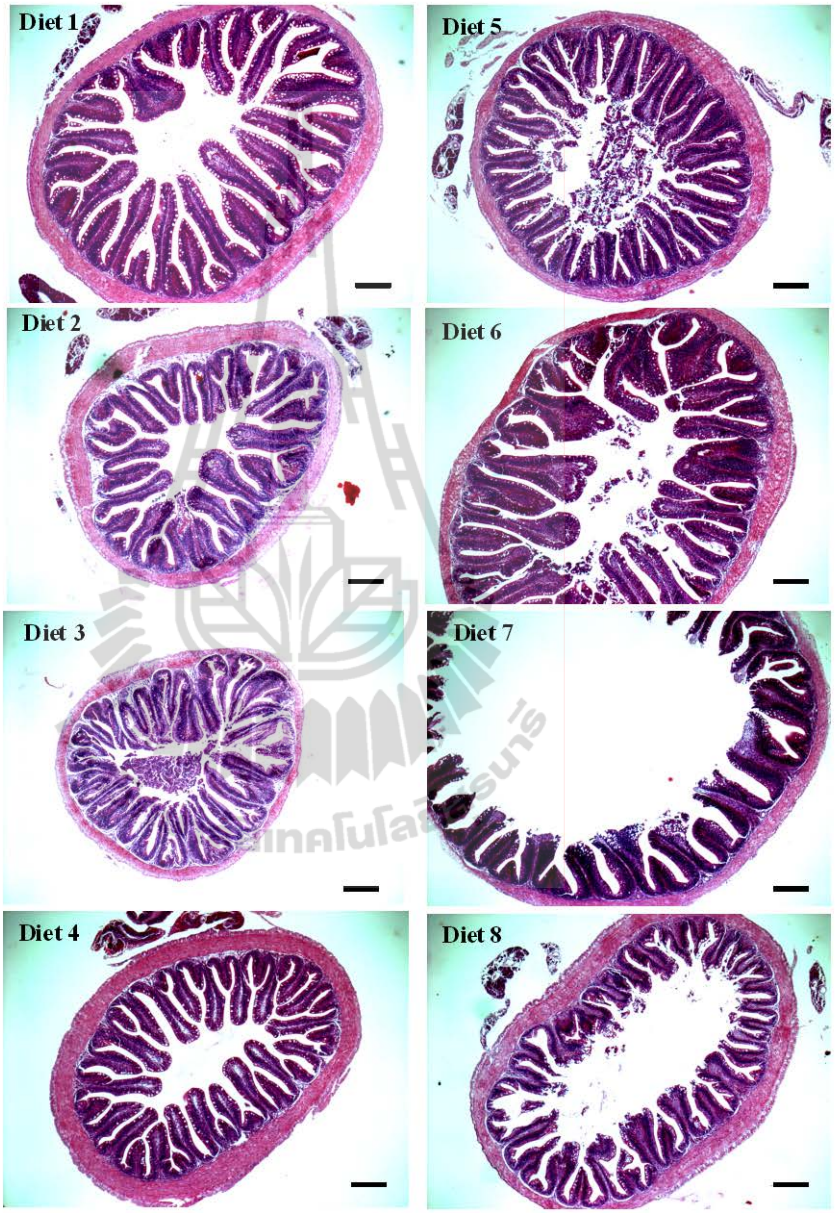

Figure 3.4 Representative hematoxylin-eosin-stained section of jejunum with different levels of RWS in practical fish diets for juvenile sex reversal Nile tilapia. The bar represents 100 µm.

Table 3.8 Villi height, epithelium thickness and No. goblet cells in part of the intestine (Duodenum and Jejunum) of Nile tilapia fed experimental diets for 6 weeks (mean ± SD, n = 3)[1]

Diet	Duodenum			Jejunum		
	villi height (µm)	epithelium thickness (µm)	No. goblet cells	villi height (µm)	epithelium thickness (µm)	No. goblet cells
1	612.5 ± 68.6	49.7 ± 3.8	40.5 ± 9.1^{a}	511.3 ± 31.5^{a}	31.7 ± 3.5	43.0 ± 7.1^{a}
2	726.6 ± 32.6	58.3 ± 1.5	35.5 ± 3.5^{ab}	508.5 ± 5.7^{a}	30.7 ± 3.5	35.5 ± 0.7^{a}
3	764.0 ± 4.2	48.0 ± 4.6	40.0 ± 5.7^{a}	441.8 ± 27.9^{a}	30.3 ± 2.5	31.5 ± 6.4^{ab}
4	890.3 ± 29.3	47.7 ± 6.7	40.0 ± 5.7^{a}	482.3 ± 43.5^{a}	35.0 ± 7.0	38.5 ± 5.0^{a}
5	651.8 ± 97.2	56.0 ± 5.2	38.0 ± 1.4^{ab}	456.8 ± 51.3^{a}	34.3 ± 6.8	36.5 ± 3.5^{a}
6	652.3 ± 55.9	56.7 ± 5.0	31.5 ± 2.1^{ab}	396.0 ± 44.6^{a}	32.3 ± 2.3	30.5 ± 2.1^{ab}
7	508.3 ± 66.8	54.3 ± 4.0	20.5 ± 0.7^{b}	217.5 ± 7.1^{b}	22.3 ± 2.5	17.5 ± 2.1^{b}
8	650.0 ± 12.0	49.7 ± 5.5	38.5 ± 2.1^{ab}	391.7 ± 27.6^{a}	31.7 ± 3.2	26.5 ± 2.1^{ab}

[1]Mean with different superscript in each column differed significantly from each other (P<0.05).

3.5 Discussion

Rice wine residual, a by-product of rice wine production, is high in nutritional value. Previously, RWS had been considered to be an industrial waste that required special treatment. Fish production is an industry that mainly uses FM as the protein source for feed. Based on its high protein composition and richness in essential amino acids required for fish growth, RWS would have potential for use as a protein source and might be able to partially replace FM in fish diets. In the present study, we investigated a maximum level of RWS for use as the protein source in the diet for juvenile Nile tilapia. The production of Nile tilapia in tropical areas generally requires 4 months to grow juvenile fish to reach a harvestable size. During the process, diet formulation changes are implemented to increase productivity and reduce cost (http://www.leepattana.com/web/eng/product_list.php). A high quality of diet is normally required during the 4-6 week period for growing juvenile fish (35-40 to 100 g). The experimental diets that were prepared as floating pellets have been used primarily for industrial fish farming. We also evaluated several haematological factors and blood biochemistry, as well as a histological analysis of intestines to determine whether RWS had any detrimental effects when used as feed stuff. Therefore, we provide valuable information on the effect of incorporating RWS in fish feed on the growth performance, fillet composition and health status of fish.

The results of the present study showed that an RWS inclusion level of up to 22.5 % had no effects on growth response as represented by weight gain and SGR compared to Diet 1. A regression analysis provided a model for feed manufacturers to use to estimate the growth response of Nile tilapia. Our findings demonstrated the maximum growth performance that is predicted for Nile tilapia at the juvenile stage

fed RWS (on an isonitrogenous and isocaloric basis). Neither FCR nor PER was affected by feeding different inclusion levels of RWS. A similar feed efficiency, but poorer growth responses, in experimental feed containing high RWS can be explained by the less palatability of the diets when the feed contains a high level of RWS. It was reported that different starch and protein sources affected the physical quality of extruded fish diets (Øverland et al. 2009; Sørensen et al., 2010). It should be noted that other factors, including differences in amino acids and fatty acids among experimental diets, would affect the growth performance, because feed ingredients, such as soybean meal, rice bran, cassava chips and corn meal, varied slightly in order that all diets can be provided in the form of floating pellets, isonitrogenous, and isocaloric. When compared with the use of fermentation by-products, such as Distiller's dried grains with soluble (DDGS) in the diet for Nile tilapia, the inclusion level of RWS here was similar. Lim et al. (2007) reported that up to 20.0 % DDGS could be incorporated in the Nile tilapia diet without adverse effects on growth performance when using DDGS as a replacement for a mixture of soybean meal and canola meal. Several authors have reported that FM can be replaced entirely by animal by-products (El-Sayed 1998; Cavalheiro et al., 2007; Hernández et al., 2010). Although RWS is inferior to animal by-products for use as a replacement for FM, it is similar to plant protein sources. The partial replacement of dietary FM with plant-based protein has been reported in a range of 10.0-45.0 % (Richter et al., 2003; Soltan et al., 2008).

