EFFECT OF VARIOUS FEED ADDITIVES ON THE PERFORMANCE OF AQUATIC ANIMALS

水棲動物における各種飼料添加物の効果に関する研究

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2016

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

Doctor of Philosophy (Ph.D.)

in Fisheries Science

Major Subject: Aquatic Animal Nutrition

The United Graduate School of Agricultural Sciences

Kagoshima University, Faculty of Fisheries

Kagoshima, Japan

September 2016

APPROVAL OF DISSERTATION

The Dissertation titled

Effect of Various Feed Additives on the Performance of Aquatic Animals

Submitted to the United Graduate School of Agricultural Sciences,
Kagoshima University, Japan
by

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In partial fulfillment of the requirements for the degree of

Doctor of Philosophy (Ph.D.)

in Fisheries Science

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September 2016

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ENGLISH ABSTRACT

Aquaculture industry is playing an increasing role to supply comparatively safer animal protein for human consumption, as intensive aquaculture expanded, diseases occurred more frequently. The need for enhanced disease resistance, feed efficiency and growth performance of cultured organisms is substantial for various sectors of this industry. Moreover, the application of antibiotics and chemotherapeutics to control these diseases caused many other problems such as the spread of drug resistant pathogens, suppression of aquatic animal's immune system, environmental hazards and food safety problems. Nowadays, we have learned more sustainable ways to manage gut microflora and fish performance using functional feed additives to modulate the health of farmed animals. Therefore, this study revealed the use of substantially important and promising additives in aqua feed. The options available to regulate fish performances include the use of probiotics, prebiotics and immunostimulants. The red sea bream, *Pagrus major* and amberjack, *Seriola dumerili* are important cultured species in East Asia countries, particularly Japan due to their high market value, desirable taste and recent supply shortage. The current research was conducted to evaluate the effects of various feed additives on the performances of red sea bream and amberjack. In the first part three studies were conducted to determine the supplemental effects of heat-killed *Lactobacillus plantarum* (HK-LP), *Pediococcus pentosaceus* (PP) and β-glucan (BG) on red sea bream. Six diets were prepared to contain HK-LP at 0, 1, 10, 100, 1000, and 2000 mg kg^{-1} diet. After 56 days, the fish fed diet HK-LP at 1000 mg kg^{-1} resulted in a significant increase in growth performance, feed utilization, immune response and stress resistance when compared to the control diet. To study the supplemental effects of PP on red sea bream, fish fed five diets containing (0, 1.6×10^{10}, 1.6×10^{11}, 1.6×10^{12}, and 3.2×10^{12} cells g^{-1}). After 56 days, fish fed PP supplemented diets resulted in significantly higher growth performance, immune response and stress resistance in a

dose dependent manner than the control group. Same trend has been reported for fish fed diet containing BG at 250, 500 or 1000 mg of BG kg^{-1} diet. Considering these promising results, we suggest that the supplementation of HK-LP, PP and BG improves growth and health condition of red sea bream. In the second part, seven diets were formulated to contain 0%, 15%, 30% and 45% SBM together with HK-LP at 0.0 and 0.1%, and fed for amberjack. Fish fed a diet containing 30% SBM with HK-LP grew significantly faster than other groups with notable feed utilization. Further, feed utilization significantly increased in fish fed diets supplemented with HK-LP except for fish fed diet contains 45% SBM. Interestingly, immune response significantly enhanced in fish fed diets containing 15% and 30% SBM with HK-LP. In conclusion, the addition of HK-LP to amberjack diets appeared to improve SBM utilization, immune response and stress resistance. In the third part, dietary HK-LP and BG had a significant interaction on enhancing the growth, digestibility and immune responses of red sea bream, where, diets were formulated to contain three levels of HK-LP (0.025, 0.05 and 0.1% of dry diet) combined with two levels of BG (0 and 0.1% of dry diet) according to a 3×2 factorial design. A diet without HK-LP and BG supplementation was used as a negative control. Fourth part, red sea bream was fed with basal diet supplemented with *L. rhamnosus* (LR), *Lactococcus lactis* (LL), and *L. rhamnosus* + *Lactococcus lactis* (LR+LL) at 10^6 cell g^{-1} diet. Feeding a mixture of LR and LL significantly increased growth performance, intestine bacteria count, immune response and enhanced oxidative status. In the last part, we evaluated the effects of dietary HK-LP and vitamin C (VC) on the performances of red sea bream. Beside the basal diet, four diets with 0 or 1 g HK-LP kg^{-1} combined with 0.5 or 1 g VC kg^{-1} were fed to red sea bream. fish fed with both supplements showed higher growth, humoral and mucosal immune responses, anti-oxidative status, mucus secretion and stress resistance as well as

decreased plasma triglyceride and total cholesterol levels than the fish fed control diet with significant interaction between HK-LP and VC. The overall findings of this research suggest that these additives have beneficial effects on growth performances and health condition of red sea bream and amberjack.

(学位第３号様式)

学 位 論 文 要 旨

氏 名	マハムド アブドルハミッド オムラン ダウッド
題 目	水棲動物における各種飼料添加物の効果に関する研究 (Effect of Various Feed Additives on the Performance of Aquatic Animals)

水産養殖産業は、人間の食品として、比較的安全な動物性タンパク質を供給するという役割を果たし、増え続ける消費に対し、集約的養殖を拡大してきたが、それに伴い疾病の発生も頻繁に生じている。養殖の現場からは、養殖種の疾病に対する抵抗性、飼料効率及び成長の向上に対する要望が多く寄せられている。さらに、抗生物質や薬剤療法による疾病の管理は、薬剤耐性菌の増加、水生動物の免疫システムの抑制、環境汚染や食品の安全性の問題の原因となる。近年は、養殖魚の健康を改善するために、機能性飼料添加物を用いて腸内細菌叢や魚の状態を管理する持続的な方法が研究されている。本研究は、養魚飼料において重要なまたは効果が期待できる添加物の利用性を明らかにするために行った。加えて、プロバイオティクス、プレバイオティクス及び免疫賦活物質の添加による魚の健康状態の維持の可能性も検討した。マダイ及びカンパチは東アジア諸国、特に日本では高い市場価値や味の良さから重要な養殖種である。本研究では、マダイ及びカンパチにおける各種飼料添加物の効果を検討した。まず、*Lactobacillus plantarum* 加熱死菌体(HK-LP), *Pediococcus pentosaceus* (PP) 及びβ-グルカン (BG)のマダイに対する添加効果を明らかにするために、3つに飼育実験を行った。HK-LPは、飼料中に0mg、1mg、10mg、100mg、1000mg及び2000mg添加した6種類の試験試料を用いて、56日間の飼育実験を行い、2000mg添加区が対照区にと比較して優位に高い成長、飼料効率、免疫応答及び環境ストレス耐性を示した。次に、PPの添加量を変えた5種類の試験飼料（無添加、$1.6x10^{10}$、$1.6x10^{11}$、$1.6x10^{12}$、$3.2x10^{12}$細胞/g　飼料）を用いて、マダイにおけるPPの添加効果を調べた。56日後の成長、免疫応答及び環境ストレス耐性は、無添加区に比べ優位に高く、添加量に応じて向上していた。また、β-グルカンの添加効果（無添加、250mg、500mg、1000mg/kg飼料）を調べた試験でもPPと同様の結果が得られた。以上の結果から、HK-LP,PP及びBK添加はマダイの成長と健康状態を改善することが明らかになった。

次に、飼料中大豆粕（SBM）添加量を4段階（0%、15%、30%及び45%）、HK-LP添加量を2段階（0%、0.1%）に変えた7種類の試験飼料を用いてカンパチの成長と健康状態に及ぼす影響を検討した。HK-LPを添加した30%SBM飼料区が、他の試験区に比べ優位に高い成長を示し、特に飼料効率が向上していた。飼料効率は45%SBM区を除き、HK-LP添加によって有意に向上していた。また、15%及び30%SBM添加区は、HK-LP添加によって、免疫応答も優位に高い値を示した。また、HK-LPを3段階（0.025%、0.05%及び0.1%）、BG添加量を2段階（0%及び0.1%）に変えて作製した試験飼料を用いて、HK-LPとBGがマダイの成長、消化吸収率及び免疫応答に有意な相乗効果を示すことを明らかにした。*L. rhamnosus* (LR)、*Lactococcus lactis* (LL)または混合添加区 (LR+LL) を10^6細胞/g飼料添加した試験飼料を用いて、LR+LL区の成長、消化管細菌数、免疫応答及び酸化ストレス耐性が向上することを明らかにした。最後に、マダイにおけるHK-LPとビタミンCの交互作用を調べ、両者の添加により、成長、免疫応答、抗酸化ストレス、粘液分泌量及びストレス耐性が向上すること、血漿中トリグリセリドと総コレステロール量に交互作用が見られることを明らかにした。

本研究の結果から、これらの飼料添加物は海産養殖種の成長と健康状態の向上に有益であることが示された。

List of Publications, Conferences Presentations and Academic Awards

A. Publications in Peer Reviewed Journals:

Dawood, **M.A.O.,** Koshio, S., Ishikawa, M., Yokoyama, S., 2015. Effects of Partial Substitution of Fish Meal by Soybean Meal with or without Heat-Killed *Lactobacillus plantarum* (LP20) on Growth Performance, Digestibility, and Immune Response of Amberjack, *Seriola dumerili* Juveniles. BioMed Res. Int. Article ID 514196, 2015.

Dawood, **M.A.O.,** Eldakar, A., Mohsen, M., Abdelraouf, E., Koshio, S., Ishikawa, M., Yokoyama, S., 2014. Effects of Using Exogenous Digestive Enzymes or Natural Enhancer Mixture on Growth, Feed Utilization, and Body Composition of Rabbitfish, *Siganus rivulatus*. Journal of Agricultural Science and Technology, B 4, 180-187.

Dawood, **M.A.O.,** Koshio, S., Ishikawa, M., Yokoyama, S., 2015. Effects of heat killed *Lactobacillus plantarum* (LP20) supplemental diets on growth performance, stress resistance and immune response of Red sea bream, *Pagrus major*. Aquaculture, 442, 29-36.

Dawood, **M.A.O.,** Koshio, S., Ishikawa, M., Yokoyama, S., 2015. Interaction effects of dietary supplementation of heat-killed *Lactobacillus plantarum* and β-glucan on growth performance, digestibility and immune response of juvenile red sea bream, *Pagrus major*. Fish & Shellfish Immunology, 45(1), 33-42.

Dawood, **M.A.O.,** Koshio, S., Ishikawa, M., Yokoyama, S., 2016. Effects of dietary inactivated *Pediococcus pentosaceus* on growth performance, feed utilization and blood characteristics of red sea bream, *Pagrus major* juvenile. Aquaculture Nutrition, 22(4), 923-932.

Dawood, **M.A.O.,** Koshio, S., Ishikawa, M., Yokoyama, S., Elbasuini, M.F., Hossain, M.S., Nhu, T.H., Moss, A.S., Dossou, S., Wei, H., 2015. Dietary supplementation of β-glucan improves the innate immune response, stress resistance and growth performance of Red Sea bream, *Pagrus major*. Aquaculture Nutrition, in press.

Dawood, **M.A.O.,** Koshio, S., Ishikawa, M., Yokoyama, S., El-Basuini, M.F., Hossain, M.S., Nhu, T.H., Dossou, S., Moss, A.S., 2016. Effects of dietary supplementation of *Lactobacillus rhamnosus* or/and *Lactococcus lactis* on the growth, gut microbiota and immune responses of red sea bream, *Pagrus major*. Fish & Shellfish Immunology, 49, 275-285.

Dawood, **M.A.O.,** Koshio, S., Ishikawa, M., Yokoyama, S., 2016. Immune responses and stress resistance in red sea bream, *Pagrus major*, after oral administration of heat-killed *Lactobacillus plantarum* and vitamin C. Fish & Shellfish Immunology, 54, 266-275.

Dawood, **M.A.O.,** Koshio, 2016. Recent advances in the role of probiotics and prebiotics in carp aquaculture: A review. Aquaculture, 454, 243-251.

El-Basuini, M.F., El-Hais, A.M., **Dawood, M.A.O.**, Abou-Zeid, A.E.S., EL-Damrawy, S.Z., Khalafalla, M.M.E.S., Koshio, S., Ishikawa, M., Dossou, S., 2016. Effect of different levels of dietary copper nanoparticles and copper sulphate on growth performance, blood biochemical profiles, antioxidant status and immune response of red sea bream (*Pagrus major*). Aquaculture, 455, 32-40.

Dawood, **M.A.O.,** Koshio, S., 2016. Vitamin C supplementation to optimize growth, health and stress resistance in aquatic animals. Reviews in Aquaculture, in press.

Hossain, M.S., Koshio, S., Ishikawa, M., Yokoyama, S., Sony, N.M., **Dawood, M.A.O.**, Kader, M.A., Bulbul, M. and Fujieda, T., 2016. Efficacy of nucleotide related products on growth, blood chemistry, oxidative stress and growth factor gene expression of juvenile red sea bream, *Pagrus major*. Aquaculture, 464, 8-16.

El-Basuini, M.F., El-Hais, A.M., **Dawood, M.A.O.**, Abou-Zeid, A.E.S., EL-Damrawy, S.Z., Khalafalla, M.M.E.S., Koshio, S., Ishikawa, M., Dossou, S., 2016. Effects of dietary copper nanoparticles and vitamin C supplementations on growth performance, immune response and stress resistance of red sea bream, *Pagrus major*. Aquaculture Nutrition, in press.

Dawood, M.A.O., Koshio, S., Ishikawa, M., El-Sabagh, M., Yokoyama, S., Wang, W.L., Yukun, Z., Olivier, A., 2016. Physiological response, blood chemistry profile and mucus secretion of red sea bream (*Pagrus major*) fed diets supplemented with *Lactobacillus rhamnosus* under low salinity stress. Fish Physiology and Biochemistry, in press.

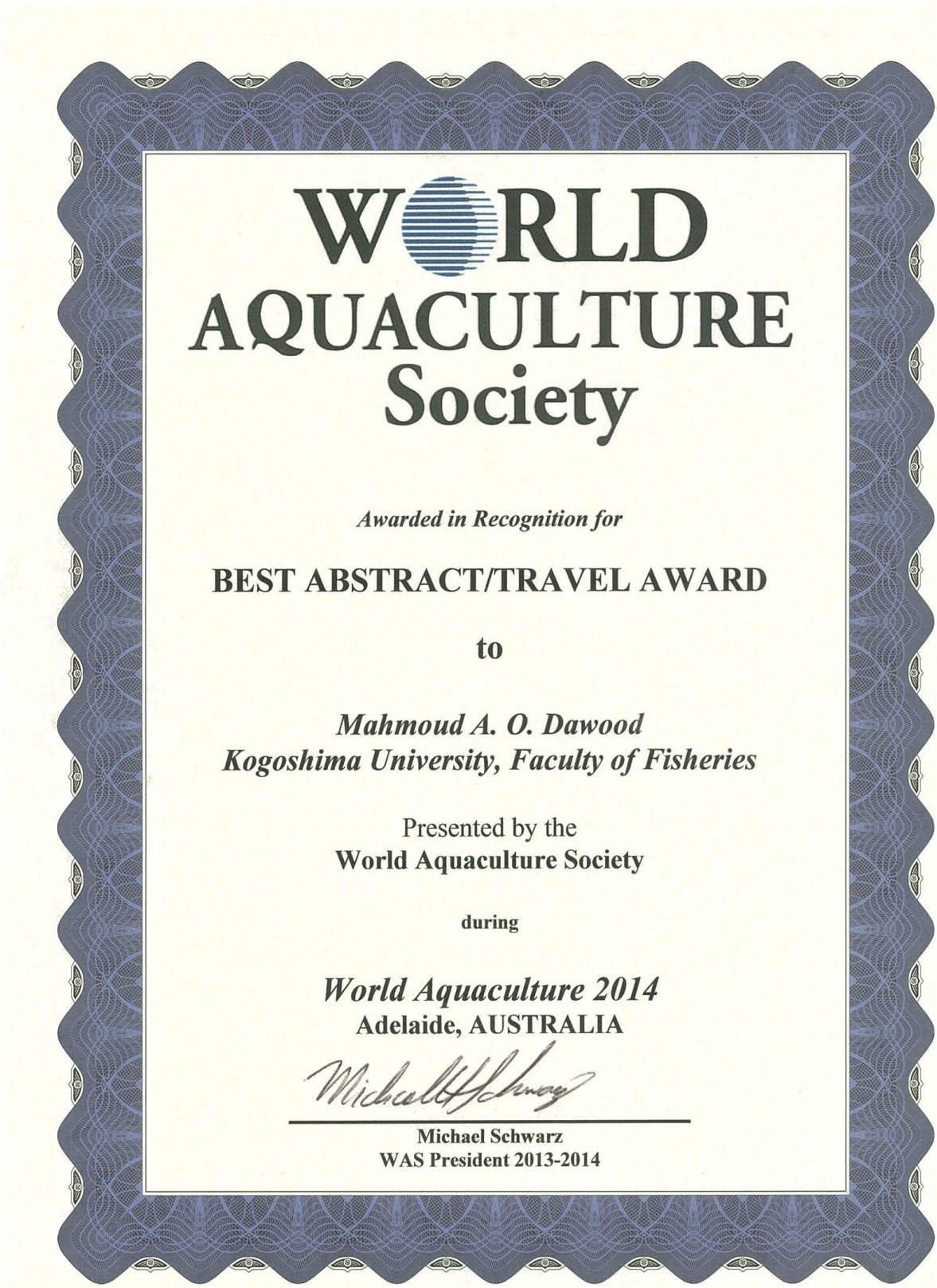
Dawood, M.A.O., Koshio, S., Ishikawa, M., El-Sabagh, M., Esteban, M.A., Zaineldin, A.I., 2016. Probiotics as an environment-friendly approach to enhance red sea bream, *Pagrus major* growth, immune response and oxidative status. Fish & Shellfish Immunology, in press.

B. Conference Abstracts

1. **Dawood**, **M.A.O.,** Koshio, S., Ishikawa, M., Yokoyama, S., 2013. Effect of Dietary Exogenous Digestive Enzymes on Growth Performance, Feed Utilization and Body Composition of Rabbitfish, *Siganus rivulatus.* Book of abstracts in Asian Pacific Aquaculture, Vietnam. 10-13 December, 2013.
2. **Dawood**, **M.A.O.,** Koshio, S., Ishikawa, M., Yokoyama, S., 2014. Effects of the partial substitution of fish meal by soybean meal with or without heat killed *Lactobacillus plantarum* (LP20) on the growth performance, stress resistance and immune response of amberjack, *Seriola dumerili* juveniles. Book of abstracts in World Aquaculture Adelaide. Australia. 7-12 June, 2014.
3. **Dawood**, **M.A.O.,** Koshio, S., Ishikawa, M., Yokoyama, S., 2015. Interaction effects of dietary supplementation of heat-killed *Lactobacillus plantarum* and β-glucan on growth performance, digestibility and immune response of juvenile red sea bream, *Pagrus major.* Book of abstracts in Aquaculture America 2015. New Orleans, Louisiana. USA. 19-22 February, 2015.
4. **Dawood**, **M.A.O.,** Koshio, S., Ishikawa, M., Yokoyama, S., 2015. Efficiency of heat killed *Lactobacillus plantarum* supplemental diets on red sea bream, *Pagrus major.* World Aquaculture 2015 Jeju. Korea. 26- 30 May, 2015.
5. El Basuini M.F., Abou-Zeid A.E., **Dawood**, **M.A.O.**, EL-Damrawy S.Z., Khalafalla M.M.E., Koshio, S., Ishikawa, M., Dossou S., 2016. The effect of dietary nano-copper and copper sulfate on red sea bream juveniles. the 7th World Fisheries Congress in Busan, Korea. 23-27 May, 2016.

C. Academic Awards

Best Abstract/Travel Award, 2014: World Aquaculture Society, World Aquaculture 2014, Adelaide, Australia.



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ACKNOWLEDGEMENT

All praises are due to the Almighty **Allah** who enables me to perform higher education and to complete as well as to submit the dissertation. I am greatly acknowledged to the Egyptian government for financial support which had given me the opportunity to carry out doctoral course in Japan.

I would like to express deepest sense of gratitude, gratefulness, sincere appreciation and profound regards to my mentor Dr. Shunsuke Koshio, Professor, Faculty of Fisheries, Kagoshima University, Japan for his affectionate encouragement, worthy inspiration, scholastic guidance and constructive criticism throughout the research work and during producing this dissertation.

With profound regard, I also would like to thank Dr. Manabu Ishikawa, Associate Professor and Dr. Saichiro Yokoyama, Assistant Professor for their sincere encouragement, valuable suggestions and hands-on training on laboratory procedures and analytical equipment operations.

I am greatly indebted to Professor Dr. Hiroto Maeda for introducing me to my supervisor in Kagoshima University and encouraging appreciation during my research. Thanks are given to House Wellness Foods Corp., Itami and Kyushu Medical Company, Fukuoka, Japan for supplying bacterial preparations used for the research.

I feel proud to express my heartfelt gratitude to my Egyptian lab mates Mr., Mohamed Fouad, Mr., Amr Zain and all the foreign students: Sakhawat, Nhu, Wei, Amina, Serge and Japanese students in the lab for their assistance, co-operation and inspiration throughout the study period. Special thanks to "Kagoshima Muslim community" for offering me a favorable environment to practice my religion properly.

Last but not the least, I gratefully acknowledge with regards and deepest sense of gratitude to my Father, Mother, Brothers, Sisters, Wife, Daughter, Sons and all of my family members who lived in Egypt for their sacrifice, continuous mental support and keeps me always cheerful.

Mahmoud A. O. Dawood

ABBREVIATIONS

ACP	:	Alternative complement pathway	WG	:	Weight gain
ADC	:	Apparent digestibility coefficient	LA	:	Lysozyme activity
BA	:	Bactericidal activity	LAB	:	Lactic acid bacteria
BAP	:	Biological anti-oxidant potential	LL	:	*Lactococcus lactis*
BG	:	β-glucan	LR	:	*Lactobacillus rhamnosus*
BUN	:	Blood urea nitrogen	MDA	:	Malondialdehyde
CA	:	Crude ash	MRS	:	Man, Rogosa, and Sharpe
CAA	:	Crystalline amino acid	NBT	:	Nitroblue tetrazolium
CAT	:	Catalase enzyme activity	OD	:	Optical density
CFU	:	Colony forming unit	PA	:	Protease activity
CL	:	Crude lipid	PBS	:	Phosphate buffered saline
CORT	:	Relative value of cortisol	PER	:	Protein efficiency ratio
CP	:	Crude protein	PG	:	Protein gain
DM	:	Dry matter	PP	:	*Pediococcus pentosaceus*
d-ROMs	:	Reactive oxygen metabolites	PR	:	Protein retention
FBW	:	Final body weight	RaRBC	:	Rabbit red blood cells
FER	:	Feed efficiency ratio	S.D.	:	Standard deviation
FI	:	Feed intake	S.E.M.	:	Standard error of mean
FM	:	Fish meal	SBM	:	Soybean meal
Glu	:	Glucose	SGR	:	Specific growth rate
GOT	:	Glutamyl oxaloacetic transaminase	SOD	:	Sodium oxide dismutase
GPT	:	Glutamic-pyruvate transaminase	SR	:	Survival rate
HBSS	:	Hank's buffered salt solution	TAA	:	Total amino acid
Hct	:	Hematocrit level	T-Bil	:	Total bilirubin
HK-LP	:	Heat killed *Lactobacillus plantarum*	T-Cho	:	Total cholesterol
HPLC	:	High performance liquid chromatography	TG	:	Triglyceride
HSI	:	Hepatosomatic index	TSA	:	Trypto Soya agar
VSI	:	Viscerosomatic index	TSP	:	Total serum protein
VC		Vitamin C			

Chapter I: General Introduction

1. General introduction

1.1 Aquaculture and feed additives

Aquaculture industry is playing an increasing role to supply comparatively safer animal protein for human consumption, as intensive aquaculture expanded, diseases occurred more frequently. The need for enhanced disease resistance, feed efficiency and growth performance of cultured organisms is substantial for various sectors of this industry (EL-Haroun *et al.*, 2006). Moreover, the application of antibiotics and chemotherapeutics to control these diseases caused many other problems such as the spread of drug resistant pathogens, suppression of aquatic animal's immune system, environmental hazards and food safety problems (Bachere, 2003; Dawood *et al.*, 2015a, b, c, and e).

1.2 Alternatives for the antibiotics

Increasing with the demand for environmentally friendly aquaculture, works on finding out the alternatives for the antibiotics are urgently needed (Miranda and Zemelman, 2002). World Health Organization (WHO) encourages using of natural functional feed additives to substitute or minimize the use of chemicals through the global trend to go back to nature. The investigation and application of a wide range of dietary supplements, such as probiotics, prebiotic and other additives, are increasingly popular in aquaculture research and practice (Dawood *et al.*, 2015a). Nowadays, we have learned more sustainable ways to manage gut microflora and fish performance using functional feed additives to modulate the health of farmed animals. Additives in aquafeed are nutritive/nonnutritive ingredients that are supplemented in small amounts (alone or in combination) for a specific purpose, such as to improve the quality of fish as a final product, to preserve the physical and chemical quality of the diet, or to

maintain the quality of the aquatic environment. Moreover, growing interest in raising fish in an antibiotic-free environment, driven by consumer interest in the quality and safety of seafood, has hastened the search for safe dietary immunostimulants as an alternative to antibiotics. However, the importance of these additives received little attention outside the purview of aquaculture researchers. This study revealed the use of substantially important and promising additives in aquafeed (Barrows, 2000). The options available to regulate fish performances include the use of probiotics, prebiotics and immunostimulants.

1.2.1 Probiotics

The administration of probiotics to fish seems to be a very promising control measure for the fish farms (Dawood and Koshio, 2016a; Kesarcodi-Watson *et al.*, 2008). Some concerns may arise in aquaculture due to the oral delivery of probiotics that may introduce live bacteria into the environment. Thus, the use of inactivated bacteria shed a light on such a safety-related issue since they can no longer interact with other aquatic organisms (D´ıaz-Rosales *et al.*, 2006). Currently, the definition of probiotics includes the metabolites of live or dead bacterial cells, which function as immunostimulants for modification of enzyme activity or microflora in gastrointestinal tracts that have beneficial effects on host health (Naidu *et al.*, 1999; Salminen *et al.*, 1999). Several works reported increased disease resistance (Giri *et al.*, 2014; Son *et al.*, 2009) and stronger immune responses (Giri *et al.*, 2013; Salinas *et al.*, 2005) in fish fed live bacteria supplemented diets. On the other hand, dietary supplementation of inactivated bacteria stimulated fish innate immune parameters (Biswas *et al.*, 2013a; Cerezuela *et al.*, 2012a; Dawood *et al.*, 2015a, b, c, d, and e; D´ıaz-Rosales *et al.*, 2006) and increased disease resistance (Biswas *et al.*, 2013b; Pan *et al.*, 2008). Inactivated

probiotic preparations appear as an interesting alternative to live probiotics, which could potentially cause safety problems in open aquatic environments. A comparative study between killed and live bacteria on immune responses showed that heat killed *Clostridium butyrium* retain interesting immunomodulating properties on Chinese drum, *Miichthys miiuy* (Pan *et al.*, 2008), and heat killed bacterins of *V. vulnificus* induced a better antibody response than that induced by formalinised bacterins in flounder, *Paralichthys olivaceus* (Park *et al.*, 2001). At present, lactic acid bacteria (LAB) as dietary supplements have been widely applied to enhance the immunity and disease resistance of fish (Standen *et al.* 2013), most notably *Lactobacillus* spp., *Carnobacterium* spp. and *Enterococcus faecium*. *Lactobacillus plantarum* is a gram-positive, heterofermentative lactic acid bacterium. The bacterium has a high adapting capacity to many environmental conditions, and is effective in suppressing the growth of pathogenic and spoilage microorganisms by secreting bacteriocin in food. In aquaculture, administration of live *L. plantarum* induced immune modulation, enhanced the growth performance, immune ability, and increased disease resistance in fish (Giri *et al.*, 2013; Giri *et al.*, 2014; Son *et al.*, 2009). Heat-killed *L. plantarum* (HK-LP) was used as an immunostimulant to induce interleukin-12 production and antitumor effect in mice, and to enhance gamma interferon production, which stimulates a substance that suppresses virus reproduction and other T-cells and activates macrophages cells (Murosaki *et al.*, 1998, 2000). Daily intake of HK-LP as an immunostimulant enhanced immunity response in healthy adults (Hirose *et al.*, 2006) and broiler chickens (Khonyoung and Yamauchi, 2012). Moreover, daily intake of HK L-137 stimulates innate immunity for production of type I interferon (IFN) in humans and pigs (Arimori *et al.*, 2012). In aquatic animals, Tung *et al.* (2010) concluded that the stress resistance is higher in larval and post larval kuruma shrimp, *Marsupenaeus*

japonicas fed a diet containing HK-LP. Recently, Biswas *et al.* (2013a) reported the potential effects of HK-LP (strain 06CC2) isolated from the Mangolian dairy products as novel immunostimulant to fish. In the light of these observations, it could be hypothesized that HK-LP might be effective in responses of growth and biological defense systems of fish species. At the same time, the collective data on the effects of HK-LP should be investigated to find the effective use of HK-LP for marine species (Dawood *et al.*, 2015a, b, c, d, and e). *Pediococcus pentosaceus* plays a beneficial role in the host gut environment by producing antibacterial substances such as lactic acid, acetic acid, hydrogen peroxide, and bacteriocin that suppress growth of competing bacteria (Castex *et al.*, 2008; Maeda *et al.*, 2013). Studies have begun to assess the probiotic potential of *Pediococcus* sp. for cobia *Rachycentron canadum* (Xing *et al.*, 2013), grouper *Epinephelus coioides* (Huang *et al.* 2014), rainbow trout *Oncorhynchus mykiss* Walbaum (Merrifield *et al.*, 2011), kuruma shrimp *Marsupenaeus japonicus* (Maeda *et al.*, 2013) and red sea bream *Pagrus major* (Dawood *et al.*, 2015c).

1.2.2 Prebiotics

Prebiotics, on the other hand, are indigestible substances that allow specific changes in the composition and/or activity of gastrointestinal microbiota, which has a positive effect on the nutrition and health status of the host (Song *et al.*, 2014). β-glucans (BG) as one of prebiotics has been used extensively in different fish species. β-glucans is known to have a potent stimulatory effect on the immune system of mammals, crustaceans and fish. In aquaculture, β-glucans have successfully been used to enhance the immune responses (Siwicki *et al.*, 1994; Ortun˜o *et al.*, 2002), as well as growth (Lara-Flores *et al.*, 2003), of various fish species and thus may serve as an excellent health promoter for fish culture. Glucans are capable of enhancing innate immunity by

activating macrophages, increasing their capacity to kill pathogens (Jorgensen and B. Robertsen, 1995). They have also been shown to enhance other non-specific immune factors such as lysozyme and complement activities (Paulsen *et al.*, 2001; Kühlwein *et al.*, 2014). When administered orally, glucans have a proven protective effect in several fish species and against a variety of bacterial pathogens including *Aeromonas hydrophila* (Kwak *et al.*, 2003; Selvaraj *et al.*, 2005), and *Edwardsiella tarda* (Sahoo and Mukherjee, 2002).

Several reports have shown that, use of β-glucans enhanced the growth performance in a range of cultured species including *Labeo rohita* (Misra *et al.*, 2006), sea cucumber, *Apostichopus japonicas* (Min *et al.*, 2011), koi, *Cyprinus carpio* koi (Lin *et al.*, 2011), red sea bream *Pagrus major* (Dawood *et al.*, 2015e), and mirror carp, *Cyprinus carpio* L. (Kühlwein *et al.*, 2014).

1.3 Methods for increasing soybean meal's (SBM's) inclusion rates in soy-sensitive species

The utilization of plant protein is limited by the deficiencies in essential amino acids and minerals and the presence of antinutritional factors, toxins, metabolites and complex carbohydrates (NRC, 1993). Methods for increasing SBM's inclusion rates in soy-sensitive species such as red sea bream and amberjack are required, and one of the methods is to apply dietary supplementation of functional compounds. Several authors have reported that the dietary administration of different bacterial forms enhanced the secretion of intestinal enzymes and characterization of these enzymes provides some information regarding the digestive capacity of fish to hydrolyze carbohydrate, protein and lipid of feed ingredients, leading to better growth performance and feed efficiency

(Dawood *et al.*, 2014; Dawood *et al.*, 2015d; Tovar-Ramirez *et al.*, 2002; Mohapatra *et al.*, 2012; Ray *et al.*, 2012; Lemieux *et al.*, 1999).

1.4 Interaction effects of dietary supplementation of Probiotics and Prebiotics

The mixture of probiotics and prebiotics that beneficially affects the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract is defined as synbiotics (Soleimani *et al.*, 2012). Limited data is available regarding the application of synbiotics in fish (Ai *et al.*, 2011; Dawood *et al.*, 2015a; Ye *et al.*, 2011; Zhang *et al.*, 2013; Rodriguez-Estrada *et al.*, 2009; Lin *et al.*, 2012). Recently, researchers showed great interested in the interactions, especially synergistic actions between probiotics and prebiotics. Synergistic actions between isomaltooligosaccharides and *Bacillus*, mannan oligosaccharides and *Bacillus sp.*, fructo oligosaccharides (FOS) and *B. subtilis*, chitosan oligosaccharides and *Bacillus coagulans*, FOS and *Bacillus licheniformis* have been revealed in studies on shrimp (*L. vannamei*) (Li *et al.*, 2009), European lobster larvae (*Homarus gammarus* L.) (Daniels *et al.*, 2010), sea cucumber (*A. japonicus*) (Zhang *et al.*, 2010), koi (*Cyprinus carpio* koi) (Lin *et al.*, 2012) and triangular bream (*M. terminalis*) (Zhang *et al.*, 2013), respectively.

Gibson, (2004) suggested that a prebiotic could be selectively fermented by the intestinal microbiota and stimulate selectively the growth and/or activity of intestinal bacteria. It was demonstrated that probiotics given in the form of synbiotics to the host yielded significantly better results than that given in individual form (Rodriguez-Estrada *et al.*, 2009). Prebiotics in the synbiotics can enhance the survival of probiotics in gastrointestinal tract (Roberfroid, 2000; Bielecka *et al.*, 2002), and thus improving the quick reproducibility of probiotics in vivo and perform beneficial effects.

1.5 Efficiency of dietary supplementation of vitamin C

Vitamin C has been known to be an important micronutrient correlating with an enhanced aquatic animal performance (Dawood *et al.*, 2016b; Dawood and Koshio, 2016b; Gao and Koshio, 2014; Shahkar *et al.*, 2015). Vitamin C (ascorbic acid, AA) is an essential micronutrient for normal growth and physiological function of most aquatic animals (Lim and Lovell 1978; Ren *et al.*, 2007). Many fish and crustacean species have a limited ability to synthesize vitamin C (Lightner *et al.*, 1979), due to the absence of L-gulonolactone oxidase that is responsible for AA biosynthesis (Ai *et al.*, 2006; Fracalossi *et al.*, 2001). So that, signs of deficiency are observed when vitamin C excluded from the diet. AA is a strong antioxidant that is capable of scavenging reactive oxygen species (Bae *et al.*, 2012). It participates in synthesis of steroid hormones and collagen (Cavalli *et al.*, 2003; Hunter *et al.*, 1979; Hayat *et al.*, 2013; Lightner *et al.*, 1979), tolerance to toxicants and environmental stressors (Agrawal *et al.*, 1978; Ishibashi *et al.*, 1992). It has also been proposed to be potentially beneficial in increasing immune response (Shahkar *et al.*, 2015; Kim and Kang, 2015; Roosta *et al.*, 2014) and reducing oxidative damage to tissues (Huang *et al.*, 2015; Liang *et al.*, 2015), even though it's exact mechanism has not been demonstrated. Vitamin C also promotes beneficial effects on serum bactericidal activity, phagocytic activity, antibody levels and lysozyme activity (Ren *et al.*, 2005). Phagocytic activity of cells of the immune system in fish produces reactive oxygen radicals that are potent microbicidal factors (Secombes *et al.*, 1988). However, there is still limited information on the relationship between dietary AA and its antioxidant effects with different degrees of lipid oxidation for fish species. Vitamin C also facilitates the absorption of iron (Hsu and Shiau 1999), thus preventing the anemia often observed in case of vitamin C deficiency in fish. Moreover, vitamin C may play a role in reproduction of cultured fish species. Reduced

reproductive performance has been reported in female tilapia (*Oreochromis mossambicus*) and rainbow trout (*Salmo gairdneri*) fed vitamin C deficient diets (Sandnes *et al.,* 1984). Furthermore, supplementation of AA in egg, larval development and broodstock showed enhanced immune function and overall health (Shahkar *et al.*, 2015).