The proximate composition of fillets did not differ among treatment diets. Similar results were found in the body composition when FM was replaced by up to 20.0 % DDGS (Lim et al., 2007). However, the replacement of FM by a plant-based protein (cowpea protein concentrate and plant protein mixture) or animal protein source

(shrimp industry waste, shrimp meal, blood meal, meat and bone meal, and poultry by-product) that exhibited a change in body composition was recorded in Nile tilapia (Olvera-Novoa et al., 1997; El-Sayed 1998; Cavalheiro et al., 2007; Soltan et al., 2008). For example, while the substitution of FM by cowpea protein concentrate increased the whole-body protein content, FM substitution with the others did not. Accumulation of whole-body lipid was observed in fish fed diet with FM replaced by cowpea protein concentrate, plant protein mixture and poultry by-product; however, it was not influenced by the replacement of FM by the others.

Hematological and blood chemical parameters could be used as indices to reflect the nutritional status of the fish. The hematocrit (week3) and hemoglobin (week 6) declined significantly in fish that were fed diets that contained a high incorporation level of RWS in comparison with the experimental diet 1 without RWS. However, all hematological values are in the ranges that were reported by Bittencourt et al. (2003). A possible reason for the reduction of haemoglobin may be related to the absorption of dietary iron. Therefore, additional research on the supplementation of dietary iron is necessary to improve the inclusion level of RWS in fish feed.

The BUN has been reported to be the blood parameter to indicate quality and quantity of dietary protein (Eggum, 1970). The BUN decreased significantly with increasing RWS incorporation level to 37.5 % (only week 3). By the end of week 6, the plasma glucose in fish increased as the RWS content increased. Several factors affected the glucose levels in fish. These include stress, environmental factors and diet composition (Chen et al., 2003). In addition, gluconeogenesis, which is generated by an excess of dietary protein level, affected the increment of blood glucose. In the present study, although dietary glucose did not vary among experimental diets, the

total reducing sugar increased as the amount of RWS incorporation increased. Consequently, the higher level of plasma glucose might have been influenced by some sugar and polysaccharide content in RWS. A regression model of glucose provided a model with which to predict the effect of RWS on the glucose level in the blood. The blood cholesterol was reduced with increasing RWS in experimental diets. Several factors have been reported to affect the level of blood cholesterol, including cholesterol metabolism and diet consumed (Tocher et al., 2008). The reduction of FM content in diet might directly affect the decrease in cholesterol (Chen et al., 2003). Our present study suggested that decrease in dietary cholesterol as a result of the reduction in FM content in experimental diets affected the blood cholesterol in tested fish. In this regard, it can be noted that hypocholesterolemic effects were reported in previous studies that involved the replacement of FM by plant-based protein diet. Because plant-based protein contain anti-nutritional compounds with actions of non-starch polysaccharides and non-starch polysaccharides in diets attributed a trend of reduced digestibility of fat and protein to the possible effect of increased viscosity of intestinal contents on diffusion and mixing of digestive enzymes (Borgeson et al., 2006; Soltan et al., 2008; Lim and Lee, 2009).

It has been shown that supplementation of *Saccharomyces cerevisiae* and its components has variable effects as an immunostimulant, depending on *S. cerevisiae*'s component, dosage, stage and condition of rearing (Whittington et al., 2005; Abdel-Tawwab et al., 2008; Shelby et al., 2009). RWS is high not only in the protein source obtained from *S. cerevisiae,* but also in the *S. cerevisiae* cellular component. Therefore, we also hypothesized that the inclusion of RWS in feed would have the

effect of stimulating the immune system in Nile tilapia. We considered the lysozyme activity in an effort to interpret the effects of RWS on the humoral, non-specific immune response. The results obtained in this study showed that a graded inclusion of RWS had no effect on lysozyme activity. Our results are in agreement with the effects of the supplementation of yeast component, including β-glucan and oligosaccharide on the lysozyme level of Nile tilapia (Whittington et al., 2005; Shelby et al., 2009). However, El-Boshy et al. (2010) reported that dietary *S. cerevisiae* or β-glucan significantly enhanced lysozyme activity in Nile tilapia. In fact, the immune system of fish involves both an innate and an acquired immunity system. Thus, further investigation is needed to determine whether RWS influences non-specific humoral, as well as cell-mediated immunity, and to clearly understand its effect on fish health.

Several factors, such as nutritional components, stress and disease, affects intestinal morphology. Intestinal morphology affects the physiological and metabolism of nutrient absorption. The replacement of FM by plant protein was shown to induce enteritis in salmon and Atlantic cod (Uran et al., 2008; Olsen et al., 2007). In this study, to investigate whether RWS affected the intestinal integrity, we undertook intestinal morphometry in the jejunum and duodenum parts. The gut surface area determined by gross morphological features, such as the villus height and epithelium thickness, influences ingestion and absorption and, consequently, affects the net utilization of dietary nutrients. An increase in villus height and reduction in epithelium thickness facilitate the absorption process. Globet cells, which are distributed along the villi, play an important role in synthesizing and secreting mucin into the mucus layer to destroy pathogen (Blomberg et al., 1993). The results obtained in the present study demonstrated that a high incorporation level of RWS (>

30.0 %) tended to have negative effects on the intestinal morphology, although the most significant effect was observed in the jejunum part. In addition, a regression equation generated the predicted model of the effects of RWS on intestinal morphology. These negative effects by RWS on the intestinal morphometry were related to growth performance traits.

3.6 Conclusion

The present study demonstrated that RWS can be incorporated in a practical diet and partially replace FM (on isonitrogenous and isocaloric basis) without any significant, adverse effects on the growth performance, carcass composition and health status. Our findings provide the first evidence of the potential benefit of utilizing the by-product from the rice wine industry as an alternative protein source for aquafeeds. This useful information will be providing for agricultural countries that are located mainly in Asia where their socio-economies particularly depend on aquaculture and rice production. Moreover, the each parameter result in term of science and commercial with RWS found that RWS had the potential better than commercial diet when used RWS at the levels 22.5%.

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CHAPTER IV

GROWTH PERFORMANCE AND RESISTANCE TO *STREPTOCOCCUS INIAE* OF JUVENILE NILE TILAPIA (*OREOCHROMIS NILOTICUS*) FED DIETS SUPPLEMENTED WITH GROBIOTIC®-A AND BREWTECH® DRIED BREWERS YEAST

4.1 Abstract

This study was conducted to evaluate the effect of feeding diets supplemented with two commercial products of brewers yeast: a prebiotic GB (GroBiotic®-A), a mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products; and BY (Brewtech®), partially autolyzed brewers yeast. A basal diet which was formulated to contain 32% crude protein and 6% lipid (control) was supplemented with 1% and 2% of BY or GB. Each diet was fed to Nile tilapia in quadruplicate aquaria for 12 weeks. Weigh gain, feed intake, survival and whole body proximate composition of fish were not significantly affected by dietary treatments. Serum total protein, total immunoglobulin, and lysozyme activity were unaffected by dietary treatments. However, serum hemolytic complement activity (SH50) of fish fed with 1% BY was significantly higher than those of fish fed with the control diet (P<0.05). In order to evaluate the effect of dietary BY or GB on resistance of fish to *Streptococcus iniae*, fish from each treatment diet was injected with *S. iniae*. The

result showed that agglutinating antibody titer to *S. iniae* was unaffected by dietary treatments. Furthermore, cumulative mortality at 20 days post-challenge with *S. iniae* of fish fed with 1% and 2% of BY or GB was lower than that of fish fed with control diet. However, there was not significant difference (P>0.05).