Red sea bream (*Pagrus major*) is one of the most economically cultured warm water marine fish in Japan as well in the world. Until now, there are no studies on the application of probiotics and prebiotics for red sea bream. Similarly, the amberjack (*Seriola dumerili*) is one of the most important cultured species in Japan because of its delicacy and comparatively higher market value. It is distributed throughout the tropical and subtropical seas except the Pacific Ocean. There have been no studies about dietary soybean meal and the effects of bacterial cells have been undertaken on amberjack to date. Therefore, the overall objectives of the present study were:

1- To date few studies have attempted to quantify the value of commercially available additives in improving growth performance of cultured Japanese species.

2- To investigate the effect of the oral administration of heat killed *Lactobacillus plantarum*, *Pediococcus pentosaceus* and β-glucan on the growth, survival, immune response, and stress resistance of juvenile red sea bream.

3- To determine the effects of the partial substitution of fish meal by soybean meal with or without HK-LP on growth, digestibility, blood chemistry, immune responses and stress resistance of amberjack, *Seriola dumerili* juveniles.

4- To determine the interaction effects of dietary supplementation of HK-LP and BG on the growth, blood chemistry, and innate immunity of red sea bream, *Pagrus major*.

5- To evaluate the use of two probiotic species at the same time in the diet and their effect on growth, nutrient utilization, gut microbial population and immune response of fish.

6- To clarify the possible interactions between dietary HK-LP and vitamin C on growth performance, blood chemistry profile, immune response and tolerances against stress of red sea bream, and the result would suggest new avenues for the alleviation of stress and prevention of fish diseases.

Chapter II: General Materials and Methods

2. General Materials and Methods

2.1 Preparation of test diets

Protein source was brown fishmeal which contained 67% of crude protein and 8% of crude lipid and casein which contained 87% of crude protein. The lipid sources were Pollack liver oil and soybean lecithin. Activated gluten was used as a binder to produce pellet diet. A-starch and dextrin was the carbohydrate source in the diets. Cellulose powder was used to adjust to 100% total proportion. Feed additives were thoroughly mixed with lipid sources (Pollack liver oil and soybean lecithin) with a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan), before adding other ingredients. Then, added to the dry ingredients and mixed in a food mixer for 15 min. the required amount was mixed with water (35–40% of the dry ingredients), and then added to the premixed ingredients and mixed for another 15 min. The pH of the diets was adjusted to the range of 7.0–7.5 with 4 N sodium hydroxide. The mixture was then passed through a meat grinder with an appropriate diameter (1.2–2.2 mm) to prepare pellets, which were then dried in a dry-air mechanical convection oven (DK 400, Yamato Scientific, Tokyo, Japan) at 50 °C for about 120 min. to approximately 11% moisture, the test diets were stored at −28 °C in a freezer until use.

2.2 Experimental system

The feeding trials were carried out at the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. The feeding trial was conducted in 200 and 100-L polycarbonate tanks in a flow through sea water system where each tank was equipped with an inlet, outlet, and continuous aeration. The tanks were maintained under natural light/dark regime. The seawater was pumped from the deep basin of Kagoshima bay, Japan; gravel filtered and supplied to the system. A flow rate of 1.5 L

min^{-1} was maintained throughout the experimental period. During the experimental period, the monitored water quality parameters were checked regularly. The detected ranges are considered within optimal values for the cultured fish species.

2.3 Feeding protocol

All fish were fed the respective test diets visually near satiation level by hand twice a day, 7 days per week for 56 days. Any uneaten feed left was removed one hour after feeding and dried using a freeze drier, weighing and finally subtracted from the total amount of supplied diets to calculate the actual feed intake. The water quality was checked regularly. All fish were weighed in bulk at 2 weeks' interval to determine growth, check their health condition and ration was adjusted according to mean fish weight.

2.4 Sample collection

A pooled sample of 10-20 fish at the beginning was stored at −20 °C for whole body analysis. At the end of the feeding trial, all fish were fasted for 24 h prior to final sampling. Then the total number, individual body weight and length of fish from each tank were measured. Blood was taken from the caudal vein of fish using heparinized disposable syringes. Hematocrit was determined using the micro hematocrit technique. Plasma samples were obtained by centrifugation at 3000×g for 15 min ×4 °C using a high-speed refrigerated micro centrifuge (MX-160; Tomy Tech USA Inc., Tokyo, Japan) and kept at −80 °C. In addition, non-heparinized disposable syringes were used to collect blood for serum analysis. Serum samples were obtained by centrifugation at 3000×g for 15 min4 °C to collect serum. Liver was dissected out, then, weighed to get hepatosomatic index (HSI). Fish for skin mucus were washed with distilled water

individually, and skin mucus was collected from body surface (200 mm^2) by using a small piece of sterilized cotton and immediately suspended in 1 ml of phosphate-buffered saline (PBS, pH=7.4) and centrifuged (2000×g, 10 min., 4 °C) (MX-160, Tomy Seiko Co., LTD, Tokyo, Japan), and the supernatant in the under tube was transferred into another centrifugal tube (510-GRD, QSP, San Diego, CA, USA) and kept at -80°C until further analysis were performed.

2.5 Analytical procedure

2.5.1 Proximate composition

The experimental diets and fish whole body were analyzed for moisture, crude protein, total lipid and ash, in triplicate, using the following standard methods (AOAC, 1990, 1998):

2.5.1.1 Moisture

Fish whole body were dried to a constant weight using the freeze dryer (Eyela freeze dryer FD-1, Tokyo Rikakikai Co. Ltd., Japan). Diet samples were also dried to a constant weight using mechanical convection oven at 135°C. Loss in weight represented moisture content.

Moisture (%) = {(weight before drying -weight after drying/weight before drying)} × 100

2.5.1.2 Ash

Samples were taken in crucibles with cover slightly ajar and placed in a muffle furnace at 550°C until sample weight became constant. Ash contents were calculated as follow:

Ash (%) = (weight of ash/ sample weight) × 100

2.5.1.3 Crude protein

Approximately 0.2 g of sample and 2 g of catalizer (K_2SO_4:$CuSO_4$; 9:1) were digested with 10 ml of concentrated H_2SO_4 and 5 ml of 30% H_2O_2 for 90 min at 420°C. Then digested samples were distilled in 50 ml of 30-40% NaOH using Kjeldahl distilling apparatus (Kjeltec System 1007, Tecator, Sweden). Approximately 150 ml distillate in H_3BO_3 solution mixed with methylene blue and methyl red indicators in ethanol was titrated with 0.1N H_2SO_4 to neutral pH. Percent of N was calculated for obtaining crude protein (%) using the following formula:

Nitrogen (%) = {14.008 × (A-B) × 0.1 × F}/ {Sample weight (g) × 10}

Where, A = Amount (ml) of H_2SO_4 solution titrated for sample

B = Amount (ml) of H_2SO_4 solution titrated for blank

F = Factor of 0.1 N sulfuric acid solution

Crude protein (%) = % N × 6.25

2.5.1.4 Crude lipid

Soxhelt method was contributed to analyze the crude lipid. 2 g sample was weighed and placed in an extraction thimble and covered with cotton. 125 ml of diethyl ether was used for extracting fat from the sample using Soxhelt extraction apparatus for 5-6 hours. The flask with the extracted fatwas then dried in rotary vacuum evaporator (Eyela SB 1100, CCA 1111). Flasks were then dried in oven (110°C) for constant weight. Crude lipid was then calculated as follows:

Crude lipid= [weight of fat (g)/ dry sample weight (g)] × 100

2.5.2 Amino acids

Total amino acid (TAA) concentrations were analyzed using high performance liquid chromatography (HPLC, Shimadzu Corp. Kyoto, Japan) according to Kader *et al.* (2010, 2012). To determine TAA, samples were prepared as follows: 2 mg samples

were spiked with known amount of norleucine as an internal standard and hydrolyzded with 4 N methanesulfonic acid at 110°C for 22 h. The pH of the hydorlysate was adjusted to 2.2, filtered and stored at 4°C. The chromatographic separation and analysis of the amino acids were performed with the HPLC unit with an ion exchange resin column.

2.5.3 Blood parameters

Hematocrit was determined using the micro hematocrit technique. Plasma chemical parameters were measured spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP- 4430, Arkray, Inc. Kyoto, Japan). Biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were also measured spectrophotometrically from blood plasma with an automated analyzer FRAS4, Diacron International s.r.l., Grosseto, Italy by following Morganti *et al.* (2002). Briefly for BAP measurement, 50 µl of R2 reagent (ferric salt) was transferred to R1 reagent (a particular thiocyanate derivative), mixed gently and read on a photometer at a wave length of 505 nm. Then, 10 µl plasma sample was added to the cuvette, mixed gently and read on the previous photometer. In case of d-ROMs, 20 µl plasma was added to 1 ml R2 reagent (a buffer, pH 4.8) and mixed gently. Then, 10 µl R1 reagent (an alkylamine) was added and incubated for 1 min at 37 °C. The resulting deep red coloring was photometrically detected at a wavelength of 505 nm. Free radicals were expressed in Carratelli Units (U.Carr). One Carratelli Unit is equal to a hydrogen peroxide concentration of 0.08% mg. Total serum protein (TSP) was determined by SPOTCHEM™ EZ model SP-4430 system (Tatsumi *et al.*, 2000).

2.5.4 Immune parameters

2.5.4.1 Lysozyme activity

Lysozyme activity was determined with turbidometric assays (Lygren *et al.*, 1999). Ten microliters of serum or mucus samples was put into well of microplate, then added 190 µl of substrate (0.2 mg *Micrococcus lysodeikticus*, lyophilized cell, Sigma, USA)/ml PBS, pH 7.4, their absorbance were measured after 1 and 5 min, which were incubated with gentle shaking at constant room temperature, at 450 nm with ImmunoMini NJ-2300 (System Instruments, Tokyo, Japan). A unit of enzyme activity was defined as the amount of enzyme that causes a decrease in absorbance of 0.001/min.

2.5.4.2 Bactericidal activity

A bactericidal activity was measured according to Iida *et al.* (1989). Samples were diluted 3, 4 and 5 times with a Tris buffer (pH 7.5). The dilutions were mixed with a bacterial suspension (0.001 g/ml, *Escherichia coli*, IAM1239 cell line, Kagoshima, Japan) and incubated at 25 $^{\circ}$C for 24 h in micro tube rotator (MTR-103, AS ONE, Osaka, Japan). 50 µl of the reaction solutions were incubated on TSA (Trypto-Soya agar, Nissui Pharmaceutical Co. Ltd., Japan) at 25 $^{\circ}$C for 24 h (on X-plate). Colony forming unit (CFU) was counted by the plate counting method as described by Ren *et al.* (2007).

2.5.4.3 Peroxidase content

The total peroxidase content in serum was measured according to Salinas *et al.* (2008), with some modifications. Briefly, 15 µl of serum were diluted with 35 µl of Hank's Buffered salt solution (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. Then, 50 µl of peroxidase substrate (3, 30, 5, 50-tetramethylbenzidine hydrochloride) (TMB; Thermo Scientific Inc., USA) was added. The serum mixture was incubated for 15 min. The colour-developing reaction in serum samples was stopped by adding 50 µl

of 2 M sulphuric acid and the OD (450 nm) was measured in a plate reader. PBS was used as a blank instead of serum.

2.5.4.4 Serum alternative complement pathway

Serum alternative complement pathway (ACP) activity was assayed according to Yano (1992). Briefly, a series of volumes of the diluted serum ranging from 0.1 to 0.25 ml were dispensed into test tubes and the total volume made up to 0.25 ml with barbitone buffer in the presence of ethyleneglycol-bis (2-aminoethoxy)-tetraacetic acid (EGTA) and Mg^{2+}, then 0.1 ml of rabbit red blood cells (RaRBC) was added to each tube. After incubation for 90 min at 20 °C, 3.15 ml 0.9% NaCl was added. Following this, the sample was centrifuged at 1600 ×g for 10 min at 4 °C to eliminate unlysed RaRBC. The optical density of the supernatant was measured at 414 nm. A lysis curve was obtained by plotting the percentage of haemolysis against the volume of serum added. The volume yielding 50 % haemolysis was determined and in turn used for assaying the complement activity of the sample (ACH_{50} value = units/ml) for each experimental group.

2.5.4.5 Nitro blue tetrazolium assay

The nitro blue tetrazolium (NBT) assay as described by Anderson and Siwicki (1995) with some modifications by Kumari and Sahoo (2005). Briefly, Blood (0.1 ml) was placed in microtiter plate wells, to which an equal amount of 0.2% NBT solution (Sigma, USA) was added and incubated for 30 min at room temperature. A sample of NBT blood cell suspension (0.05 ml) was added to a glass tube containing 1m l N, N-dimethylformamide (Sigma, USA) and centrifuged for 5 min at 3000 rpm. Finally, the optical density of supernatant was measured at 540 nm. Dimethylformamide was used as the blank.

2.5.5 Antioxidant enzymes activities and lipid peroxidation

Serum superoxide dismutase (SOD) activity was measured by the percentage reaction inhibition rate of enzyme with WST (Water Soluble Tetrazolium dye) substrate and xanthine oxidase using a SOD Assay Kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's instructions. The percent inhibition was normalized by mg protein and presented as SOD activity units. One unit of SOD is defined as the amount of the enzyme in 20 µl of sample solution that inhibits the reduction reaction of WST-1 with superoide anion by 50%. The catalase (CAT) enzyme activity was performed using spectrophotometric determination of hydrogen peroxide (H_2O_2) which form stable complex with ammonium molybdate (Goth, 1991). The serum (50 µl) was added to the 1.0 ml substrate (65 µmol per ml H_2O_2 in 60 mmol l^{-1} phosphate buffer, pH 7.4) and incubated for 60 s at 37 °C. The enzymatic reaction was stopped with 1.0 ml of 32.4 mmol l^{-1} ammonium molybdate ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$). A yellow complex of ammonium molybdate and hydrogen peroxide was formed. The absorbance of this yellow colour was measured at 405 nm with a spectrophotometer (HACH, DR/4000U) against a blank (serum was replaced with distilled water). One-unit CAT decomposes 1 µmol of hydrogen peroxide per minute under assay conditions. CAT activities are expressed as kilo unit per liter. The malondialdehyde (MDA) concentration was used as a marker of lipid peroxidation in fish serum and measured using Colorimetric TBARs Microplate Assay Kit (Oxford Biomedical Research, Inc., USA) according to the manufacturer's instructions. The absorbance was measured at 532 nm. The MDA level was expressed as nmol per ml serum.

2.5.6 Amount of secreted mucus

Amounts of secreted mucus were quantitated by the method described in Kakuta *et al.* (1996). Secreted mucus from 3 fish in each test group with a constant area on the body surface (200 mm^2 on dorsal side) was collected by a small piece of sterilized cotton, and immediately suspended in 1 ml of phosphate buffered saline (PBS, pH = 7.4) and centrifuged (3000×g for 5 min under 4 $^{\circ}$C). Total protein in the mucus supernatant was determined by the method of Lowry *et al.* (1951). Amounts of secreted mucus were expressed as mean relative values on the basis of total protein in the mucus sample of HK-LP or BG free group (Yokoyama *et al.*, 2006).

2.5.7 Low salinity stress test

Tolerance against exposure to low salinity seawater was examined. After the feeding trial, fish were randomly selected, pooled and transferred from usual seawater (3.5 %) directly into a 30-L black tank containing low-salinity water (0.2 %). The city water was de-chlorinated by strongly aerating for at least 24 h and mixed with seawater, and then used as low salinity water. Salinity of the test solution was confirmed with a reflect photometer. The passing of time to reach 50% death was calculated according to Moe *et al.* (2004) as follows: time to death (min) was converted to $\log_{10}$ values. When the fish were exposed to low-salinity water at first, they were still alive. Therefore, the survival was assumed to be 100%, which was converted to a log value [$\log_{10}(100) = 2$]. The calculation was conducted based on the data as every 10 minutes. These values of log survival rate were plotted against the time of death to determine the duration of 50% mortality of fish in each treatment.

The equation is as follows:

$Y = a X + b$

Where Y = $\log_{10}$ (survival), X = time to individual death of fish (min). LT_{50} (X) was obtained when Y = 1.7 as $\log_{10}$ (50) = 1.7. This test was conducted in duplicates for each experimental treatment.

2.5.8 Digestibility assessment

A digestibility trial was conducted at the end of the feeding period by pooling the remaining fish from the same treatments and distributing randomly into triplicates tanks. The fish were fed a diet containing chromium oxide (Wako Pure Chemical Industries, Ltd) as the inert marker at a level of 0.5% (Cr_2O_3) to the previous test diets formulations and fed to the fish under the same condition as the feeding experiment apart from the difference in stocking density. After 5-day adaptation of the diets, feces were collected at 3-h intervals between the morning and afternoon feeds using a siphon. Feces collection continued for two weeks until a sufficient amount of feces had been collected for analysis. Feces were freeze-dried immediately and kept at −20 °C until analysis. Concentration of chromium oxide in diets and feces was determined according to Furukawa and Tsukahara (1966).

2.5.9 Intestinal bacterial counts

Another nine fish were sampled in each treatment after cease of feeding for 24 h to enumerate the total intestinal microbiota and lactic acid bacteria (LAB). The fish were transferred from the rearing tank onto a clean bench. The surface of the fish body was sterilized with 70% ethanol before opening the ventral surface with sterile scissor. Intestinal tract of fish was removed, weighed and washed thoroughly three times with PBS. Samples were serially diluted with PBS and 100 µl of the solution was spread onto triplicate TSA (Trypto-Soya agar, Nissui Pharmaceutical Co. Ltd., Japan) media

to determine total bacterial populations. DeMan, Rogosa and Sharpe (MRS, Merck) were also used to detect viable LAB species.The agar plate inoculated with each dilution was incubated for 3 to 5 days at 25°C. Colony forming units (CFU ml^{-1}) were determined for viable bacterial populations (Nikoskelainen *et al.*, 2003).

2.6 Statistical analysis

All data were presented as means values ± standard error of mean (S.E.M., n=3). Data from each group were compared to control group using Tukey's test (one-way ANOVA). Differences between them were considered significant when P<0.05. Two-way ANOVA was employed to test the interaction effects of dietary feed additives excluding the control diet.

Chapter III:

Efficiency of probiotics and prebiotics as feed additives for cultured fish species

Experiment I: Effects of heat killed *Lactobacillus plantarum* (LP20) supplemental diets on growth performance, stress resistance and immune response of red sea bream, *Pagrus major*

Effects of heat killed *Lactobacillus plantarum* (LP20) supplemental diets on growth performance, stress resistance and immune response of red sea bream, *Pagrus major*

3.1.1 Abstract

A feeding trial was carried out to determine effects of heat-killed *Lactobacillus plantarum* (HK-LP) for red sea bream (*Pagrus major*). Five dietary levels of a commercial product containing 20% HK-LP at 1, 10, 100, 1000, and 2000 mg kg^{-1} diets were supplemented to the basal diet (control), respectively. Triplicate groups of fish (initial weight: 11 g) were stocked in 100-L polycarbonate circular tanks at a density of 12 fish per tank under the flow-through system, and were fed the respective test diets for 56 days. At the end of a feeding trial, the results showed that the fish fed the diet at 10, 100, 1000 and 2000 mg kg^{-1} HK-LP significantly grew faster than control group. Similarly, significantly improved feed intake, feed efficiency ratio, protein retention, and apparent digestibility coefficients were also found at 1000 mg kg^{-1} HK-LP group than those in HK-LP free group. Some parameters such as serum lysozyme activity, total serum protein, mucus secretion, and the tolerance against low salinity stress were improved in fish fed 1000 mg kg^{-1} HK-LP compared to those in HK-LP free group. This study demonstrated that HK-LP enhanced non-specific immune defense system of red sea bream, providing them with higher resistance to the stress and better immune response.

Keywords: Heat killed bacteria, *Lactobacillus plantarum*, Red sea bream, Growth performance, Blood parameters, Immune response

3.1.2 Introduction

The success of modern aquaculture requires good management practices. In aquaculture, antibiotics and chemotherapy have been applied to prevent disease outbreaks and control proliferation of pathogens, causing proliferation of bacteria resistant to drugs for a long time (Miranda and Zemelmen, 2002). More recently, the administration of probiotics to fish seems to be a very promising control measure for the fish farms (Kesarcodi-Watson *et al.*, 2008). Some concerns may arise in aquaculture due to the oral delivery of probiotics that may introduce live bacteria into the environment. Thus, the use of inactivated bacteria shed a light on such a safety-related issue since they can no longer interact with other aquatic organisms (D´ıaz-Rosales *et al.*, 2006). Currently, the definition of probiotics includes the metabolites of live or dead bacterial cells, which function as immunostimulants for modification of enzyme activity or microflora in gastrointestinal tracts that have beneficial effects on host health (Naidu *et al.*, 1999; Salminen *et al.*, 1999). Several works reported increased disease resistance (Giri *et al.*, 2014; Son *et al.*, 2009) and stronger immune responses (Giri *et al.*, 2013; Salinas *et al.*, 2005) in fish fed live bacteria supplemented diets. On the other hand, dietary supplementation of inactivated bacteria stimulated fish innate immune parameters (Biswas *et al.*, 2013a; Cerezuela *et al.*, 2012a; D´ıaz-Rosales *et al.*, 2006) and increased disease resistance (Biswas *et al.*, 2013b; Pan *et al.*, 2008). Inactivated probiotic preparations appear as an interesting alternative to live probiotics, which could potentially cause safety problems in open aquatic environments. A comparative study between killed and live bacteria on immune responses showed that heat killed *C. butyrium* retain interesting immunomodulating properties on Chinese drum, *M. miiuy* (Pan *et al.*, 2008), and heat killed bacterins of *V. vulnificus* induced a better antibody

response than that induced by formalinised bacterins in flounder, *P. olivaceus* (Park *et al.*, 2001).

Lactobacillus plantarum is a gram-positive, heterofermentative lactic acid bacterium. The bacterium has a high adapting capacity to many environmental conditions, and is effective in suppressing the growth of pathogenic and spoilage microorganisms by secreting bacteriocin in food. In aquaculture, administration of live *L. plantarum* induced immune modulation, enhanced the growth performance, immune ability, and increased disease resistance in fish (Giri *et al.*, 2013; Giri *et al.*, 2014; Son *et al.*, 2009). Heat-killed *L. plantarum* (HK-LP) was used as an immunostimulant to induce interleukin-12 production and antitumor effect in mice, and to enhance gamma interferon production, which stimulates a substance that suppresses virus reproduction and other T-cells and activates macrophages cells (Murosaki *et al.*, 1998, 2000). Daily intake of HK-LP as an immunostimulant enhanced immunity response in healthy adults (Hirose *et al.*, 2006) and broiler chickens (Khonyoung and Yamauchi, 2012). Moreover, daily intake of HK L-137 stimulates innate immunity for production of type I interferon (IFN) in humans and pigs (Arimori *et al.*, 2012). In aquatic animals, Tung *et al.* (2010) concluded that the stress resistance is higher in larval and post larval kuruma shrimp, *Marsupenaeus japonicas* fed a diet containing HK-LP. Recently, Biswas *et al.* (2013a) reported the potential effects of HK-LP (strain 06CC2) isolated from the Mangolian dairy products as novel immunostimulant to fish. In the light of these observations, it could be hypothesized that HK-LP might be effective in responses of growth and biological defense systems of fish species. At the same time, the collective data on the effects of HK-LP should be investigated to find the effective use of HK-LP for marine species.

Red sea bream (*Pagrus major*) is one of the most economically cultured warm water marine fish in Japan as well in the world. Until now, there are no studies on the application of probiotics for red sea bream. Therefore, this study aims to investigate the effect of the oral administration of HK-LP on the growth, survival, immune response, and stress resistance of juvenile Red sea bream.

3.1.3 Materials and methods

3.1.3.1 *Preparation of HK-LP*

HK-LP Prep (LP20) was made by House Wellness Foods Corp. (Itami, Japan), and it contains 20% HK-LP and 80% dextrin in dried-weight basis. The concentration of HK-LP in the dry product is 2×10^{11} cfu/g. HK-LP Prep was prepared based on the method previously described (Murosaki *et al.*, 1998). The product was stored at −20 °C until use.

3.1.3.2 *Test fish and experimental system*

The feeding trial was carried out at the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. The juvenile red sea bream, *Pagrus major*, were purchased from a private fish hatchery (Ogata Suisan Co, Kumamoto, Japan). The fish were acclimatized for two weeks in the laboratory condition and reared in a 500-L tank with flow-thru system. During this period, a commercial diet (50% crude protein; Higashimaru, Japan) was supplied to the fish. The feeding trial was conducted in 100-L polycarbonate tanks (filled with 80 L of water) in a flow through sea water system where each tank was equipped with an inlet, outlet, and continuous aeration. The tanks were maintained under natural light/dark regime. The seawater was pumped from the deep basin of Kagoshima bay, Japan; gravel filtered and supplied to the system. A flow rate of 1.5 L min^{-1} was maintained throughout the experimental period. During the

experimental period, the monitored water quality parameters (mean ± S.D.) were: water temperature 26.1±1.7 °C; pH 8.1±0.5 and salinity 33.1±0.5. These ranges are considered within optimal values for juvenile red sea bream.

3.1.3.3 *Test diets*

The formulation and chemical composition of the experimental diets are shown in Tables 3.1.1 and 3.1.2 respectively, which followed (Yokoyama *et al.,* 2005; Ren *et al.*, 2008) with slight modification. Protein source was brown fishmeal which contained 67% of crude protein and 8% of crude lipid and casein which contained 87% of crude protein. The lipid sources were Pollack liver oil and soybean lecithin. Activated gluten was used as a binder to produce pellet diet. α-starch and dextrin was the carbohydrate source in the diets. HK-LP Prep was supplied in the diets with six levels: 0 (as control diet), 1, 10, 100, 1000, and 2000 mg kg^{-1} diet. Cellulose powder was used to adjust to 100% total proportion. HK-LP Prep was thoroughly mixed with lipid before adding other ingredients. Pollack liver oil, soybean lecithin and HK-LP Prep were premixed with a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan), added to the dry ingredients and mixed for another 15 min. The diets were prepared by thoroughly mixing all the dry ingredients in a food mixer for 15 min. the required amount was mixed with water (35–40% of the dry ingredients), and then added to the premixed ingredients and mixed for another 15 min. The pH of the diets was adjusted to the range of 7.0–7.5 with 4 N sodium hydroxide. The mixture was then passed through a meat grinder with an appropriate diameter (1.2–2.2 mm) to prepare pellets, which were then dried in a dry-air mechanical convection oven (DK 400, Yamato Scientific, Tokyo, Japan) at 50 °C for about 120 min. to approximately 11% moisture, the test diets were stored at −28 °C in a freezer until use.

Table 3.1.1: Basal diet composition

Ingredients	g/kg dry diet
Brown Fish meal[1]	280
Casein[2]	280
Dextrin[3]	70
α-starch[4]	60
Soybean lecithin[5]	50
Pollack liver oil[6]	60
Vitamin mixture[7]	30
Mineral mixture[8]	30
Stay-C[9]	0.8
Activated gluten[10]	50
α-Cellulose+HK-LP Prep.[11]	89.2

[1]Nippon Suisan Co. Ltd (Tokyo, Japan), [2]Wako Pure Chemicals Industries, Inc. (Osaka, Japan), [3]Kanto Chemicals (Tokyo, Japan), [4]Asahi Chemicals (Wakayama, Japan), [5, 6]Riken Vitamin, Tokyo, Japan, [7]Vitamin mixture (g kg^{-1} diet): according to Dawood et al. (2015a) [8]Mineral mixture (g kg^{-1} diet): according to Dawood et al. (2015a), [10]A-glu SS: Glico Nutrition Company Ltd. Osaka, Japan, [11]HK-LP: Heat-killed *Lactobacillus plantarum* (LP20).

Table 3.1.2: Chemical analysis of the experimental diets (% dry matter basis)

Proximate composition	HK-LP supplemented (mg/kg)					
	0	1	10	100	1000	2000
Crude protein	50.8	50.6	51.3	50.9	51.3	51.1
Total lipid	14	14.4	14	14.2	14.4	14.2
Ash	10.7	10.7	10.7	10.8	10.9	10.8
Gross energy (KJ g^{-1}) *	20.2	20.2	20.1	20.1	20.2	20.2

*Calculated using combustion values for protein, lipid and carbohydrate of 23.6, 39.5 and 17.2 kJ g^{-1}, respectively.

3.1.3.4 *Feeding protocol*

At the beginning of the feeding trial, juveniles (average body weight, 11±0.03 g) (mean±S.D.), were randomly stocked in previously prepared eighteen tanks at a stocking density of 12 fish per tank with triplicates per dietary treatment. All fish were fed the respective test diets visually near satiation level (at 7 to 8% of body weight) by hand twice a day, 7 days per week for 56 days. Any uneaten feed left was removed one hour after feeding and dried using a freeze drier, weighing and finally subtracted from the total amount of supplied diets to calculate the actual feed intake. The water quality was checked regularly. All fish were weighed in bulk at 2 weeks' interval to determine growth, check their health condition and ration was adjusted according to mean fish weight.

3.1.3.5 *Sample collection and biochemical analysis*

A pooled sample of 10 fish at the beginning was stored at −20 °C for whole body analysis. At the end of the feeding trial, all fish were fasted for 24 h prior to final sampling. Then the total number, individual body weight and length of fish from each tank were measured. Blood was taken from the caudal vein of nine fish per treatment (three fish per each tank) using heparinized disposable syringes. Hematocrit was determined using the micro hematocrit technique. Plasma samples were obtained by centrifugation at 3000×g for 15 min ×4 °C using a high-speed refrigerated micro centrifuge (MX-160; Tomy Tech USA Inc., Tokyo, Japan) and kept at −80 °C. In addition, non-heparinized disposable syringes were used to collect blood for serum analysis. Serum samples were obtained by centrifugation at 3000×g for 15 min4 °C to collect serum. Nine fish per treatment (three fish per each tank) were randomly sampled used for collect skin mucus and liver. Liver was dissected out, then, weighed to get hepatosomatic index (HSI). Fish for skin mucus were washed with distilled water

individually, and skin mucus was collected from body surface (200 mm^2) by using a small piece of sterilized cotton and immediately suspended in 1 ml of phosphate-buffered saline (PBS, pH=7.4) and centrifuged (2000×g, 10 min., 4 °C) (MX-160, Tomy Seiko Co., LTD, Tokyo, Japan), and the supernatant in the under tube was transferred into anther centrifugal tube (510-GRD, QSP, San Diego, CA, USA) and kept at -80°C until further analysis were performed.

The experimental diets and fish whole body were analyzed for moisture, crude protein, total lipid and ash, in triplicate, using standard methods (AOAC, 1990), including moisture by oven-drying at 110 °C to constant weight, crude protein by the Kjeldahl method, crude lipid by the Soxhlet extraction method and ash by combustion in Muffle furnace at 550 °C for 4h. Plasma chemical parameters were measured spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan). Biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were also measured spectrophotometrically from blood plasma with an automated analyzer FRAS4, Diacron International s.r.l., Grosseto, Italy by following Morganti *et al.* (2002). Briefly for BAP measurement, 50 µl of R2 reagent (ferric salt) was transferred to R1 reagent (a particular thiocyanate derivative), mixed gently and read on a photometer at a wave length of 505 nm. Then, 10 µl plasma sample was added to the cuvette, mixed gently and read on the previous photometer. In case of d-ROMs, 20 µl plasma was added to 1 ml R2 reagent (a buffer, pH 4.8) and mixed gently. Then, 10 µl R1 reagent (an alkylamine) was added and incubated for 1 min at 37 °C. The resulting deep red coloring was photometrically detected at a wavelength of 505 nm. Free radicals were expressed in Carratelli Units (U. Carr). One Carratelli Unit is equal to a hydrogen peroxide concentration of 0.08% mg. Total serum protein (TSP) was determined by SPOTCHEM™ EZ model SP- 4430system (Tatsumi *et al.*, 2000).

3.3.6 *Lysozyme activity of Serum and Mucus*

Lysozyme activity of serum and mucus was determined with turbidometric assays (Lygren *et al.*, 1999). Ten microliters of serum or mucus samples was put into well of microplate, then added 190 µl of substrate (0.2 mg *Micrococcus lysodeikticus*, lyophilized cell, Sigma, USA)/ml PBS, pH 7.4, their absorbance were measured after 1 and 5 min, which were incubated with gentle shaking at constant room temperature, at 450 nm with ImmunoMini NJ-2300 (System Instruments, Tokyo, Japan). A unit of enzyme activity was defined as the amount of enzyme that causes a decrease in absorbance of 0.001/min.

3.1.3.7 *Amount of secreted mucus*

After 56 days feeding trial, amounts of secreted mucus were quantitated by the method described in Kakuta *et al.* (1996). Total protein in the mucus supernatant was determined by the method of Lowry (Lowry *et al.*, 1951). Amounts of secreted mucus were expressed as mean relative values on the basis of total protein in the mucus sample of HK-LP free group.

3.1.3.8 *Low salinity stress test*

Tolerance against exposure to low salinity seawater was examined. After the feeding trial, total 8 fish per treatment were randomly selected, pooled and transferred from usual seawater (3.5 %) directly into a 30-L black tank containing low-salinity water (0.2 %). The city water was de-chlorinated by strongly aerating for at least 24 h and mixed with seawater, and then used as low salinity water. Salinity of the test solution was confirmed with a reflect photometer. The passing of time to reach 50% death was calculated according to Moe *et al.* (2004) as follows: time to death (min) was converted to $\log_{10}$ values. When the fish were exposed to low-salinity water at first, they were still alive. Therefore, the survival was assumed to be 100%, which was converted to a log

value [$\log_{10}(100) = 2$]. The calculation was conducted based on the data as every 10 minutes. These values of log survival rate were plotted against the time of death to determine the duration of 50% mortality of fish in each treatment.

The equation is as follows:

$Y = a X + b$

Where $Y = \log_{10}$(survival), X = time to individual death of fish (min). LT_{50} (X) was obtained when $Y = 1.7$ as $\log_{10}(50) = 1.7$. This test was conducted in duplicates for each experimental treatment.

3.1.3.9 *Digestibility assessment*

A digestibility trial was conducted at the end of the feeding period by pooling the remaining fish from the same treatments and distributing randomly into triplicates tanks (3 fish per tank). The fish were fed a diet containing chromium oxide (Wako Pure Chemical Industries, Ltd) as the inert marker at a level of 1% (Cr_2O_3) to the previous test diets formulations and fed to the fish under the same condition as the feeding experiment apart from the difference in stocking density. After 5-day adaptation of the diets, feces were collected at 3-h intervals between the morning and afternoon feeds using a siphon. Feces of red sea bream very rapidly settled to the bottom of the tank and did not easily break up in the water so that nutrient and marker losses were minimized. Feces collection continued for two weeks until a sufficient amount of feces had been collected for analysis. Feces were freeze-dried immediately and kept at −20 °C until analysis. Concentration of chromium oxide in diets and feces was determined according to Furukawa and Tsukahara (1966). At the end of digestibility trial nine fish per treatment (3 fish per tank) were randomly collected, washed with distilled water and then stored at −80°C (MDF-U383, SANYO, Tokyo, Japan) for the whole body proximate analysis.