4.2 Introduction

Tilapia, because of their fast growth, enormous adaptability to a wide range of physical and environmental conditions, ability to reproduce in captivity, resistance to handling and disease, good flesh quality, feed on a low trophic level and excellent growth rate on a wide variety of natural and artificial diets, are the most successfully cultured species worldwide (Lim and Webster, 2006). They are presently cultured in virtually all types of production systems, in both fresh and salt water, and in tropical, subtropical and temperate climates. They are increasingly recognized as the species of choice for intensive aquaculture and are likely to become the most important cultured fish in the world (Fitzsimmons, 2006). According to the American Tilapia Association, global farm-raised tilapia production is expected to reach 3 million mt by 2010, compared to 2.6 million mt in 2007.

However, a major problem associated with intensive fish culture is the increased susceptibility of fish to infectious diseases, including streptococcal disease in tilapia caused by *Streptococcus iniae*. The problem of streptococcal disease is worldwide (Muzsquiz et al., 1999) and the annual loss to the aquaculture industry is estimated to be over $100 million (Shoemaker and Klesius, 2001). Commercially, antibiotics have been supplemented in aqua feeds for treatment and prevention of bacterial disease of

aquatic animals (Li and Gatlin, 2005). The use of antibiotics can lead to the emergence of antibiotic-resistant bacteria, and contamination in food products and the environment (FAO, 2002). The use of antibiotics in animal production has been banned in EU countries and is increasingly under public scrutiny and criticism in most other countries. Consequently, a wide variety of products ranging from polysaccharides, plant extracts and some nutrients have been added in fish diets as immunostimulants to stimulate immune system function, and/or their resistance to infectious diseases or serve as adjuvant to improve vaccine efficacy (Sakai, 1999; Gannam and Schrock, 2001).

Brewtech® dried brewers yeast (BY), *Saccharomyces cerevisiae*, is a natural product of the brewing industry containing various immunostimulating compounds such as β-glucans, nucleic acids and mannans. It has been shown to positively influence non-specific immune responses as well as growth of various fish species and thus may serve as an excellent health promoter for fish culture (Li and Gatlin, 2003; 2004; 2005; Waszkiewicz-Robak and Karwowska, 2004). GroBiotic®-A (GB), a commercial prebiotic consisting of a mixture of partially autolyzed brewers yeast, dairy ingredient components and dried fermentation products, has been shown to enhance the growth performance, feed efficiency and survival of hybrid striped bass to *S. iniae* and *Mycobacterium marinum* (Li and Gatlin, 2004; 2005).

Thus, this study was conducted to evaluate the effects of feeding diets supplemented with BY and prebiotic GB on growth performance, proximate body composition, immune response and resistance of juvenile Nile tilapia (*Oreochromis niloticus*) to *S. iniae* challenge.

4.3 Materials and methods

4.3.1 Experimental fish and husbandry

Juvenile Nile tilapia spawned and reared at our laboratory on commercial fry and fingerling diets were acclimated to laboratory conditions and fed the basal experimental diet without BY or GB supplementation for 2 weeks. At the end of the acclimation period, fish with an average weight of 13.35 ± 0.11 g (mean ± S.E.M.) were randomly selected and stocked in 20, 57-L glass aquaria at a density of 30 fish aquarium^{-1}. The aquaria were supplied with flow-through dechlorinated city water at an initial rate of about 0.6 L min^{-1} and increased gradually to about 1.0 L/min by the 6th week of the trial. Water flow rates were checked and adjusted twice daily to ensure proper water exchange. Water temperature was maintained constant (26 ± 1^{o}C) by a centralized water heater. The water was continuously aerated with air stones, and the photoperiod was maintained on a 12:12-h light: dark schedule. Dissolved oxygen and temperature in three randomly chosen aquaria were measured once every other day using an YSI model 58 Oxygen Meter (Yellow Spring Instrument, Yellow Spring, OH). During the trial, water temperature and dissolved oxygen averaged 25.27 ± 0.10 °C and 5.34 ± 0.06 mg L^{-1}, respectively.

4.3.2 Feed and feeding

A practical basal diet was formulated to contain approximately 32% crude protein, 6.0% crude lipid and 2,900 kcal of digestible energy (DE) kg^{-1} based on feedstuff values reported in NRC (1993). Brewtech® dried brewers yeast (BY) and GroBiotic®-A (GB)[1] provided by International Ingredient Corporation, St. Louis, MO

[1] Use of trade or manufacturer's name does not imply endorsement.

was added to the basal diets at levels of 0, 1 and 2% (Table 4.1). The levels of soybean meal, corn oil and celufil (non-nutritive filler) were adjusted to maintain equal levels of dietary protein and lipid. All diets were supplemented with vitamins and minerals in amounts to meet the known requirements of tilapia (Lim and Webster, 2006). Dry ingredients were thoroughly mixed for 10 min in a Hobart mixer (Hobart Corporation, Troy, Ohio) before the oil was added. After the oil was dispersed, approximately 280 mL of deionized water kg^{-1} of diet was added. The moist mixture was extruded through a 3-mm diameter die in a Hobart meat grinder. The resulting moist pellets were air-dried at room temperature (24^{o}C) to a moisture content of about 10%. Pellets were ground into small pieces, sieved to obtain appropriate sizes, and stored frozen in plastic bags at -20^{o}C until fed (Peres et al., 2003).

Fish in four randomly assigned aquaria were fed one of the five experimental diets twice daily (between 07.30-08.30 and 15.00-16.00 h) to apparent satiation for 12 weeks. The amount of diet consumed was recorded daily by calculating the differences in weight of diets prior to the first and after the last feeding. Once a week, aquaria were scrubbed and accumulated waste was siphoned. On cleaning days, fish were fed only in the afternoon. Feed was not offered on sampling days.

Table 4.1 Ingredients composition (%) of five experimental diets

Ingredient	Percent (%) in diets				
	1	**2**	**3**	**4**	**5**
Menhaden fish meal	8.00	8.00	8.00	8.00	8.00
Soybean meal	45.00	44.10	43.30	44.30	43.60
Corn meal	23.50	23.50	23.50	23.50	23.50
Wheat middling	13.60	13.60	13.60	13.60	13.60
Corn Oil	3.50	3.45	3.40	3.45	3.40
Carboxymethyl cellulose	3.00	3.00	3.00	3.00	3.00
Dicalcium phosphate	1.00	1.00	1.00	1.00	1.00
Vitamin premix[1]	0.50	0.50	0.50	0.50	0.50
Mineral premix[2]	0.50	0.50	0.50	0.50	0.50
Brewers yeast	0.00	1.00	2.00	0.00	0.00
GroBiotic®-A	0.00	0.00	0.00	1.00	2.00
Celufil	1.40	1.35	1.20	1.15	0.90
Ethoxyquin	(0.02% or 200 mg/kg diet)				
Total	100.00	100.00	100.00	100.00	100.00
DE (kcal/kg diet)[3]	2,900	2,900	2,900	2,900	2,900

[1]Vitamin premix, diluted in cellulose, provided by the following vitamins (mg/kg diet): vitamin A (retinyl acetate), 4,000 IU; vitamin D_3 (cholecalciferol), 2,000 IU; vitamin K (menadione sodium bisulfide), 10; vitamin E (α-tocopheryl acetate), 50; thiamin hydrochloride, 10; riboflavin, 12; pyridoxine hydrochloride, 10; D-calcium pantothenate, 32; nicotinic acid, 80; folic acid, 2; vitamin B_{12}, 0.01; biotin, 0.2; choline chloride, 400; vitamin C (as L-ascorbyl-2-polyphosphate, 45% vitamin C activity), 60.

[2]Trace mineral premix provided by the following minerals (mg/kg diet): zinc (as $ZnSO_4.7H_2O$), 150; iron (as $FeSO_4.7H_2O$), 40; manganese (as $MnSO_4.7H_2O$), 25; copper (as $CuCl_2$), 3; iodine (as KI), 5; cobalt (as $CoCl_2.6H_2O$), 0.05; selenium (as Na_2SeO_3), 0.09.

[3]DE (Digestible energy) was calculated based on feedstuff values reported in NRC (1993).

4.3.3 Growth measurements

Fish in each aquarium were removed, anesthetized with 150 mg L^{-1} tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Redmond, WA, USA), counted and group weighed every 3 weeks, following 16 h of feed deprivation. When fish were removed for weighing, aquaria were cleaned thoroughly, and three-fourths of the water drained. Weight measurements and fish counts were used for estimation of weight gain, feed efficiency ratio (FER; wet weight gain dry feed $intake^{-1}$), and survival.

4.3.4 Proximate composition of experimental fish and diets

At the end of the feeding trial, four fish from each aquarium that had been bled for immunological assays were pooled, stored in plastic bags and kept at -20^{o}C for subsequent determination of whole body proximate composition. After descaling, fish from each aquarium were finely ground in a Hobart meat grinder and analyzed in duplicate for proximate composition following the standard methods (AOAC, 1990). Moisture content was determined by drying samples in an oven at 105^{o}C until constant weight was reached. Samples used for dry matter were digested with concentrated nitric acid and incinerated in a muffle furnace at 600^{o}C overnight for measurement of ash contents. Protein was determined by combustion method using a FP-2000 Nitrogen Analyzer (Leco Corporation, St. Joseph, MI.). Lipid content of samples was determined by petroleum ether extraction using a Soxtec System (2055 Soxtec Avanti; Foss Tecator, Höganäs, Sweden). Proximate composition of experimental diets was determined in triplicate using the same procedures.

4.3.5 Immunological assays

At the end of the growth trial, four fish per aquaria were randomly chosen and anesthetized with MS-222 as previously described. Blood samples were collected from the caudal vasculature using non-heparinized tuberculin syringes and allowed to clot at 4^{o}C overnight. Serum samples were collected following centrifugation at 1,000 × g for 10 min and stored at -80°C for subsequent assays for serum protein, total immunoglobulin, lysozyme activity and spontaneous hemolytic complement activity (SH50).

Serum protein concentration was determined using the modified Biuret method. Total protein reagent (Sigma, Chemical Co., St. Louis, MO, USA) was added to each well of the microtiter plate at 250 μL well^{-1}. Then, 5 μL of serum was added to each well. After 30 min incubation at room temperature, the absorbance of the samples was read at 570 nm. Serum total protein concentrations were calculated using bovine serum albumin as an external standard.

Serum total immunoglobulin was determined following the method of Siwicki and Anderson (1993). The assay was based on the measurement of total protein content in serum prior and post precipitating the immunoglobulin molecules using 12% solution of polyethylene glycol. The difference in protein content was considered as total immunoglobulin content.

Serum lysozyme activity was determined by the method of Litwack (1955) as modified by Sankaran and Gurnani (1972). The assay is based on lysis of lysozyme sensitive gram-positive bacterium *Micrococcus lysodeikticus* (Sigma Chemical Co., St. Louis, MO) by the lysozyme present in the serum. Freeze-dried *M. lysodeikticus* suspension (0.25 mg mL^{-1}) was prepared immediately before use by dissolving in

sodium phosphate buffer (0.04 M Na_2HPO_4, pH 6.0). Serum (15 μL $well^{-1}$ in duplicate) from each of the four fish per tank was placed in a microtiter plate and 250 μL of bacterial cell suspension was added to each well. Hen egg white lysozyme was used as an external standard. The initial and final (after 30 min incubation at 37°C) absorbances of the samples were measured at 450 nm. The rate of reduction in absorbance of samples was converted to lysozyme concentration (μg mL^{-1}) using the standard curve.

Spontaneous hemolytic complement activity (SH50) was determined using the method reported by Sunyer and Tort (1995) and modified for using in microtiter plates as described in Lim et al., (2009). Briefly, sheep red blood cells (SRBC) in Alsever's solution (Remel, Inc., Lanexa, KS) were added to tilapia serum that had been serially diluted in cold phosphate buffered saline (PBS) solution (0.85% PBS, 0.1% gelatin, 0.15mM $CaCl_2$, and 0.5mM $MgCl_2$) in a round bottom microtiter plate. The plates were incubated at room temperature for 1 h with occasional shaking. After incubation, plates were centrifuged at 800 × g (2,000 rpm) for 10 min at 4°C, and the supernatant pipetted into a new microplate (flat-bottom microtiter). Hemolysis was evaluated spectrophotometrically at 415 nm and converted to percent hemolysis based on distilled water controls. The 50% lysis point (SH50) was calculated by linear regression of each serum sample and expressed as the log dilution.

4.3.6 Disease challenge

S. iniae (ARS 98-60) was obtained from Aquatic Animal Health Research Laboratory, USDA-ARS, Auburn, AL, USA, was isolated from hybrid striped bass (*Morone chrysops* × *Morone saxatilis*) with natural streptococcal disease and reisolated from experimentally infected Nile tilapia, was used to challenge tilapia by

intraperitoneal (IP) injection. The isolate was identified as *S. iniae* by the methodology described by Shoemaker and Klesius (1997). Frozen stock-culture of *S. iniae* was grown in tryptic soy broth (TSB; Difco Laboratories, Sparks, MD) for 24 h at 28°C. The concentration of the culture was adjusted to an optical density of 1.0 at 540 nm, using a spectrophotometer, to give an estimated *S. iniae* concentration of 1×10^9 colony-forming units (CFU) mL^{-1}. After sampling for immunological assays and measurement of proximate body composition, the number of tilapia remaining in the original aquaria was adjusted to twenty, and all fish in each aquarium were challenged by IP injection with 100 μL of *S. iniae* culture containing 1×10^5 CFU mL^{-1} (10^4 CFU $fish^{-1}$). After injection, the fish were returned to their respective aquaria. Each group of fish continued to be fed twice daily with the same experimental diet that was assigned in the growth trial. Fish were monitored for mortality and dead fish removed and recorded twice daily for 20 days following injection.

4.3.7 Agglutination antibody titer

At the end of the challenge trial (day 21), blood samples were collected from the caudal vasculature of four surviving fish and sera collected following centrifugation and stored at −80 °C. Agglutinating antibody titer against *S. iniae* in pre- and post-challenge sera was determined by modifying the method of Chen and Light (1994) as described in Yildirim-Aksoy et al. (2007). Formalin-killed *S. iniae* cells were adjusted to an optical density of 0.8 at 540 nm and added to plasma serially diluted in PBS in a 96-round-bottomed microtiter plate and mixed. Positive plasma from a *S. iniae* infected fish and negative (PBS) were used as assay controls. The plates were covered with plastic film and incubated at room temperature for 16 h. The agglutination end point was established as the last serum dilution where cell

agglutination was visible after incubation as compared to the positive control. Agglutination titers were reported as $\log_{10}$ of the reciprocal of this serum dilution. Baseline fish, sampled prior to disease challenge, were negative for *S. iniae*.

4.3.8 Data analysis

Data were analyzed by one-way analysis of variance (ANOVA). Duncan's multiple range tests were used to determine differences between treatment means. Differences were considered significant at the $P < 0.05$. All analysis was performed using the SAS program version 9 (Statistic Analysis Systems, SAS Institute, Inc., Cary, NY, 2001).

4.4 Results

4.4.1 Proximate composition of the experimental diets

The proximate composition of BY and GB supplement in five experimental diets that are presented in Table 4.2.

4.4.2 Growth performance

Mean final weight gain, feed intake, feed efficiency ratio (FER) and survival after 12 weeks of feeding with diets containing various levels of BY and prebiotic GB are given in Table 4.3. Weight gain, feed intake and survival were not significantly affected by dietary treatment. However, fish fed the diet supplemented with 1% GB had numerically lower weight gain and significantly lower FER than the group fed other diets. There were no significant differences among FER values of fish in other treatments.