3.1.3.10 *Evaluation of growth performance parameters*

The following variables were evaluated:

Weight gain (%) = (final weight – initial weight) ×100/initial weight; Specific growth rate (SGR %, day^{-1}) = {(Ln (final weight) –Ln (initial weight)) / duration (56 days)} ×100; Survival (%) =100× (final no. of fish/ initial no. of fish); Feed intake (FI, g $fish^{-1}$ 56 $days^{-1}$) = (dry diet given − dry remaining diet recovered)/ no. of fish; Feed efficiency ratio (FER) = live weight gain (g) /dry feed intake (g); Protein efficiency ratio (PER) = live weight gain (g)/dry protein intake (g)

Protein gain (PG, g kg weight $gain^{-1}$) = {(final weight (g) × final whole body protein content (%)/100) − (initial weight (g) × initial whole body protein content (%)/100)}/ (weight gain (g)) ×1000; Protein retention (PR, % of intake) = (protein gain (g kg weight $gain^{-1}$) ×100)/ protein intake (g kg weight $gain^{-1}$); Condition factor (CF) = weigh of fish (g)/ (length of fish) ^{3} (cm) ^{3} ×100; Hepatosomatic index (HSI, %) =weight of liver /weight of fish×100; Apparent digestibility coefficient (ADC, %) =100-[(%Cr_2O_3 in diet/% Cr_2O_3in feces) × (% nutrient in feces/% nutrient in diet)].

3.1.3.11 *Statistical analysis*

All data were subjected to statistical verification using package super ANOVA 1.11, Abacus Concepts, Berkeley, California, USA. Probabilities of $P<0.05$ were considered significant. Significance differences between means were evaluated using the Tukey Kramer test.

3.1.4 Results

3.1.4.1*Growth parameters and nutrient utilization*

Growth performance, nutrient utilization and survival of red sea bream juveniles fed test diets for 56 days are shown in Table 3.1.3. All treatments showed high survival

rates between 91.7% and 100% and there were no significant differences among all groups (P>0.05). Growth performance and nutrient utilization of fish in all groups generally increased during the trial; however, the increase was not always statistically significant. The highest values of final body weight, %WG (weight gain), and SGR (specific growth rate) were obtained in fish fed a diet containing 1000 mg kg^{-1} HK-LP. But the values were not significantly different from those of fish fed a diet containing 100 mg kg^{-1} HK-LP (P<0.05). FI (feed intake), FER (feed efficiency ratio), PER (protein efficiency ratio), PG (protein gain), and PR (protein retention) showed the similar trends. The poorest growth performances and nutrient utilization were obtained in HK-LP free group although the significant differences on the parameters measured were not detected from those in fish fed a diet containing 1 mg kg^{-1} HK-LP.

3.1.4.2*Whole body proximate analysis*

The whole body proximate compositions of red sea bream are shown in Table 3.1.4. All the fish showed some changes in the analyzed parameters compared to those of the initial values. However, there were no significant differences (P>0.05) in the final whole body proximate compositions among all groups of fish fed the different experimental diets. No differences were also detected in CF and HSI of fish among treatments (Table 3.1.4).

3.1.4.3 *Blood parameters and responses against stress*

Table 3.1.5 represents the blood parameters of red sea bream after 8 weeks of feeding trial. Hematocrit was significantly higher in fish fed diet containing 1000 mg kg^{-1} HK-LP than 1 mg kg^{-1} HK-LP group (P<0.05), however no significant differences were detected between other experimental groups. Plasma total protein was significantly higher in diet groups at 100, 1000 and 2000 mg kg^{-1} HK-LP than 0 and 1 mg kg^{-1} HK-LP groups (P<0.05). The supplementation level of 1000 mg kg^{-1} HK-LP recorded a

significantly highest value than the other supplemented levels (P<0.05). GOT and T-Cho of fish fed HK-LP free diet was significantly higher than other experimental groups (P<0.05). Similarly, TG of fish fed HK-LP free diet was significantly higher than that fed diet with supplementation level 1000 mg kg^{-1} HK-LP (P<0.05), however no significant differences were observed in other groups.

Figure 3.1.1 shows the results of low salinity stress test. The fish that received a diet containing 1000 mg kg^{-1} HK-LP showed significantly higher tolerance against low salinity stress than those of other treatments (P<0.05). Furthermore, 10, 100, and 2000 mg kg^{-1} HK-LP groups showed significantly higher tolerance against low-salinity stress than HK-LP free group (P<0.05). Oxidative status of fish was analyzed from plasma (Fig. 3.1.2). Reactive oxygen metabolites (d-ROMs) were affected by the supplementation levels of HK-LP, in which 1000 mg kg^{-1} HK-LP supplemented level recorded a significantly lowest value than those of the other supplemented groups (P<0.05). On the other hand, biological antioxidant potential (BAP) was found highest in 1000 mg kg^{-1} HK-LP group and lowest in HK-LP free group. Fig. 3.1.2 shows the pattern of combined effects of d-ROMs and BAP. The 100, 1000 and 2000 mg kg^{-1} HK-LP groups were located in zone A, 1 and 10 mg kg^{-1} HK-LP groups in zone B and HK-LP free group zone C, respectively.

3.1.4.4 *Non-specific immune responses*

Table 3.1.6 shows non-specific immune parameters. There was no significant difference in mucus lysozyme activity among all treatment groups. However, serum lysozyme activity and TSP results showed that those of the fish at 1000 mg kg^{-1} HK-LP were significantly higher than HK-LP free group (P<0.05), and there was no significant difference in serum LA and TSP of fish fed diet containing 1000 mg kg^{-1} HK-LP and other supplemented groups except HK-LP free group.

3.1.4.5 *Amounts of secreted mucus*

Significantly higher amounts of secreted mucus were observed (Fig. 3.1.3) in all HK-LP supplemented groups than that in HK-LP free group. Although the highest concentration was found in fish fed 2000 mg kg^{-1} HK-LP, the value was not significantly different from those of fish fed 100 and 1000 mg kg^{-1} HK-LP ($P>0.05$), but that was significantly higher than those from fish fed 1 and 10 mg kg^{-1} HK-LP, respectively ($P<0.05$).

3.1.4.6 *Digestibility*

The apparent digestibility coefficients of dry matter (ADC_{DM}), protein ($ADC_{Protein}$), and lipid (ADC_{Lipid}) were found to be significantly different ($P<0.05$) with highest being in 1000 mg kg^{-1} HK-LP group than HK-LP free group (83% for DM, 91.3% for protein, and 88.7% for lipid) (Table 3.1.7).

Table 3.1.3: Growth parameters and nutrient utilization in red sea bream fed test diets for 56 days [Within a row, values with different letters are significantly different (P<0.05)]

Parameters	HK-LP supplemented (mg/kg)					
	0	1	10	100	1000	2000
In wt[1]	11.0±0.03	11.0±0.03	11.0±0.01	11.0±0.01	11.1±0.06	11.0±0.02
Fn wt[2]	53.8±0.6^{a}	57.6±1.3^{ab}	59.8±1.3^{b}	61.1±0.9^{b}	67.2±1.3^{c}	60.1±0.9^{b}
WG[3]	389.8±6.8^{a}	423.9±12.6^{ab}	442.9±12.1^{b}	457.2±8.09^{bc}	507.7±10.8^{c}	445.6±8.74^{b}
SGR[4]	2.84±0.03^{a}	2.96±0.04^{ab}	3.02±0.04^{b}	3.07±0.03^{bc}	3.22±0.03^{c}	3.03±0.03^{b}
FI[5]	45.0±0.3^{a}	45.1±0.4^{a}	47.1±0.3^{b}	48.1±0.4^{b}	52.1±0.2^{c}	46.7±0.3^{b}
FER[6]	0.95±0.01^{a}	1.04±0.03^{ab}	1.04±0.03^{ab}	1.04±0.02^{ab}	1.08±0.03^{b}	1.05±0.02^{ab}
PER[7]	1.87±0.02^{a}	2.04±0.07^{ab}	2.02±0.05^{ab}	2.05±0.04^{ab}	2.1±0.06^{b}	2.06±0.03^{ab}
PG[8]	157.1±5.0^{ab}	157.7±3.4^{ab}	152.7±4.2^{a}	153.3±0.71^{a}	170.5±2.6^{b}	163.0±2.5^{ab}
PR[9]	36.0±1.3^{a}	36.0±0.9^{a}	36.5±0.7^{ab}	37.5±0.1^{ab}	45.5±0.8^{c}	38.9±0.6^{b}
SR[10]	91.7	91.7	94.5	97.2	100	94.4

[1]In wt: initial mean weight (g), [2]Fn wt: final mean weight (g), [3]WG: percent weight gain (%), [4]SGR: specific growth rate (% day^{-1}), [5]FI: feed intake (g dry diet fish^{-1} 56 days^{-1}), [6]FER: feed efficiency ratio, [7]PER: protein efficiency ratio, [8]PG: protein gain (g kg body weight gain^{-1}), [9]PR: protein retention (% of intake), [10]SR: survival rates (%).

Table 3.1.4: Effects of dietary administration of HK-LP on the whole body proximate analysis (wet weight basis) and somatic parameters in juvenile red sea bream [Within a row, values with different letters are significantly different (P<0.05)]

Parameters	Initial[1]	HK-LP supplemented (mg/kg)					
		0	1	10	100	1000	2000
Moisture (%)	77.8	68.2±0.2	67.8±0.5	68.8±0.2	68.0±0.1	67.5±0.6	67.4±0.1
CP (%)[2]	14.2	15.5±0.4	15.6±0.3	15.1±0.3	15.3±0.1	16.2±0.1	16.0±0.2
CL (%)[3]	2.75	10.5±0.5	10.8±0.4	10.4±0.3	10.6±0.6	11.3±0.2	10.9±0.1
CA (%)[4]	4.54	4.41±0.02	4.43±0.02	4.54±0.2	4.59±0.03	4.54±0.1	4.52±0.1
CF (%)[5]	_	2.42±0.1	2.5±0.1	2.52±0.1	2.45±0.1	2.46±0.1	2.57±0.1
HSI (%)[6]	_	1.53±0.1	1.56±0.2	1.67±0.1	1.60±0.1	1.37±0.1	1.64±0.1

[1]Initial values are not included in the statistical analysis. [2]CP: crude protein, [3]CL: crude lipid, [4]CA: crude ash, [5]CF: condition factor (%), [6]HSI: hepatosomatic index.

Table 3.1.5: Blood parameters in juvenile red sea bream fed test diets for 56 days*

Parameters	HK-LP supplemented (mg/kg)					
	0	1	10	100	1000	2000
Hematocrit (%)	49.3±0.67^{ab}	47.3±1.45^{a}	49.7±0.88^{ab}	49.3±2.19^{ab}	52±1.15^{b}	50.3±1.2^{ab}
Total protein (g/dl)	4.07±0.09^{a}	4.03±0.09^{a}	4.27±0.12^{ab}	4.33±0.03^{b}	4.7±0.06^{c}	4.33±0.03^{b}
Total albumin (g/dl)	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00
Total bilirubin (mg/dl)	0.3±0.06	0.2±0.00	0.27±0.03	0.23±0.03	0.2±0.00	0.2±0.00
Glucose (mg/dl)	45.0±1.53	44.0±1.16	48.3±0.88	43.7±0.88	48.3±0.33	46.3±1.20
GOT (IU/l)[1]	99.0±1.53^{b}	70.7±5.61^{a}	72.0±0.58^{a}	70.7±2.33^{a}	71.3±0.67^{a}	77.7±1.45^{a}
GPT (IU/l)[2]	<10.00	<10.00	<10.00	<10.00	<10.00	<10.00
BUN (mg/dl)[3]	5.33±0.33	6.67±0.88	6.00±1.00	6.00±0.58	7.00±0.58	7.00±0.58
TG (mg/dl)[4]	120.3±0.9^{b}	117.0±4.0^{ab}	111.3±1.2^{ab}	111.7±4.4^{ab}	107.7±1.2^{a}	113.7±5.8^{ab}
T-Cho (mg/dl)[5]	260.0±4.6^{b}	194.3±1.2^{a}	195.3±2.3^{a}	202.7±4.1^{a}	197.3±3.8^{a}	205.3±6.0^{a}
d-ROMs[6]	89.7±2.3^{bc}	111.0±7.0^{c}	135.3±2.9^{d}	87.7±1.3^{b}	66.0±2.1^{a}	83.7±7.2^{b}
BAP[7]	2208±45^{a}	2917±51^{b}	2942±99^{b}	3026±57^{b}	3394±60^{c}	2835±28^{b}

*Values are means of triplicate groups±SEM. Within a row, values with different letters are significantly different (P<0.05). Absence of letters indicates no significant difference between treatments.

[1]GOT: glutamyl oxaloacetic transaminase, [2]GPT: glutamic-pyruvate transaminase, [3]BUN: blood urea nitrogen, [4]TG: triglyceride, [5]T-Cho: total cholesterol, [6]d-ROMs: reactive oxygen metabolites, [7]BAP: biological antioxidant potential.

Table 3.1.6: Non-specific immune parameters in red sea bream juveniles fed diets containing different level HK-LP for 56 days*

Parameters	HK-LP supplemented (mg/kg)					
	0	1	10	100	1000	2000
Serum LA[1]	15.0±1.4[a]	33.3±7.9[ab]	36.7±4.6[ab]	40.8±15.6[ab]	44.2±6.8[b]	26.7±4.4[ab]
Mucus LA	39.2±8.8	40.8±4.6	48.3±3.6	48.3±4.2	54.8±5.3	46.7±0.8
TSP (g/dL) [2]	3.5±0.1[a]	3.9±0.2[ab]	3.8±0.1[ab]	3.83±0.1[ab]	4.53±0.1[b]	4.07±0.3[ab]

*Values are means of triplicate groups±SEM. Within a row, values with different letters are significantly different (P<0.05). Absence of letters indicates no significant difference between treatments.

[1]LA: lysozyme activity (unit/mL), [2]TSP: total serum protein (g/dL).

Table 3.1.7: Apparent digestibility coefficients (ADC) in red sea bream when measured at the end of feeding trial*

Parameters	HK-LP supplemented (mg/kg)					
	0	1	10	100	1000	2000
ADC $_{Protein}$	88.8±0.3[a]	89.8±0.5[ab]	89.1±0.1[ab]	89.3±0.8[ab]	91.3±0.1[c]	90.4±0.2[bc]
ADC $_{Lipid}$	85.0±0.1[a]	85.3±1.1[a]	85.4±0.6[ab]	86.6±1.1[ab]	88.7±0.6[b]	86.4±0.2[ab]
ADC $_{Dry\ matter}$	80.7±0.3[a]	80.6±0.2[a]	81.8±0.3[ab]	80.8±0.9[a]	83.0±0.1[b]	82.3±0.1[ab]

*Values are means of triplicate groups±SEM. Within a row, values with different letters are significantly different (P<0.05).

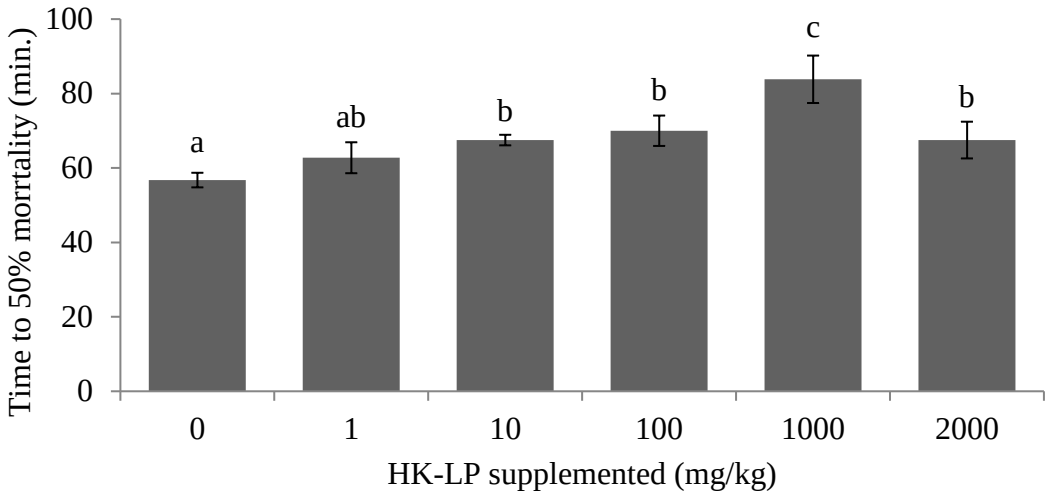


Fig. 3.1.1: Time to 50 % mortality (min.) after low salinity stress test when red sea bream fed diets containing different levels of HK-LP for 56 days. Values are means±pooled SEM from duplicate groups. Values with different letters are significantly different (P<0.05).

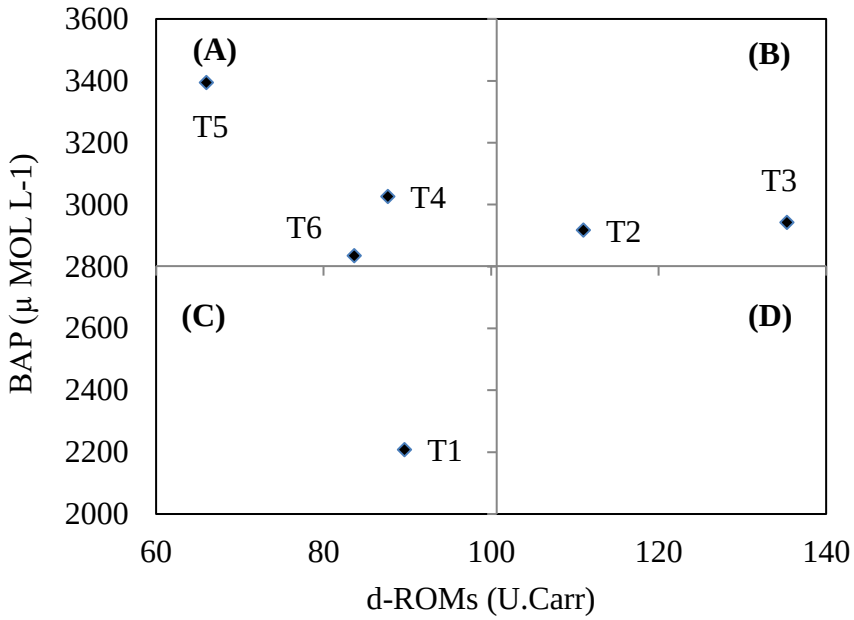


Fig. 3.1.2: Oxidative stress parameters in red sea bream fed test diets for 56 days. Values are means of triplicate groups. T1: HK-LP free, T2: HK-LP 1 mg kg^{-1}, T3: HK-LP 10 mg kg^{-1}, T4: HK-LP 100 mg kg^{-1}, T5: HK-LP 1 000 mg kg^{-1}, T6: HK-LP 2000 mg kg^{-1}.