Table 4.2 Proximate composition (%) of five experimental diets

Ingredient	Percent (%) in diets				
	1	**2**	**3**	**4**	**5**
Menhaden fish meal	8.00	8.00	8.00	8.00	8.00
Soybean meal	45.00	44.10	43.30	44.30	43.60
Corn meal	23.50	23.50	23.50	23.50	23.50
Wheat middling	13.60	13.60	13.60	13.60	13.60
Corn Oil	3.50	3.45	3.40	3.45	3.40
Carboxymethyl cellulose	3.00	3.00	3.00	3.00	3.00
Dicalcium phosphate	1.00	1.00	1.00	1.00	1.00
Vitamin premix[1]	0.50	0.50	0.50	0.50	0.50
Mineral premix[1]	0.50	0.50	0.50	0.50	0.50
Brewers yeast	0.00	1.00	2.00	0.00	0.00
GroBiotic®-A	0.00	0.00	0.00	1.00	2.00
Celufil	1.40	1.35	1.20	1.15	0.90
Total	100.00	100.00	100.00	100.00	100.00
Ethoxyquin	(0.02% or 200 mg/kg diet)				
Determined nutrition content (% as is)					
Dry matter	90.51	90.89	90.70	89.37	89.13
Protein	32.32	332.5	332.23	32.42	33.73
Fat	6.04	6.17	6.09	6.36	6.31
Ash	6.95	6.76	6.80	6.66	6.75

[1] Table 4.1

Table 4.3 Mean final weight gain, dry matter feed intake, feed efficiency ratio (FER) and survival of Nile tilapia fed diets containing various levels of dried brewers yeast and GroBiotic®-A for 12 weeks

Treatment	Weight gain[1] (g)	Feed intake/fish[1] (DM basis) (g)	FER[2]	Survival (%)
Control	85.28	113.86	0.75[a]	95.85
1% Brewers yeast	85.02	112.66	0.76[a]	92.50
2% Brewers yeast	84.88	115.41	0.74[a]	97.50
1% GroBiotic®-A	77.31	119.15	0.66[b]	91.68
2% GroBiotic®-A	85.39	115.97	0.74[a]	91.68
Pooled SEM	2.47	3.60	0.022	4.08

[1]Values are means of four replicates per treatment. Means in the same column with different superscripts are significantly different at $P<0.05$.

[2] FER = weight gain (g) dry feed fed $(g)^{-1}$.

4.4.3 Whole body proximate composition

Whole proximate body composition (moisture, protein, lipid, and ash) did not differ among treatments (Table 4.4). However, fish fed diets supplemented with dried BY or GB tended to accumulate more body lipid the fish fed than that control diet.

Table 4.4 Whole body proximate composition of Nile tilapia fed diets containing different levels of brewers yeast and GroBiotic®-A for 12 weeks

Treatment	Percent (%) wet weight basis			
	Moisture[1]	Protein[1]	Lipid[1]	Ash[1]
Control	73.40	15.02	6.77	3.53
1% Brewers yeast	73.57	15.21	7.17	3.30
2% Brewers yeast	72.68	15.06	7.06	3.68
1% GroBiotic®-A	73.02	14.93	7.36	3.51
2% GroBiotic®-A	72.96	14.91	7.26	3.55
Pooled SEM	0.46	0.15	0.25	0.15

[1]Values are means of two determinations of pooled samples of four fish per tank and four tanks per treatment. No significant differences were observed among treatment means at $P<0.05$.

4.4.4 Immune response

Serum protein, total immunoglobulin and lysozyme activity were unaffected by dietary treatments (Table 4.5). Serum spontaneous hemolytic complement activity (SH50) of fish fed the 1% BY diet was significantly higher than those of the groups fed the control diet and diets supplemented with 2% BY or GB. There were no significant differences among SH50 values of fish fed fish fed the control diet and diets supplemented with 2% BY and 1 or 2% GB. Serum antibody titers against *S. iniae* at 21 days post-challenge were not significantly affected by dietary treatments, although the values were numerically higher in fish fed 2% BY and 1% GB diets.

4.4.5 Bacterial challenge

The mean number of days at which the first mortality occurred after *S. iniae* challenge and cumulative mortality at day 20 post-challenge with *S. iniae* was not significantly affected by dietary treatments (Table 4.6 and Fig. 4.1). The non-significant differences among these data were due to large variations among replicate values leading to large experimental errors such as time for culturing, concentration of bacterial challenge and density of fish per aquria. Numerically, however, fish fed diets supplemented with 1% BY and 2% BY or GB had substantially lower cumulative mortality (50%) than that of the control treatment (66.25%).

Table 4.5 Mean number of days to first mortality and cumulative mortality of Nile tilapia at 20 days post-challenge with *Streptococcus iniae*

Treatment	Days to fist mortality[1]	Cumulative mortality[1] (%)
Control	2.25	66.25
1% Brewers yeast	2.25	50.00
2% Brewers yeast	2.00	50.00
1% GroBiotic®-A	2.75	58.80
2% GroBiotic®-A	2.00	50.00
Pooled SEM	0.27	7.40

[1]Values are means of four replicates per treatment. No differences were observed among treatment means at $P<0.05$

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Table 4.6 Mean serum protein, total immunoglobulin, lysozyme, spontaneous hemolytic complement (SH50) and agglutinating antibody (Ab) titer to *S. iniae* in Nile tilapia fed diets containing different levels of brewers yeast and GroBiotic®-A for 12 weeks

Treatment	Serum protein (mg mL^{-1})	Total immunoglobulin (mg mL^{-1})	Lysozyme (µg mL^{-1})	SH50[1] (units mL^{-1})	Ab titer[2] (log_{10})
Control	40.34	2.66	11.69	67.11[b]	1.26
1% Brewers yeast	39.42	2.20	12.70	130.19[a]	1.06
2% Brewers yeast	38.60	2.38	11.45	62.74[b]	1.43
1% GroBiotic®-A	39.79	2.01	9.36	89.46[ab]	1.46
2% GroBiotic®-A	39.30	2.20	10.51	78.99[b]	0.75
Pooled SEM	0.91	0.56	2.28	12.50	0.43

[1]Values are means of two determinations per fish (except Ab titer), four fish per tank and four tanks per treatment. Means in the same column with different superscripts are significantly different at $P<0.05$.

[2] Values are means of one determination per fish, four fish per tank and four tanks per treatment, measured at 21 days post-injection challenge.

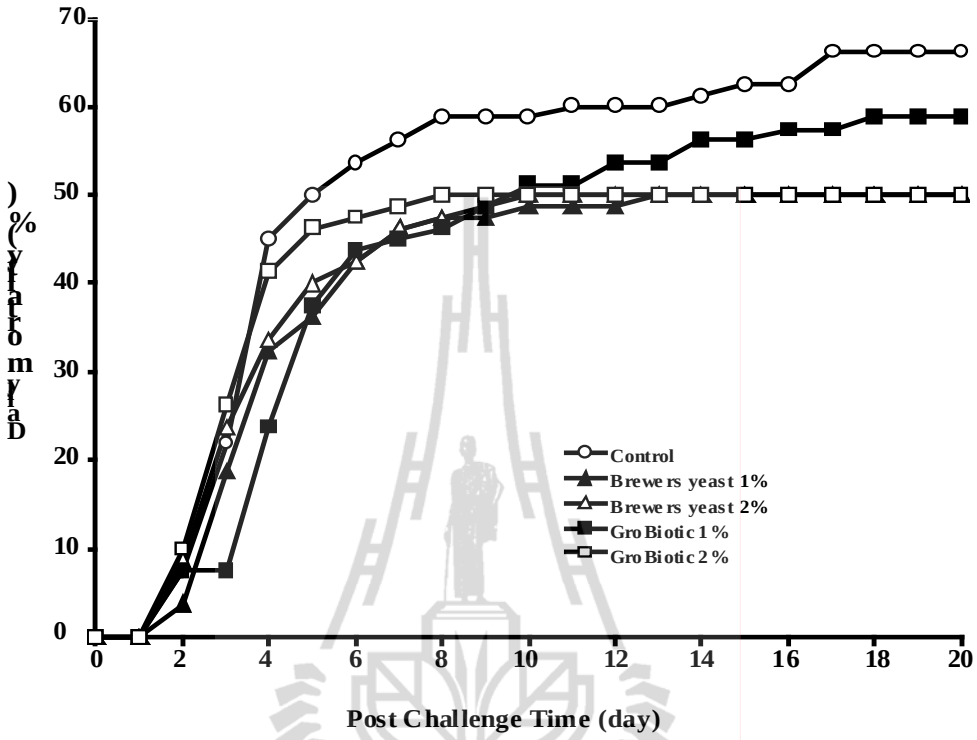

Figure 4.1 Daily cumulative mortality of Nile tilapia after 20 days of challenge with *Streptococcus iniae.*

4.5 Discussion

There have been numerous studies evaluating the influence of prebiotics such as yeast and its subcomponents (including β-glucans, nucleic acids and mannan oligosaccharides), as dietary supplements on fish growth performance, immune function and disease resistance (Duncan and Klesius, 1996; Sakai, 1999; Gannam and Schrock, 2001; Oliva-Teles and Gonçalves, 2001; Li and Gatlin, 2003; 2004; 2005; Waszkiewicz-Robak and Karwowska, 2004; Burr et al., 2005; Wittington et al., 2005; Gatesoupe, 2007; Welker et al., 2007; Shelby et al., 2009; Marrifield et al., 2010; Ringø et al., 2010). However, the beneficial effects of dietary prebiotics on growth performance and resistance to infectious diseases are not consistent. Lara-Flores et al.

(2003) and Abdel-Tawwab et al. (2008) reported that dietary supplementation of brewers yeast, *S. cerevisiae*, significantly improved growth and feed efficiency of Nile tilapia. In contrast, Li and Gatlin (2003) did not observed significant change in weight gain and feed efficiency of juvenile hybrid striped bass fed diets supplemented with 1, 2 or 4% BY. A later 7-week study by the same authors (Li and Gatlin, 2004) showed a trend of growth improvement in juvenile fish fed diets supplemented with 1 and 2% BY or GB. However, only fish fed the GB-containing diets had significant increased feed efficiency. With sub-adults of the same species, Li and Gatlin (2005) obtained generally increased performance of fish fed diets supplemented with 1 and 2% BY and 2% GB at 4 or 6 weeks, but significantly enhanced growth and feed efficiency were obtained after 12 and 16 weeks of feeding, respectively. Results of our 12-week feeding study, however, showed that dietary supplementation of BY or GB at 1 or 2% had no effect on weight gain, feed intake or survival of Nile tilapia, but a significant reduction in feed efficiency was observed in fish fed the 1% GB diet. The decrease in feed efficiency obtained in this treatment cannot be explained but was likely not related to dietary supplementation of GB. Because mixed sex tilapia were used in this study, higher proportion of female may have been stocked in this treatment. It is a common knowledge that, in tilapia, females grow slower and convert feed less efficiently than males. This is evident as fish in this treatment consumed slightly more feed but gained less weight and accumulated slightly more fat than other groups of fish. Other research showed that the growth and feed efficiency of Nile tilapia (Shelby et al., 2009) and channel catfish (Welker et al., 2007) were unaffected by dietary supplementation of yeast or yeast subcomponents consisted mainly β-glucan or oligosaccharides.

Li and Gatlin (2003) reported no significant changes in whole body composition in juvenile hybrid striped bass fed diets supplemented with 1, 2 or 4% BY for 8 weeks. In the present study, the result showed that no significant differences in whole body composition (moisture, protein, lipid and ash) of tilapia after 12 weeks of feeding diets supplemented with 1 and 2% BY or GB. However, the group fed the 1% GB diet that consumed more feed, gained less and converted feed less efficiently tended to accumulate more body lipid. In contrast, Abdel-Tawwab et al. (2008) obtained significant increased in body protein and ash in Nile tilapia fed diets supplemented with 0.1 to 0.5% and 0.5% yeast, respectively. Body total lipid content, however, significantly decreased in fish fed 0.2 and 0.5% yeast diets. They indicated that this alteration in body composition was attributed to increased feed intake, better nutrient utilization, high nutrient digestibility and increased nutrient deposit.

Even though cumulative mortality of tilapia 20 days following challenge with *S. iniae* was not significantly influenced by dietary treatments, fish fed diets supplemented with 1% BY and 2% BY or GB had substantially lower mortality (50.0%) than those of the groups fed the 1% GB (58.8%) and the control diet (62.3%). Mortality of fish fed diets containing 2% GB or BY and 1% BY ceased 8, 10 and 13 days post-challenge, respectively (Fig. 4.1). For the groups fed the control diet and the diet with 1% GB, mortality continued until day 17 and 18, respectively. Although bacterial count was not performed in this study, the earlier cessation of mortality and lower total mortality in fish fed 1% BY and 2% BY or GB were likely due to lighter infection rate. Li and Gatlin (2003) reported that, after 9 weeks of feeding, exposure of juvenile hybrid striped bass to *S. iniae* resulted in reduced signs of disease and no mortality in fish fed 2 and 4% BY diets, while 20 and 10% mortality were obtained in

fish fed the control and 1% BY, respectively. In a later study, they obtained significantly enhanced survival after bath exposure with *S. iniae* in hybrid striped bass fed diets with 1 and 2% BY or GB (Li and Gatlin, 2004). With sub-adults of the same species, Li and Gatlin (2005) obtained a significant reduction of mortality following *in situ* mycobacterial challenge in fish fed the 2% GB diet relative to the groups fed the control diet and 1 and 2% BY diets at the end of 21 weeks. Results of some earlier studies with prebiotics, particularly yeast and yeast by-products, in fish suggest that their inclusion in diets can enhance the resistance of several fish species against bacterial infections (Raa et al., 1990; Siwicki and Anderson, 1994; Yoshida et al., 1995).

Except for SH50 activity, serum protein, total immunoglobulin, lysozyme activity and agglutinating antibody titers against *S. iniae* at 21 days post-challenge were unaffected by dietary treatments. The SH50 of fish fed the 1% BY diet was significantly higher than those fed the control and 2% BY or GB diets. These SH50 values did not appear to follow the trend observed for cumulative mortality following *S. iniae* challenge as fish fed diets with 1% BY and 2% BY or GB had the same mortality (50%). Welker et al. (2007) obtained no significant differences in immune function (SH50, lysozyme activity, superoxide anion production and macrophage bactericidal activity) and resistance to *E. ictaluri* challenge in catfish fed diets supplemented with *S. cerevisiae* or yeast sub-components (glucan and mannan). Duncan and Klesius (1996) reported that channel catfish fed the β-glucan-containing (0.2%) diet had enhanced macrophage and neutrophil migration and phagocytosis, whereas fish fed the *S. cerevisiae*-diet (2.7%) had enhanced phagocytic activity of peritoneal exudate cells. This, however, had no effect on the resistance of fish to *E.*

ictaluri infection. Similarly, Ainsworth et al., (1994) reported no improved resistance to *E. ictaluri* challenge in catfish fed 0.1% β-glucan, but obtained increased antibody titers to *E. ictaluri.* In hybrid striped bass, Li and Gatlin (2003) reported improved resistance to *S. iniae* and neutrophil oxidative radical production and extracellular superoxide anion production of head kidney phagocytic cells, but serum lysozyme activity was not affect by dietary inclusion of 1, 2 and 4% BY. In later studies, they observed a trend of increasing neutrophil oxidative radical production and intracellular superoxide anion production of head kidney macrophages in fish fed 1 and 2% BY or GB, while extracellular superoxide anion production of head kidney macrophages of fish fed 1 and 2% BY and 1% GB was significantly higher than that of fish fed the control diets. All groups of fish fed BY or GB diets exhibited significantly enhanced survival following immersion challenge with *S. iniae* (Li and Gatlin, 2004). In sub-adults of the same species, they obtained significantly improved survival against *in si*tu mycobacterial infection in fish fed the 2% GB diet even though fish in this treatment had significantly lower serum peroxidase and extracellular superoxide anion production of head kidney macrophages obtained (Li and Gatlin, 2005). Jorgensen and Robertsen (1995) observed a marked increase in respiratory burst activity of head kidney macrophages of Atlantic salmon 4 to 7 days after treatments with 0.1-1μg mL^{-1} of glucan. Despite the stimulatory effect of glucan on respiratory burst activity, they reported that these macrophages did not show enhanced bactericidal activity against the avirulent or virulent strain of *Aeromonas salmonicida*.