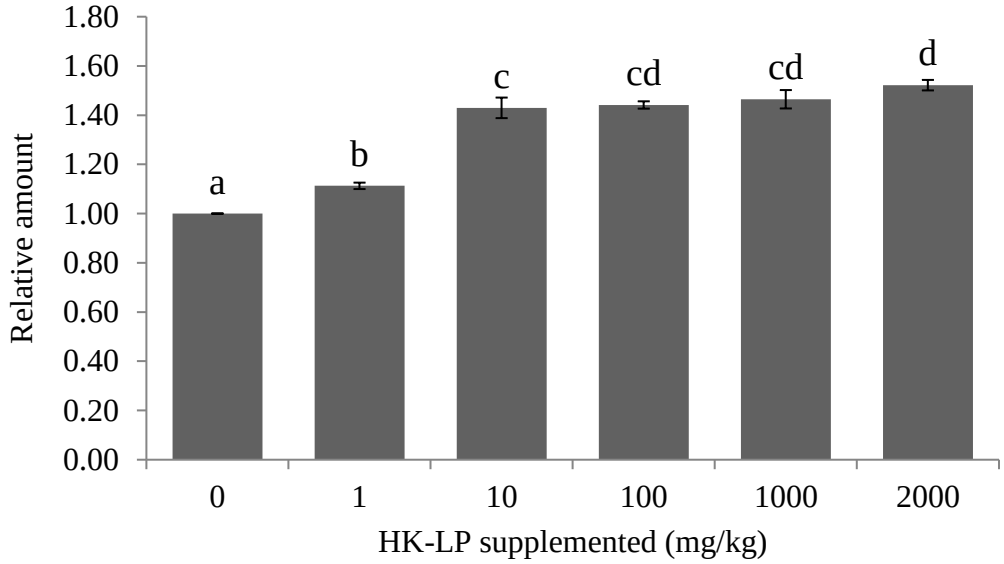


Fig. 3.1.3: Relative amounts of mucus secretion on body surface of red sea bream juvenile. Values are expressed as mean relative values±pooled SEM on the basis of amount of mucus in 0 mg kg^{-1} HK-LP supplemented group (n=9 fish). Values with different letters are significantly different (P<0.05).

3.1.5 Discussion

Since the first use of probiotics in aquaculture, a growing number of studies have demonstrated that bacterial compounds act as an immunostimulant in fish and shrimp (Biswas *et al.*, 2013a; Hoseinifar *et al.*, 2011; Kesarcodi-Watson *et al.*, 2008; Tung *et al.*, 2010; Pan *et al.*, 2008), in which specific cell compounds or non-living cells were commonly used in those studies. To our knowledge, this study was the first study to investigate the effects of HK-LP as beneficial bacteria cells on different performances of red sea bream juveniles.

The obtained data showed that the supplementation of HK-LP with 10, 100, 1000, and 2000 mg kg^{-1} in diets improved the growth performances of red sea bream juveniles. Similar benefits were previously reported in fish fed components derived from bacteria and yeast (Hoseinifar *et al.*, 2011; Rodriguez-Estrada *et al.*, 2013; Taoka *et al.*, 2006a). Rodriguez-Estrada *et al.* (2013) also reported that enhanced weight gain and feed efficiency were generally observed in rainbow trout fed diets supplemented with heat killed *Enterococcus faecalis* (at 0.25-0.5%) compared to fish fed basal diets. Additionally, Tung *et al.* (2010) observed that Kuruma shrimp, *Marsupenaeus japonicus* fed a diet containing HK-LP (at 0.1%) displayed significantly increased growth performance compared to the control fed group. From previous studies, it has been suggested that enhanced growth performance of fish fed dietary HK-LP might be attributed to elevated health status, digestibility, stimulation of gastric development and/or enzymatic secretion (Khonyoung and Yamauchi, 2012; Rodriguez-Estrada *et al.*, 2013; Tovar-Ramirez *et al.*, 2002). The results in the present study suggested that tested fish utilized experimental diets effectively by HK-LP supplementation resulting in increasing feed intake (FI), which would be one of the reasons for the faster growth in the groups. This leads to the fact that higher FI would increase the amounts of protein

and energy available for increasing the fish growth. Furthermore, the higher PG and PR values also indicate better nutrient and protein retentions, particularly in 1000 mg kg^{-1} HK-LP group than the other groups. The results in the present study are in accordance with previous studies that demonstrated the application (at different species and levels) of beneficial bacteria improved feed utilization and growth rates (Rodriguez-Estrada *et al.*, 2013; Taoka *et al.*, 2006a).

The enhanced growth performance and feed utilization of fish fed dietary HK-LP might be also attributed to the enhanced digestibility coefficients. In the present study, the highest growth performances in 1000 mg kg^{-1} HK-LP group might be partly due to the improved crude protein, lipid and dry matter digestibility coefficients in this group. Similar observations were also reported in other fish species, in which the nutrient digestibility increased considerably with the use of beneficial bacteria-supplemented diet (Rodriguez-Estrada *et al.*, 2013). Khonyoung and Yamauchi (2012) reported that the presence of beneficial bacterial cells in the intestine improved microbial balance and, therefore, improved nutrient digestion and feed utilization. The modulation of the intestinal microbial communities, as demonstrated by elevated lactic acid bacteria (LAB) levels, might also help during the digestion process in gastrointestinal tract (GI) of fish (Yanbo and Zirong, 2006). Frouël *et al.* (2008) reported that the gut microflora might be influenced by the liberation of molecule that is contained in the heat killed forms of *Lactobacillus* in the digestive tract of European sea bass (*Dicentrarchus labrax*). However, further comprehensive studies utilizing molecular methods are required to validate this hypothesis.

Blood parameters are important tools for the indication of the physiological stress response as well as the general health condition of fish (Hoseinifar *et al.*, 2011; Kader *et al.*, 2010). Concurrent to the plasma component levels, the hematocrit and plasma

total protein values increased in case of 1000 mg kg^{-1} HK-LP group, implying improved health status. Similar results were also observed on rainbow trout fed diets with beneficial bacteria cells (Panigrahi *et al.*, 2010; Rodriguez-Estrada *et al.*, 2013). Moreover, lower triglyceride and cholesterol contents in fish fed 1000 mg kg^{-1} HK-LP diet showed that the optimum availability of HK-LP in fish diets maintained low level of plasma triglycerides and cholesterol in fish. Panigrahi *et al.* (2010) reported that lipid components were important as energy reserves and found to be influenced by beneficial bacteria supplementation. The glutamyl oxaloacetic transaminase (GOT) and glutamic-pyruvate transaminase (GPT) parameters are often used to evaluate the function of liver, while parameter components are secreted into blood and the values increased or decreased depending on the damage of liver cells (Lemaire *et al.*, 1991). A decreased GOT in fish fed the diets containing HK-LP showed the release of GOT into blood was at minimal level, indicating the clinical healthy signs of fish.

Fish antioxidant defence system is strongly related to immune system, and the previous studies reported the enhancement of antioxidant status in fish after beneficial bacteria administration, contributing to enhance resistance towards infections (Reyes-Becerril *et al.*, 2008a, 2008b; Tovar-Ramírez *et al.*, 2010). Oxidative stress will affect diseases and pathological status in fish (Martinez-Alvarez *et al.*, 2005). It is caused by the consequence of an imbalance between oxidants and antioxidants, in which the oxidant activity exceeds the neutralizing capacity of antioxidants (Celi *et al.*, 2010). Recently, d-ROMs and BAP were reported to be reliable parameters for determining the oxidative stress condition of fish (Gao *et al.*, 2012; Kader *et al.*, 2010). In the present study, the combined data of d-ROMs and BAP values confirmed that fish fed supplemented diets with 100, 1000, and 2000 mg kg^{-1} HK-LP were in better conditions whereas HK-LP free group implied a comparatively higher oxidative conditions. One of the interesting

findings in the present study is that 1000 mg kg^{-1} HK-LP group showed the highest tolerance against low salinity stress test when comparing with other groups. Yokoyama *et al.* (2005) used the similar stress test for assessing the healthy status, where inducing stress affected the survival and growth of fish. It has been reported that the stress responses tended to increase the energy demand at the expense of anabolic processes (Kubilay and Ulukoy, 2002). The results of the present study confirmed a higher tolerance against low-salinity stress in fish is positively linked to the less oxidative stress conditions of fish.

In the present study, supplementation of HK-LP cells enhanced immune parameters, including total serum protein (TSP), serum lysozyme activity (LA), and skin mucus production. Other inactivated bacteria with beneficial properties have demonstrated a capacity to modify the immune parameters of Gilthead seabream (Salinas *et al.*, 2008), Nile tilapia (Taoka *et al.*, 2006a), Rainbow trout (Rodriguez-Estrada *et al.*, 2013) and beluga (Hoseinifar *et al.*, 2011). The immunomodulating activity of nonviable bacteria may be due to the existence of certain microbial components, such as capsular polysaccharides, peptidoglycans, and lipoteichoic acids, which are potent stimulators of fish immune system (Secombes *et al.*, 2001; Nayak, 2010). Lysozyme is an important defence molecule of fish innate immune system (Saurabh and Sahoo, 2008), and although the LA among various fish species can be very different, the ranges of LA in the present study were similar to those of the previous studies on red sea bream, *Pagrus major* (Ji *et al.*, 2007). Furthermore, we found the clear increase in LA for fish fed HK-LP supplemental diets than HK-LP free group. Similarly, Pan *et al.* (2008) observed significant increase in LA of Chinese drum, *M. miiuy* fed beneficial bacteria cells. The lowest LA was found in HK-LP free group, which together with other blood parameters (TSP, GPT), implied a less-healthy condition of the fish fed with this diet. Skin mucus

is the first line of defense mechanisms against parasite and bacterial infection with its enzymatic systems (Fast *et al.*, 2002; Hjelmeland *et al.*, 1983), and prevents fish body from being directly exposed to pollutant and stressor through environmental water. In the present study, higher amounts of mucus secretion were observed in all HK-LP supplemented groups compared to that of HK-LP free group. In agreement with our findings, Salinas *et al.* (2008) and Rodriguez-Estrada *et al.* (2013) reported the capacity of lactic acid bacteria and inactivated *Enterococcus faecalis* to modify the epidermal mucus production in fish.

According to the result of this study, intake of HK-LP (LP20) enhanced growth performance, nutrient utilization, and non-specific immune responses of red sea bream juveniles, *Pagrus major* and it also induced higher resistance to the stress such as low-salinity stress. Therefore, the use of HK-LP (LP20) is more environmental friendly approach compared to the use of antibiotics and chemicals in fish culture operation.

Experiment II: Effects of dietary inactivated *Pediococcus pentosaceus* on growth performance, feed utilization and blood characteristics of red sea bream, *Pagrus major* juvenile

3.2.1 Abstract

A feeding trial was carried out to determine effects of dietary supplements of inactivated *Pediococcus pentosaceus* (PP) on growth, feed utilization and blood characteristics of red sea bream. Five diets containing different concentrations [0 (PP0), 1.6×10^{10} (PPI), 1.6×10^{11} (PPII), 1.6×10^{12} (PPIII), and 3.2×10^{12} (PPIV) cells g^{-1}] of PP were fed to fish for 56 days. At the end of the feeding trial, the obtained results revealed a significant increase ($P < 0.05$) in weight gain, specific growth rate and skin mucus secretion in all PP supplemented groups when compared with control group. Moreover, fish fed PPIII and PPIV diets resulted in significantly higher final body weight, feed efficiency ratio and bactericidal activity than those of fish fed PP0 diet ($P < 0.05$). Further, fish fed the PPIII diet also had a higher feed intake, protein efficiency retention, hematocrit, mucus lysozyme activity (LA), plasma and serum proteins than those of PP0 fed group ($P < 0.05$). Similarly, fish fed PPIV diet resulted in a significant increase in serum LA, peroxidase activity and tolerance against low salinity stress test ($P < 0.05$). Considering these promising results, we suggest that the supplementation of PP Prep improves growth and health conditions of *Pagrus major*.

Keywords: Probiotic, *Pediococcus pentosaceus,* Growth performance, Blood characteristics, Immunity, *Pagrus major*

3.2.2 Introduction

Every year, the production of red sea bream, *Pagrus major*, increases to match its high demand due to its great market and nutritional value. However, fish grown in intensive aquaculture systems are often exposed to stressful conditions which have a negative impact on their growth and immunity (Wang *et al.*, 2008a, b). Recently, several studies have demonstrated that probiotics can improve growth performance, feed utilization, digestibility of dietary ingredients, disease resistance and immunostimulation of aquatic animals (Balca´zar *et al.*, 2006; Diaz-Rosales *et al.*, 2009; Sáenz de Rodrigáñez *et al.*, 2009; Ferguson *et al.*, 2010; Merrifield *et al.*, 2011; Xing *et al.*, 2013; Wang, 2011). Probiotics have been defined as live microorganisms that impart positive effects on a host animal by improving the microflora of its gastrointestinal tract (GIT) (Fuller, 1989). Currently, probiotics are defined as the metabolites of live or dead bacterial cells, which function as immunostimulants for modification of enzyme activity or microflora in gastrointestinal tracts (Naidu *et al.*, 1999; Salminen *et al.*, 1999). Some concerns may arise in fish aquaculture due to the oral delivery of probiotics, which may introduce live bacteria into the environment. Consequently, the use of inactivated bacteria, which would no longer interact with the aquatic environment, was considered (Salinas *et al.*, 2008). Dietary supplementation of several inactivated beneficial bacteria improved the growth performance, immunity and disease resistance of fish (Irianto and Austin, 2003; D´ıaz- Rosales *et al.*, 2006; Rodriguez-Estrada *et al.*, 2013; Salinas *et al.*, 2006; Choi and Yoon, 2008; Pan *et al.*, 2008; Panigrahi *et al.*, 2010). Therefore, it can be reasonably hypothesized that heat inactivated bacteria might be an effective alternative for live bacteria supplementation in fish diets. At present, lactic acid bacteria (LAB) as dietary supplements have been widely applied to enhance the immunity and disease resistance of fish (Standen *et al.*, 2013), most notably *Lactobacillus* spp.,

Carnobacterium spp. and *Enterococcus faecium*. However, there remains a distinct lack of literature regarding *Pediococcus pentosaceus*. LAB plays a beneficial role in the host gut environment by producing antibacterial substances such as lactic acid, acetic acid, hydrogen peroxide, and bacteriocin that suppress growth of competing bacteria (Castex *et al.*, 2008; Maeda *et al.*, 2013). Studies have begun to assess the probiotic potential of *Pediococcus* sp. for cobia *Rachycentron canadum* (Xing *et al.*, 2013), grouper *Epinephelus coioides* (Huang *et al.*, 2014), rainbow trout *Oncorhynchus mykiss* Walbaum (Merrifield *et al.*, 2011) and kuruma shrimp *Marsupenaeus japonicus* (Maeda *et al.*, 2013). However, the mechanisms behind the positive effects on a host animal are not clearly understood. Therefore, this study aims to investigate the effect of the oral administration of inactivated *P. pentosaceus* on growth performance, feed utilization and blood characteristics of the juvenile red sea bream. Furthermore, stress resistance under low salinity stress condition was monitored.

3.2.3 Materials and methods

3.2.3.1 *Preparation of bacteria*

Heat-killed *Pediococcus pentosaceus* preparation strain D3268 (PP) Prep was kindly provided by Kyushu Medical Company, Fukuoka, Japan. The bacterial Prep previously isolated from the content of the intestine of wild-captured kuruma shrimp *Marspenaeus japonicus*, Nagasaki prefecture, Japan (Maeda *et al.*, 2013). For culture, the strain was grown in Man, Rogosa, and Sharpe (MRS) agar (Becton Dickinson and Company, NJ, USA) and glucose yeast extract peptone (GYP) agar medium at 37 °C for 24 hours without shaking. The bacterial cells were centrifuged at 8000×*g* for 5min at 4 °C. Then, the collected cells were resuspended in a certain amount of 20% (w/v) skim milk. The suspended cells were lyophilized followed by heat treatment at 70 °C for 20 min in order

to inactivate live cells. The bacterial cell count was performed before the heat-treatment (1.6×10^{12} cells/g). MRS agar was used to calculate the number of bacteria with a conventional agar plate method. The dried products were stored at −20 ˚C until use.

3.2.3.2 *Diet preparation*

The formulation and chemical composition of the experimental diets are shown in Tables 3.2.1 and 3.2.2 respectively. PP Prep was supplied in the diets with five levels: the basal diet (PP0) was used as control diet. Diets 1–4 were formulated to be PPI (PP0+ PP, 1.6×10^{10} cells g^{-1}), PPII (PP0+ PP, 1.6×10^{11} cells g^{-1}), PPIII (PP0+ PP, 1.6×10^{12} cells g^{-1}), PPIV (PP0+ PP, 3.2×10^{12} cells g^{-1}). Cellulose powder was used to adjust to 100% total proportion. PP Prep was thoroughly mixed with lipid before adding other ingredients. Pollack liver oil, soybean lecithin and PP Prep were premixed with a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan), added to the dry ingredients and mixed for another 15 min. The diets were prepared by thoroughly mixing all the dry ingredients in a food mixer for 15 min. The required amount was mixed with water (35–40% of the dry ingredients), and then added to the premixed ingredients and mixed for another 15 min. The pH of the diets was adjusted to the range of 7.0–7.5 with 4 N sodium hydroxide. The mixture was then passed through a meat grinder with an appropriate diameter (1.2–2.2 mm) to prepare pellets, which were then dried in a dry-air mechanical convection oven (DK 400, Yamato Scientific, Tokyo, Japan) at 50 ˚C for about 120 min. to approximately 11% moisture. The test diets were stored in a freezer at −28 ˚C until use.

Table 3.2.1: Basal diet composition

Ingredients	g/kg dry diet
Brown Fish meal[1]	280
Casein[2]	280
Dextrin[3]	70
α-starch[4]	60
Soybean Lecithin[5]	50
Pollack liver oil[6]	60
Vitamin mixture[7]	30
Mineral mixture[8]	30
Stay-C[9]	0.8
Activated gluten[10]	50
α-Cellulose+ *Pediococcus pentosaceus* Prep.[11]	89.2

[1] Nihon Suisan Co. Ltd (Tokyo, Japan), [2] Wako Pure Chemicals Industries, Inc. (Osaka, Japan),[3] Kanto Chemicals (Tokyo, Japan),[4] Asahi Chemicals (Wakayama, Japan),[5, 6] Riken Vitamin, Tokyo, Japan,[7] Vitamin mixture (g kg^{-1} diet): as shown in the last srudy,[8] Mineral mixture (g kg^{-1} diet): as shown in the last srudy,[10] Glico Nutrition Company Ltd. Osaka, Japan. Commercial name "A-glu SS",[11] *Pediococcus pentosaceus* Prep: preparation of heat-killed *Pediococcus pentosaceus* D3268 made by Kyushu Medical, (Fukuoka, Japan).

Table 3.2.2: Chemical analysis of the experimental diets

Ingredients	Experimental diet				
	PP0	PPI	PPII	PPIII	PPIV
Proximate composition (gkg^{-1} dry matter basis)					
Crude protein	507.9	512.9	508.8	511.04	512.8
Total lipid	139.8	139.6	141.9	142	143.6
Ash	107.3	106.6	107.7	107.8	108.5
Gross energy (KJ g^{-1}) *	20.2	20.1	201	20.2	20.1

* Calculated using combustion values for protein, lipid and carbohydrate of 23.6, 39.5 and 17.2 kJ g^{-1}, respectively. Carbohydrate was calculated by the difference: 1000− (protein+lipid+ash+moisture).

3.2.3.3*The feeding trial and sample collection*

Juvenile red sea bream was obtained from a private fish hatchery (Ogata Suisan Co, Kumamoto, Japan), and transported alive to the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. Prior to the feeding trial, all fish were acclimated to the indoor rearing conditions for 2 weeks and fed a commercial pelleted fish feed (Higashimaru, Kagoshima, Japan). A pooled sample of 20 fish at the beginning was stored at −20 ˚C for whole body analysis. A total of 225 fish (initial body weight 6 ± 0.2 g) were divided into 15 round tanks (100 L) with ambient temperature filtered seawater at the same density (15 fish $tank^{-1}$) with triplicate tanks. The water flow to the tanks was 1.5 L min^{-1}, artificial aeration and natural light/dark regime was applied in the trial. The temperature, pH and salinity of the tank water during the trial were 28.3± 1.2 ˚C, 7.9 ± 0.7 and 33.1 ± 0.5 g L^{-1}, respectively. Fish were fed test diets at 08:00 and 16:00 h to apparent satiation level for 56 days. Uneaten diets were collected every day, dried and weighed to determine feed intake (FI). All fish were weighed in bulk at 10 days' interval to determine growth, check their health condition and ration was adjusted according to mean fish weight. The health condition of fishes was checked visually through their movements, infectious diseases symptoms and body appearance all over the body and fins of the fish. At the end of feeding trial, all fish were fasted for 24 h prior to final sampling. Then the total number, individual body weight and length of fish from each tank were measured. Three fish from each replicate tank were randomly collected and stored at −20 ˚C for final whole body analysis. Blood was taken from the caudal vein fifteen fish per treatment (five fish per tank) using heparinized (1800 IU mL^{-1}) needle (25 G × 1") and syringes (1 mL). Plasma samples were obtained by centrifugation at 3000×*g* for 15min at 4˚C using a high-speed refrigerated micro centrifuge (MX-160; Tomy Tech USA Inc., Tokyo,

Japan) and kept at −80 ˚C. In addition, non-heparinized disposable syringes were used to collect blood for serum analysis. Serum samples were obtained by centrifugation at 3000×*g* for 15 min 4˚C to collect serum. Nine fish per treatment (three fish per each tank) were randomly sampled for collect skin mucus, liver and viscera. Viscera and liver were dissected out, then weighed to get viscera somatic index (VSI) and hepatosomatic index (HSI) respectively. Fish for skin mucus were randomly selected and their surfaces were washed with distilled water individually, and skin mucus was collected from body surface (200mm^2) by using a small piece of sterilized cotton and immediately suspended in 1 ml of phosphate-buffered saline (PBS) (pH=7.4) and centrifuged (2000×*g*, 10 min., 4˚C) (MX-160, Tomy Seiko Co., LTD, Tokyo, Japan), and the supernatant in the under tube was transferred into another centrifugal tube (510-GRD, QSP, San Diego, CA, USA) and kept at -80˚C until further analysis was performed.

3.2.3.4 *Biochemical and blood measurements*

The ingredients, diets and fish whole body were analyzed for moisture, crude protein, crude lipid and ash, in triplicate, using standard methods (AOAC, 1990), including moisture by oven-drying at 110 ˚C to constant weight, crude protein by the Kjeldahl method, crude lipid by the Soxhlet extraction method and ash by combustion in Muffle furnace at 550 ˚C for 4h. Hematocrit was determined using the micro hematocrit technique. Plasma chemical parameters and total serum protein (TSP) were measured spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan) (Tatsumi *et al.*, 2000). Biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were also measured spectrophotometrically from blood plasma with an automated analyzer FRAS4, Diacron International s.r.l., Grosseto, Italy by following Morganti *et al.* (2002).

3.2.3.5 *Immunological measurements*

3.2.3.5.1 *Lysozyme activity of Serum and Mucus*

Lysozyme activity of serum and mucus was determined with turbidometric assays (Lygren *et al.*, 1999). One unit of lysozyme activity was defined as the amount of enzyme that causes a decrease in absorbance of 0.001/min.

3.2.3.5.2 *Serum bactericidal activity*

The serum bactericidal activity was measured according to Iida (Iida *et al.*, 1989). Serum was diluted 3, 4 and 5 times with a Tris buffer (pH 7.5). The dilutions were mixed with a bacterial suspension (0.001 g/ml, Escherichia coli, IAM1239 cell line, Kagoshima, Japan) and incubated at 25°C for 24 h by micro tube rotator (MTR-103, AS ONE, Osaka, Japan). The solutions were incubated on TSA (Trypto-Soya agar, Nissui Phatmaceutical Co. Ltd., Japan) at 25°C for 24 h. CFU (Colony Forming Unit) were counted by the plate counting method as described by Ren *et al.* (2007).

3.2.3.5.3 *Serum peroxidase activity*

The total peroxidase content in serum was measured according to Salinas *et al.* (2008), with some modifications. Briefly, 15 µl of serum were diluted with 35 µl of Hank's Buffered salt solution (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. Then, 50 µl of peroxidase substrate (3, 30, 5, 50-tetramethylbenzidine hydrochloride) (TMB; Thermo Scientific Inc., USA) was added. The serum mixture was incubated for 15 min. The colour-developing reaction in serum samples was stopped by adding 50 µl of 2 M sulphuric acid and the OD (450 nm) was measured in a plate reader. PBS was used as a blank instead of serum.

3.2.3.6 *Amount of secreted mucus*

After skin mucus was collected, amounts of secreted mucus were determined according to Yokoyama *et al. (*2006). Total protein in the collected supernatant was determined

by the method of Lowry *et al.* (1951). Then, amounts of secreted mucus were expressed as mean relative values on the basis of total protein in the mucus sample of PP0 group.

3.2.3.7 *Low salinity stress test*

Tolerance against exposure to low salinity seawater was examined. After the feeding trial, three fish from each rearing tank (total 9 fish per treatment) were randomly selected and transferred into a 100-L black tank containing low-salinity water (0.2%). The city tap water was dechlorinated by strongly aerating for at least 24 h and mixed with seawater, and then used as low salinity water. The tanks for stress test were equipped with continuous aeration and kept under ambient temperature during the stress test. Number of dead fish in each test tank was recorded every 20 min. The passing of time to reach 50% death was calculated according to Moe *et al. (*2004) and Ren *et al.* (2007).

3.2.3.8 *Statistical analysis*

All data were subjected to statistical verification using package super ANOVA 1.11, Abacus Concepts, Berkeley, California, USA. Probabilities of $P<0.05$ were considered significant. Significance differences between means were evaluated using the Turkey-Kramer test.

3.2.4 Results

3.2.4.1 *Growth parameters, nutrient utilization and whole body proximate analysis*

Growth performances and nutrient utilization of *P. major* are shown in Table 3.2.3. The highest value of final body weight, WG, and SGR was obtained in fish fed PPIII diet. However, the values were not significantly different from those of fish fed PPII and PPIV diets ($P<0.05$). Feed intake and feed efficiency (FER and PER) showed the similar trends. No differences were detected in protein gain and protein retention of fish

among treatments. Survival rates for all groups were acceptable (>90%). The whole body proximate compositions of fish at the end of the feeding trial showed some changes in the analyzed parameters compared to those of the initial values (Table 3.2.4). However, there displayed no significant differences (P>0.05) in the final whole body proximate compositions among all groups. No differences were also detected in CF, HSI and VSI of fish among treatments (Table 3.2.4).

3.2.4.2 *Blood parameters and responses against stress*

Table 3.2.5 represents the blood parameters of red sea bream after 56 days of feeding trial. Overall, dietary treatments had no effect in blood parameters of fish among different treatments except for those of hematocrit, plasma total protein, and triglyceride (TG). Hematocrit was significantly higher in fish fed PPIII diet than PP0 and PPI groups (P<0.05), however no significant differences were detected among other groups. Interestingly, plasma total protein was significantly higher in fish fed PPIII diet when compared with other groups (P<0.05). Although no differences were detected among all groups, TG of fish fed PP0 diet was significantly higher than that fed PPII diet (P<0.05). Comparatively higher levels of reactive oxygen metabolites (d-ROMs) were detected in fish fed PPIII diet when compared to other groups while biological antioxidant potential (BAP) was not affected by the dietary supplementation (Table 3.2.5). Integration of d-ROMs and BAP was used for evaluation of oxidative stress of test fish (Figure 3.2.2), it would be concluded that fish fed PPII, PPIII, and PPIV diets were in better conditions whereas fish fed PP0 and PPI diets implied a comparatively poorer conditions. The fish received PPIV diet showed significantly higher tolerance against low-salinity stress than PP0 group (P<0.05), however no significant differences were observed among other groups (Figure 3.2.1).

3.2.4.3 *Immunological parameters*

Immunological results are displayed in Table 3.2.6. Serum lysozyme activity (LA) was significantly higher in fish fed PPIV diet than the other groups, except for fish fed PPIII diet ($P<0.05$). On the other hand, mucus LA was significantly higher in fish fed PPIII diet than the other groups; however, no differences were detected with PPI and PPIV groups. Furthermore, total serum protein was significantly enhanced in case of PPIII group when compared with control group. Significantly higher serum bactericidal activity was obtained in PPIII and PPIV groups when compared with the control group ($P<0.05$). The PPIV diet provoked a maximum increase in serum peroxidase activity (compared with control) after 56 days of feeding trial.

3.2.4.4 *Amounts of secreted mucus*

Significantly higher amounts of secreted mucus were observed (Fig. 3.2.3) in all PP supplemented groups than that in control group ($P<0.05$). Although the highest concentration was found in fish fed PPIV diet, the value was not significantly different from those of fish fed PPI, PPII and PPIII diets ($P>0.05$).

Table 3.2.3: Growth parameters and nutrient utilization in red sea bream fed test diets for 56 days*

Parameters	Experimental diet				
	PP0	PPI	PPII	PPIII	PPIV
In wt[1]	6.12±0.04	5.79±0.3	5.8±0.1	6.23±0.02	6.02±0.3
Fn wt[2]	39.47±0.4^{a}	40.91±2.7^{ab}	42.98±0.1^{ab}	50.3±1.03^{c}	46.42±1.6^{bc}
WG[3]	442.12±10.8^{a}	506.18±19.7^{b}	542.72±9.1^{bc}	607.4±15.4^{d}	572.1±10^{cd}
SGR[4]	3.32±0.03^{a}	3.49±0.1^{b}	3.58±0.02^{bc}	3.73±0.03^{c}	3.65±0.03^{c}
FI[5]	40.56±0.4^{a}	41.76±1.1^{ab}	41.33±0.1^{ab}	45.97±1.3^{b}	43.72±1.8^{ab}
FER[6]	0.82±0.03^{a}	0.84±0.04^{ab}	0.9±0.03^{abc}	0.96±0.04^{c}	0.93±0.03^{bc}
PER[7]	1.62±0.01^{a}	1.64±0.1^{a}	1.77±0.03^{ab}	1.88±0.1^{b}	1.8±0.1^{ab}
PG[8]	173.55±4	173.06±4	173.51±1.8	174.65±3.1	176.49±4.3
PR[9]	35.77±1.2	37.09±1.6	36.49±0.3	41.07±1.9	39.6±2.1
Sur[10]	95.6	91.1	100	97. 8	93.3

*Values are means of triplicate groups ±S.E.M. Within a row, means with different letters are significantly different (P<0.05), means with the same letters are not significantly different (P>0.05). Absence of letters indicates no significant difference between treatments,[1] In wt: initial weight (g),[2] Fn wt: final weight (g),[3] WG: weight gain (%) = (final weight – initial weight) ×100/initial weight,[4] SGR: specific growth rate (% day^{-1}) = {(Ln (final weight)–Ln (initial weight))/ duration (56 days)} ×100,[5] FI: feed intake (g dry diet fish^{-1} 56 days^{-1}) = (dry diet given−dry remaining diet recovered)/ no. of fish,[6] FER: feed efficiency ratio = live weight gain (g) /dry feed intake (g),[7] PER: protein efficiency ratio = live weight gain (g)/dry protein intake (g),[8] PG: protein gain (g kg body weight gain^{-1}) = {(final weight (g) ×final whole body protein content (%)/100) − (initial weight (g) ×initial whole body protein content (%)/100)}/ (weight gain (g)) ×1000,[9] PR: protein retention (% of intake) = (protein gain (g kg weight gain^{-1}) ×100)/ protein intake (g kg weight gain^{-1}),[10] Sur: survival (%) =100× (final no. of fish/ initial no. of fish).

Table 3.2.4: Whole-body proximate analysis (g kg^{-1} wet weight) and somatic parameters in juvenile red sea bream fed test diets for 56 days

Parameters	Initial	Experimental diet				
		PP0	PPI	PPII	PPIII	PPIV
Moisture	785.1	662.9±2.4	659.6±1	662±1	658.1±2	654.1±3
Crude protein	142.6	168.7±3.4	169±3.3	169.3±1.6	170.7±2.6	172.1±3.7
Total lipid	30.2	119.5±0.6	121.1±4.4	114.3±0.6	119.6±4	121.3±2
Crude ash	41.9	46.4±0.5	49±1.8	48.1±0.3	47.1±1.2	49.5±0.3
CF [1]	_	2.34±0.05	2.67±0.45	2.14±0.08	2.43±0.1	2.02±0.15
HSI [2]	_	2.05±0.2	2.06±0.1	2.46±0.2	2.22±0.3	2.58±0.12
VSI [3]	_	5.72±0.87	5.93±0.2	5.19±0.45	5.93±0.86	5.74±0.75

[1] CF: condition factor (%) = Body weight (g) × 100/body length (cm) [3],[2] HSI: hepatosomatic index (%) = Liver weight (g) × 100/body weight (g),[3] VSI: viscerasomatic index (%) = Viscera weight (g) × 100/body weight (g).

Table 3.2.5: Blood parameters in juvenile red sea bream fed test diets for 56 days

Parameters	Experimental diet				
	PP0	PPI	PPII	PPIII	PPIV
Hematocrit (%)	46±0.6^{a}	46.33±2^{a}	49.67±0.9^{ab}	51.33±1.8^{b}	49.33±1.2^{ab}
Total protein (g/dl)	4.3±0.2^{a}	4.3±0.3^{a}	4.2±0.2^{a}	5.2±0.3^{b}	4.3±0.2^{a}
Total bilirubin (mg/dl)	0.43±0.03	0.53±0.15	0.5±0.06	0.53±0.09	0.43±0.09
Glucose (mg/dl)	61.3±2.2	62±4.6	61.7±2.6	60.7±1.2	61.3±1.3
GOT (IU/l)[1]	203±6.7	191.7±4.1	195.7±5.9	196.7±4.5	194±10.7
GPT (IU/l)[2]	25.3±5.3	23.3±0.9	23.7±8	21.33±2.03	23±2
BUN (mg/dl)[3]	5.3±0.3	5.7±0.7	5.3±0.3	5.7±0.7	5.3±0.3
TG (mg/dl)[4]	392±60.4^{b}	273.7±32.6^{ab}	257±14.5^{a}	269.3±41.6^{ab}	261.3±31.8^{ab}
T-Cho (mg/dl)[5]	337±9.3	311±15.6	331±14.5	321.7±17.6	324.3±25.2
d-ROMs [6]	134±7.5	123.7±5.5	138.3±3.8	147.3±7.8	132±2.3
BAP[7]	3135.7±65.3	3267.7±170.1	3429.3±336	3570.7±16.9	3514.7±260.4

Abbreviation used: [1] GOT: glutamyl oxaloacetic transaminase, [2] GPT: glutamic-pyruvate transaminase, [3] BUN: blood urea nitrogen, [4] TG: triglyceride, [5] T-Cho: total cholesterol, [6] d-ROMs: reactive oxygen metabolites, [7] BAP: biological antioxidant potential.

Table 3.2.6: Non-specific immune parameters in red sea bream juveniles fed test diets for 56 days*

Parameters	Experimental diet				
	PP0	PPI	PPII	PPIII	PPIV
LA[1] of serum (unit/mL)	17.5±4.1[a]	19.17±4.36[a]	18.75±3.08[a]	25.42±2.27[ab]	29.58±1.87[b]
LA[1] of mucus (unit/mL)	32.5±5.28[a]	37.08±1.19[ab]	34.58±1.19[a]	51.25±6.08[b]	40.83±3.46[ab]
TSP[2] (g/dL)	3.4±0.27[a]	3.54±0.19[ab]	3.84±0.09[ab]	4.1±0.06[b]	3.97±0.18[ab]
BA[3] of serum ($\times 10^7$ CFU/mL)	4.84±0.73[a]	6.97±0.35[ab]	7.85±0.31[ab]	9.48±1.25[b]	8.3±0.58[b]
Peroxidase of serum (OD 450)	1.4±0.11[a]	1.45±0.11[ab]	1.53±0.11[ab]	1.45±0.13[ab]	1.73±0.04[b]

* Values are means of triplicate groups ±S.E.M. Within a row, means with different letters are significantly different ($P<0.05$), means with the same letters are not significantly different ($P>0.05$).

Abbreviation used: [1]LA: lysozyme activity; [2]TSP: total serum protein; [3]BA: bactericidal activity.