Although our immunological measurements focused on serum components, results of earlier studies have shown that cellular immunity to be more affected by

dietary inclusion of prebiotics. However, the effects of dietary immunostimulants on cellular and humoral immune responses as well as the resistance of fish to infectious diseases vary considerably between studies, even for the same immune parameters. Differences in species, fish size, physiological status, quality of diets, source and concentration of prebiotics, feeding duration and levels, challenge method and concentration, and virulence of the pathogens are some of the factors that may have contributed to inconsistencies among various research results. Therefore, this result has not as the hypothesis may be the concentrate of BY and GB were not suitable and the time for bacterial challenge were take time to long.

4.6 Conclusion

Data of this study indicated that weight gain, feed intake, survival and whole body proximate composition of Nile tilapia following 12 weeks of feeding were not significantly affected by dietary supplementation of 1 and 2% BY or GB. The significantly lower feed efficiency observed in fish fed the 1% GB diet was probably not related to dietary treatments. Among the serum immunological components evaluated, only SH50 was significantly affected by dietary treatments. Cumulative mortality 20 days post challenge with *S. iniae* was not significantly affected by dietary treatments. However, fish fed diets supplemented with 1% BY and 2% BY or GB had substantially reduced mortality and mortality ended earlier as compared to those of other treatments.

4. 7 References

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CHAPTER V

SUMMARY

The present study demonstrated that Sato residual or lees can be incorporated in a practical diet and partially replace fish meal without any significant, adverse effects on the growth performance, carcass composition and health status. In addition, fed diets supplemented with GroBiotic®-A (GB) and Brewetech® dried brewers yeast (BY) in Nile tilapia at the juvenile stage on growth performance, proximate body composition, immune response and resistance of juvenile Nile tilapia to *Streptococcus iniae* challenge. For replacement of Sato residual or RWS found that RWS have crude protein as 38.12%, crude lipid 5.67% crude fiber 6.42, and ash 1.36% similar to soybean meal and have essential amino acid that are requiems for fish. The RWS can be partially replacing fish meal in a practical diet for juvenile Nile tilapia up to 22.5 % had no effects on growth response as represented by weight gain and SGR compared to Diet 1. By using RWS did not affect on FCR, PER, condition factor, HSI and proximate composition of fillet. RWS had affect on some hematology parameters. RWS can replace partially fish meal protein with no negative effects in lysozyme activity and blood urea nitrogen. The level of RWS had effected on the glucose level and cholesterol in the blood of fish. By using RWS did not affect on villi height and epithelium thickness of intestine in duodenum part but RWS had affected on villi height and No. goblet cells in jejunum part. Taken together, RWS (at 22.5 %) has the potential for use in juvenile Nile tilapia diet without negative effects. These

findings provide the first evidence of the potential benefit of utilizing the by-product from the rice wine industry as an alternative protein source for aqua feeds. This useful information will be provided for agricultural countries that are located mainly in Asia where their socio-economies particularly depend on aquaculture and rice production.

For fed diets supplemental with brewers yeast or GroBiotic®-A at 1 or 2% had no effects on weight gain, feed intake or survival of Nile tilapia, but a significant reduction in feed efficiency was observed in fish fed the 1% GroBiotic®-A Diet. By using brewers yeast and GroBiotic®-A did not affect on whole body proximate composition of Nile tilapia. Among the serum immunological components evaluated, only SH50 was significantly affected by dietary treatments. Cumulative mortality 20 days post challenge with *Streptococcus iniae* was not significantly affected by dietary treatments. However, fish fed diets supplemented with 1% BY and 2% BY or GB had substantially reduced mortality and mortality ended earlier as compared to those of other treatments.

APPENDICES

APPENDIX A

Regression pattern of parameters and rice wine residual as a function of incorporation level of RWS

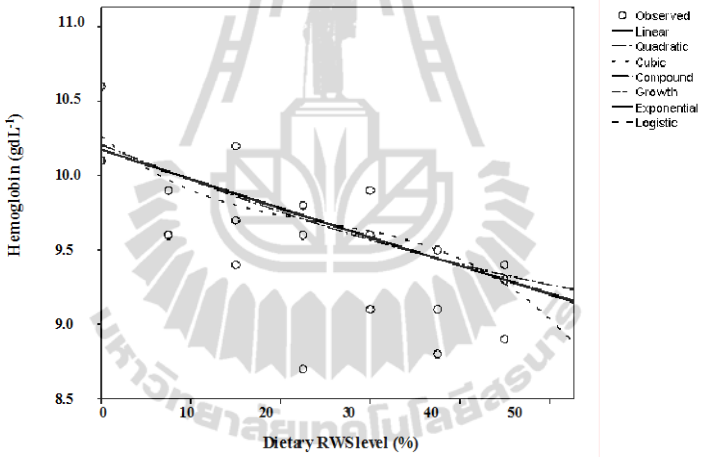

Figure A.1 Regression plot of haemoglobin and rice wine residual as a function of incorporation level of RWS. The non-linear relationship was observed between RWS and haemoglobin (y = -0.02x + 101.75, R^2 = 0.608). These plots provide illustration of the maximum haemoglobin that is predicted for Nile tilapia fed RWS.

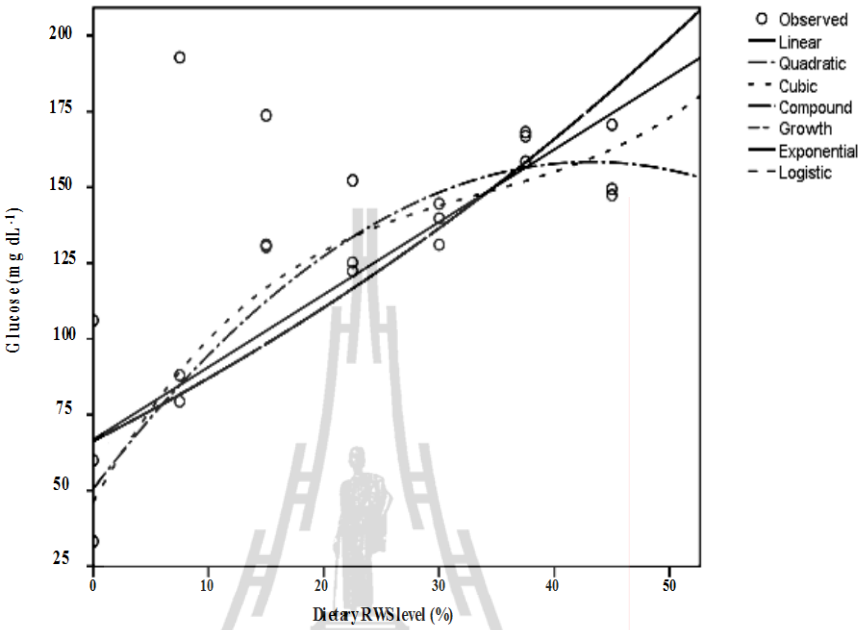

Figure A.2 Regression plot of blood glucose and rice wine residual as a function of incorporation level of RWS. The non-linear relationship was observed between RWS and blood glucose ($y = -2.310E\text{-}5x^2 + 0.02x + 6.022$, $R^2 = 0.909$). These plots provide illustration of the maximum blood glucose that is predicted for Nile tilapia fed RWS.

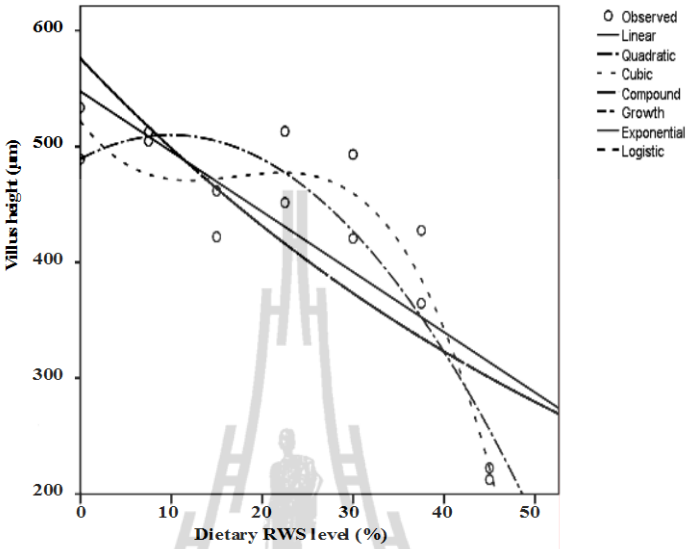

Figure A.3 Regression plot of villus height in jejunum part and rice wine residual as a function of incorporation level of RWS. The non-linear relationship was observed between RWS and villus height ($y = -1.292E\text{-}5x^3 + 0.007x^2 - 1.039x + 521.821$, $R^2 = 0.904$). These plots provide illustration of the maximum villus height that is predicted for Nile tilapia fed RWS.

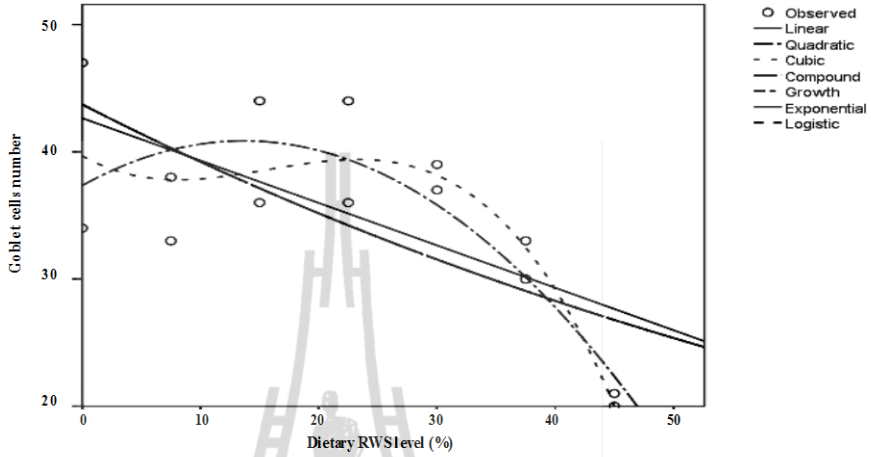

Figure A.4 Regression plot of goblet cell number in the duodenum part and rice wine residual as a function of incorporation level of RWS. The non-linear relationship was observed between RWS and goblet cell number ($y = -9.218E\text{-}7x^3 - 0.052x + 39.679$, $R^2 = 0.763$). These plots provide illustration of the maximum goblet cell number that is predicted for Nile tilapia fed RWS.

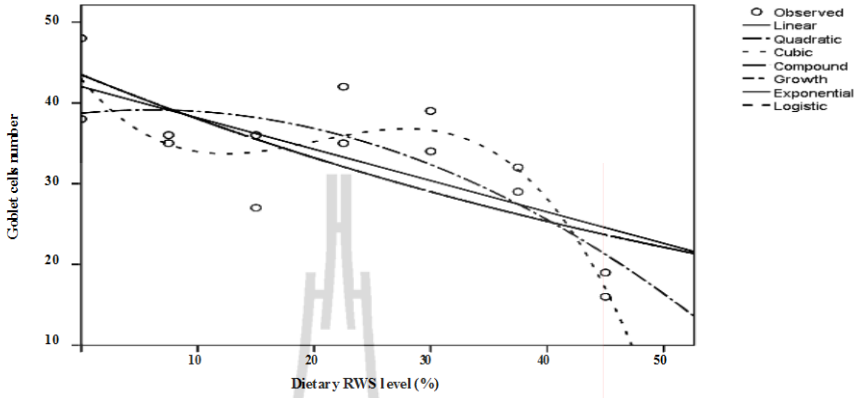

Figure A.5 Regression plot of goblet cell number in jejunum part and rice wine residual as a function of incorporation level of RWS. The non-linear relationship was observed between RWS and goblet cell number ($y = -1.679E\text{-}6x^3 + 0.001x^2 - 0.175x + 42.964$, $R^2 = 0.821$). These plots provide illustration of the maximum goblet cell number that is predicted for Nile tilapia fed RWS.

APPENDIX B

Table B.1 Ingredients composition (%) and unit cost of raw materials of eight experimental diets for juvenile Nile tilapia

Ingredient (%)	Diet								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8[1]**	**Unit cost[2]**
Rice wine residual	0	7.5	15.0	22.5	30.0	37.5	45.0	-	5
Fish meal	30.0	25.0	20.0	15.0	10.0	5.0	0	-	37
Soybean meal	27.0	27.0	28.5	30.0	30.0	31.0	32.0	-	17
Rice bran	15.0	15.0	14.5	14.0	14.0	15.0	12.0	-	7
Corn meal	14.5	14.0	10.0	9.0	6.0	4.5	4.5	-	8.50
Cassava chips	12.0	10.0	10.3	7.5	8.0	5.0	4.0	-	6
Premix	1.5	1.5	1.5	1.5	1.5	1.5	1.5	-	500
Soybean oil	0	0	0.2	0.5	0.5	0.5	1.0	-	700
Wage for fish diet									4
Cost per kilogram	29.44	27.81	26.35	24.96	23.26	21.72	20.39	25	

[1]Commercial diet

[2]Cost of raw materials (Bath/kg) on May, 31, 2011

BIOGRAPHY

Miss Kunthika Vechklang was born on April 22, 1971 in Nakhon Ratchasima, Thailand. In 1999, she studied in Department of Chemistry, Faculty of science, Nakhon Ratchasima Rajabhat University, Nakhon Ratchasima. She graduated the Bachelor's of Science in Chemistry in 1999. In 2001, she studied in Department of Biology, Faculty of Science, Mahasarakam University, Mahasarakam. She graduated the Master's of Science in Biology Education in 1999. Her master thesis topic was study of lactic acid bacteria for the development of alcoholic beverage production process. In 2006 after graduation, she got scholarship supported by Rajamangala University of Technology Isan Nakhon Ratchasima (RUMTI) Ph.D. Her Ph.D thesis was the effect of Sato residual and *Saccharomyces cerevisiae* on growth performance and immunological stimulation in juvenile sex reversal Nile tilapia (*Oreochromis niloticus)*. During Ph.D's student, she had an experience on poster presentation in title "Potential Amylase-Producing Bacteria Isolated from Dried Cassava Tuber" at the RGJ Seminar Series L on subject "Valuable products from natural resources and their application" Department of Science, September 3, 2007, Faculty of science, Mahidol University, Thailand. The topic on chapter III. The potential for rice wine residual as an alternative protein source in a practical diet for Nile tilapia (*Oreochromis niloticus)* at the juvenile stage was accepted on March, 16, 2011 to be published in Aquaculture Nutrition. However, the topic on chapter VI. Growth performance and resistance to *streptococcus iniae* of juvenile Nile tilapia (*Oreochromis niloticus*) fed diets supplemented with GroBiotic®-A and Brewtech® dried brewers yeast was submitted to be published in Journal of Applied Aquaculture.