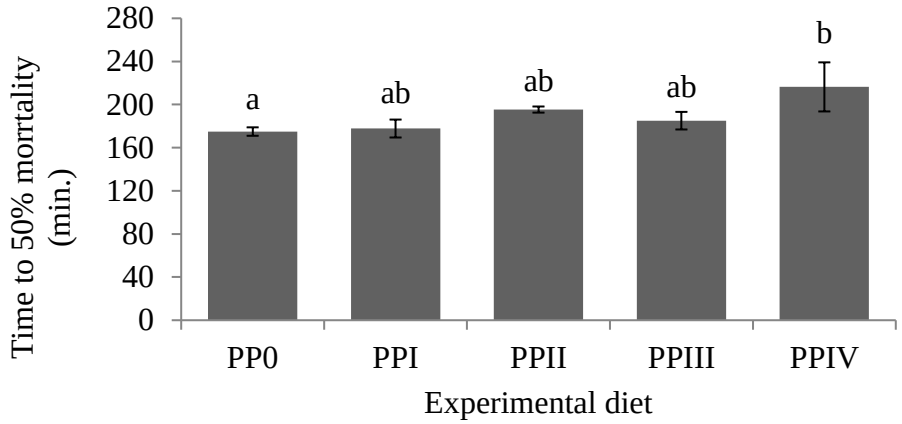


Fig. 3.2.1: Time to 50 % mortality (min.) after low salinity stress test when red sea bream fed diets containing *P. pentosaceus* Prep (PP) for 56 days. Values with different letters are significantly different ($P<0.05$). Values with the same letter are not significantly different ($P>0.05$).

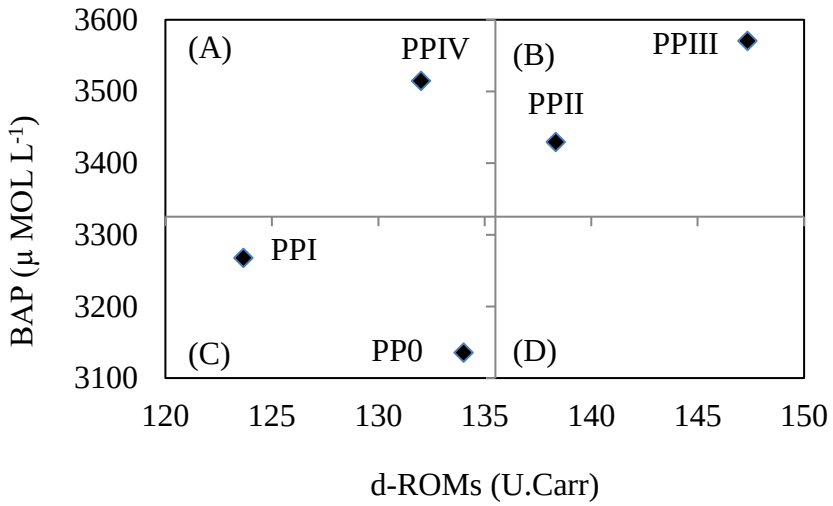


Fig. 3.2.2: Oxidative stress parameters in red sea bream fed test diets for 56 days. Zone (A): high antioxidant potential and low reactive oxygen metabolites (good condition); Zone (B): high antioxidant potential and high reactive oxygen metabolites (acceptable condition); Zone (C): low antioxidant potential and low reactive oxygen metabolites (acceptable condition); Zone (D): low antioxidant potential and high reactive oxygen metabolites (stressed condition).

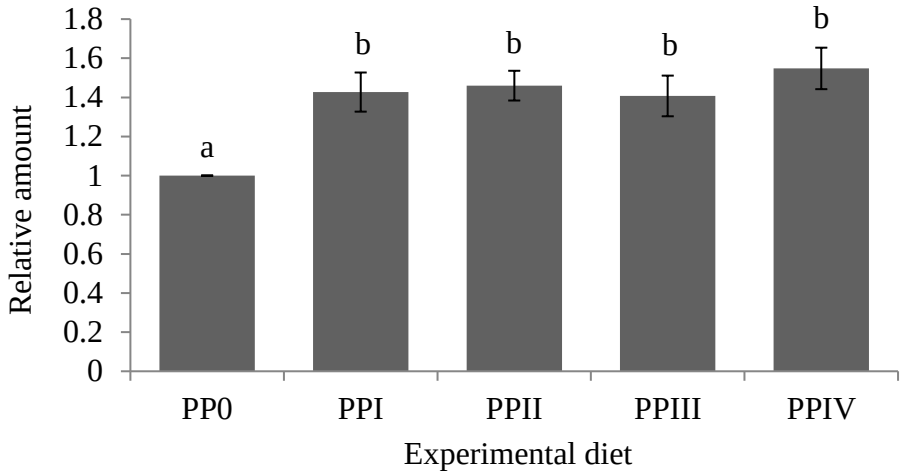


Fig. 3.2.3: Relative amounts of mucus secretion on body surface of red sea bream juvenile. Values are expressed as mean relative means ± pooled SEM (*n*=9 fish). Values with different letters are significantly different ($P<0.05$). Values with the same letter are not significantly different ($P>0.05$).

3.2.5 Discussion

The main beneficial effects of probiotics in fish farming are improvements of growth performances, immunity and tolerance against different stressors (Balca´zar *et al.*, 2006; Irianto and Austin, 2003; Gatesoupe *et al.*, 2007; Lara-Flores, 2011; Tung *et al.*, 2010; Rodriguez-Estrada *et al.*, 2013; Wang *et al.*, 2008a; Wang, 2011). In the present study, a higher growth performance and a noticeable increase in nutrient utilization of protein were observed in the experimental groups compared with the control group after 56 days of feeding, especially in the case of PPIII group. These results are in accord with previous studies that demonstrated the application (with different bacteria species and levels) of bacterial cells improves feed conversion and growth rates of Rainbow Trout *Oncorhynchus mykiss* (Bagheri *et al.*, 2008; Rodriguez-Estrada *et al.*, 2013), Cobia *Rachycentron canadum* (Xing *et al.*, 2013), Tilapia *Oreochromis niloticus* (Standen *et al.*, 2013) and grouper *Epinephelus coioides* (Huang *et al.*, 2014). The fish intestine is the direct organ for digestion, absorption and immunity, as the gut microflora is continuously exposed to PP Prep. The latter was thought to affect the production of extracellular enzymes by the microflora within the gastrointestinal (GI) tract of fish, which in turn improves nutrient absorption and utilization (Kesarocodi-Watson *et al.*, 2008; Lara-Flores *et al.*, 2003; Gatesoupe, 2007; Standen *et al.*, 2013; Tovar-Ramirez *et al.*, 2010, Wang *et al.*, 2008b). It is well known that growth response was strongly related to improved feed utilization. Our results suggested that amongst the tested fish that utilized experimental diets effectively, there were higher feed intake (FI), feed efficiency ratio (FER) and protein efficiency ratio (PER) in fish fed PPIII diet. The increased FI, FER and PER would explain the significant increased growth performance in this group. Fish fed the PP Prep supplemented diets exhibited higher survival rate. The reason for this has not been elucidated as no pathogenic challenge

was conducted, and no signs of disease were detected during the trial but perhaps PP Prep may improve general fish health conditions.

Measurements of plasma constituent levels not only are easier compared to immunological parameters or pathogen-challenge trials for evaluating disease resistance and health condition but also can be performed without killing the fish (Panigrahi *et al.*, 2010). Blood parameters obtained in the present experiment are considered to be within the normal range for juvenile red sea beam, compared to those of the previous findings (Uyan *et al.*, 2007). In the present study, the hematocrit values increased in case of fish fed PPIII diet, implying improved health status. Similar observations were reported in rainbow trout fed with *L. rhamnosus* and *E. faecalis* supplemented diets (Panigrahi *et al.*, 2010; Rodriguez-Estrada *et al.*, 2013). Furthermore, the plasma total proteins increased significantly in fish fed PPIII diet. Similar trends were recorded in *P. olivaceus* (Taoka *et al.*, 2006b). Fish fed PP-treated diets showed lower triglyceride values because optimum availability of PP Prep in fish diets maintains low level of plasma triglycerides in fish. The present results are in agreement with the findings of Panigrahi *et al.* (2010) assumed that, probiotic bacteria ferment food carbohydrate to produce short chain fatty acids in the gut, which can then cause a decrease in the systemic levels of blood lipids. Oxidative stress is considered to be involved in many performances and health status in fish (Gao *et al.*, 2012; Han *et al.*, 2014), and is the consequence of an imbalance between oxidants and antioxidants in which oxidant activity exceeds the neutralizing capacity of antioxidants (Celi *et al.*, 2010). PP Prep stimulates the tolerance of *P. major* against oxidative stress. In agreement with our results, previous studies reported enhancement in antioxidant status of fish after probiotic administration, contributing to enhanced resistance towards infections (Reyes-Becerril *et al.*, 2008b; Tovar-Ramírez *et al.*, 2010).

Inactivated bacteria with beneficial properties have demonstrated a capacity to modify the immune parameters of Gilthead Sea bream *Sparus aurata* (Salinas *et al.*, 2008), Nile Tilapia (Taoka *et al.*, 2006a), Rainbow Trout (Rodriguez-Estrada *et al.*, 2013), and red sea bream (Bo *et al.*, 2012). The immunomodulating activity of non-viable bacteria may be due to the existence of certain microbial components, such as capsular polysaccharides, peptidoglycans, and lipoteichoic acids, which are potent stimulators of the piscine immune system (Secombes *et al.*, 2001; Nayak, 2010). In the current study, we found higher increase in serum and mucus lysozyme activities (LA) in fish fed PP supplemental diets than the PP free group, which might contribute to the enhancement of the non-specific defense mechanisms. In agreement with our findings, plasma or serum LA in fish has been reported to increase after probiotic supplementation (Balc´azar *et al.*, 2007; Panigrahi *et al.*, 2004; Taoka *et al.*, 2006a; Wang *et al.*, 2008a). As an immune defense system, skin mucus has an important role and contains some enzymes to prevent the invasion of pathogens, such as lysozyme, lectine, and protease (Taoka *et al.*, 2006a, b). Our results revealed that mucus LA in the control was lower than the PPIII group, indicating improvement in antibiotic activity of fish. In line with our results, dietary feeding of probiotic supplemented diet increased the mucus LA of Japanese flounder (Taoka *et al.*, 2006b). Total serum protein (TSP) content reflects the nutritional and metabolic status of fish, and indirectly reflects the level of non-specific immunity (Ortuño *et al.*, 2001). In this study, TSP increased with PPIII diet, together with plasma total protein, which was similar with the previous study in *P. olivaceus* (Taoka *et al.*, 2006b). Serum bactericidal activity (BA) is one of the most important factors in host resistance against pathogenic bacteria. In this study, the BA in serum was enhanced by probiotic treatment, especially in the case of PPIII and PPIV groups. Similarly, Taoka *et al.* (2006a) reported that dietary compound

probiotics significantly increased the BA of tilapia. They suggested that oral administration of proper probiotic supplementation enhanced the BA during the experimental period. The peroxidase is an important enzyme that utilizes oxidative radicals to produce hypochlorous acid to kill pathogens (Nayak, 2010). In the current study, the peroxidase level in PPIV group was significantly higher than that of control group confirming other results obtained by Salinas *et al.* (2008). Therefore, we suggest that stimulation of peroxidase might be attributed to PP probiotic preparation.

Protein concentration in the skin mucus is an indicator of the amount of skin mucus excreted (Yokoyama *et al.*, 2006; Taoka *et al.*, 2006b). Fish have a unique physical barrier composed of skin and epidermal mucus that acts as the first line of defence (Palaksha *et al.*, 2008), and prevents fish body from being directly exposed to pollutant and stressor through environmental water. Results of this study showed that higher amounts of mucus secretion were observed in all fish fed PP Prep supplemented diets compared to that of fish fed PP0 diet. Similarly, oral administration of probiotic increased the secretion of skin mucus in gilthead seabream (Salinas *et al.*, 2008) and Rainbow Trout (Rodriguez-Estrada *et al.*, 2013). In contrast to our investigation, skin mucus of Japanese flounder *Paralichthys olivaceus* and Tilapia *Oreochromis niloticus* (Taoka *et al.*, 2006a, b) was decreased after probiotic feeding. These differences may be explained by different fish species, bacterial strains in the feed and the length of the studies.

It is well known that stress affects the survival and growth of fish, since stress responses tend to increase the energy demand at the expense of anabolic processes (Kubilay and Ulukoy, 2002). Our results indicated that fish fed PP Prep showed high tolerance against stress. Further research should be done to confirm the relationship between probiotics treatment and stress conditions in fish.

According to the result of this study, it is clear that intake of PP Prep enhanced growth, feed utilization, and health condition of *Pagrus major* juveniles through improvement of various immunological parameters such as lysozyme activity, bactericidal activity, peroxidase content, mucus secretion and it also induced higher resistance to the stress such as low-salinity stress. Feeding of PP Prep at a medium dose of 1.6×10^{11} to 1.6×10^{12} cells g^{-1} was ideal for optimum enhancement of the immune system. Further studies are required in order to investigate the underlying reasons for the growth promoting effects and the improved health features found in the present study.

Experiment III: Dietary supplementation of β-glucan improves growth performance, the innate immune response and stress resistance of red sea bream, *Pagrus major*

3.3.1 Abstract

A 56-day feeding trial was conducted to evaluate the effects of supplemented diets with β-glucan (BG) at four levels [0 (D1), 250 (D2), 500 (D3), and 1000 (D4) mg BG kg^{-1}] on red sea bream, *Pagrus major*. The obtained results revealed a significant increase (P<0.05) in final body weight, weight gain, specific growth rate, feed intake, body protein content, lysozyme activity, and tolerance against low salinity stress test in all BG supplemented groups when compared with BG free group. Furthermore, D4 group resulted in a significant increase in feed efficiency ratio, protein gain, protein and lipid digestibilities, serum bactericidal activity, and peroxidase content when compared with D1 group (P<0.05). Hematocrit and plasma protein content in D3 group were significantly higher than in D1 group (P<0.05). Interestingly, BG supplementation decreased glutamyl oxaloacetic transaminase (GOT) in D2 group and reactive oxygen metabolites in D2, D3 and D4 groups when compared with D1 group. Following low salinity stress test, significantly higher amounts of secreted mucus were observed in fish fed D2 and D4 diets than those from fish fed D1 diet (P<0.05). In conclusion, the supplementation of BG improves growth, stress resistance and immune response of *Pagrus major*.

Keywords: Dietary β-glucan, Red sea bream, Growth, Blood Chemistry, Stress resistance, Immunity

3.3.2 Introduction

It is well known that nutritional plans may affect fish performances (Ringø *et al.,* 2012). Therefore, alternative strategies must be developed to improve fish health and to prevent fish diseases. For this purpose, the use of feed additives such as immunostimulants are considered a promising area in aquaculture (Akhter *et al.,* 2015; Cerezuela *et al.,* 2012b; Kiron 2012). Among the most well studied dietary additives are β-glucans (Ringø *et al.,* 2012). β-glucans are wide spread substances composed of glucose as building blocks, usually isolated from cell walls of bacteria, mushrooms, algae, cereal grains, yeast and fungi (Zekovic & Kwiatowski 2005). Glucans are indigestible substances that allow specific changes in the composition and/or activity of gastrointestinal microbiota, which has a positive effect on the feed utilization and growth performance of the fish (Kühlwein *et al.,* 2014; Song *et al.,* 2014). The effectiveness of β-glucans in improving growth and feed conversion combined with enhanced health status has been proven in some studies (Kühlwein *et al.,* 2014; Sealey *et al.,* 2008; Soltanian *et al.,* 2009). Several reports have shown that, using of β-glucans enhanced the growth performance in a range of cultured species including snapper, (*Pagrus auratus*) (Cook *et al.,* 2003), rohu, *Labeo rohita* (Misra *et al.,* 2006), sea cucumber, *Apostichopus japonicas* (Min *et al.,* 2011), koi, *Cyprinus carpio* koi (Lin *et al.,* 2011), and mirror carp, *Cyprinus carpio* L. (Kühlwein *et al.,* 2014). β-glucans are also known to have a potent stimulatory effect on the immune system of aquatic animal species (Brogden *et al.,* 2014; Falco *et al.,* 2014; Ortun˜o *et al.,* 2002), as well as growth (Lara-Flores *et al.,* 2003) and thus may serve as an excellent health promoter for fish culture. They have also been shown to enhance other non-specific immune factors such as lysozyme and complement activities (Dawood *et al.,* 2015a; Falco *et al.,* 2012; Kühlwein *et al.,* 2014; Paulsen *et al.,* 2001; Pionnier *et al.,* 2013, 2014). When

administered orally, glucans have a proven protective effect in several fish species and against a variety of bacterial pathogens including *Aeromonas hydrophila* (Brogden *et al.,* 2012; Kwak *et al.,* 2003; Selvaraj *et al.,* 2005), *Aeromonas salmonicida* (Falco *et al.,* 2012), and *Edwardsiella tarda* (Sahoo & Mukherjee 2002). For red sea bream (*Pagrus major*), it is also documented that β-glucan can enhance the growth performance and innate immune response when combined with heat killed probionts (Dawood *et al.,* 2015a). However, to the authors' knowledge, the detailed supplemental effects of β-glucans on red sea bream performances are still not yet documented.

Due to its rapid growth and easy accommodation to environmental conditions, red sea bream is one of the most commercially important species grown in Japan, Korea and China. Although the biological effects of β-glucan on aquatic animals are well established, its effects on commercially important fish species, specifically on red sea bream, are not fully evaluated. Therefore, this study aims to investigate the effect of the oral administration of β-glucan on the growth performance, immune response, and stress resistance of juvenile red sea bream.

3.3.3 Materials and methods

3.3.3.1 *β-glucan preparation*

β-glucan (BG) used in this study is a commercial product containing 85 %BG produced by Daigon do (Tokyo, Japan).

6.3.2 *Test diets*

The formulation and chemical composition of the experimental diets are shown in Table 3.3.1. Four isonitrogenous (50.7% crude protein) and isolipidic (9.7% crude lipid) diets were produced using the basal formulation as the control diet (D1) and by supplementing three other diets with 0.025% (D2), 0.05% (D3) and 0.1% (D4) w/w β-

glucan. The ingredients were blended thoroughly in a mixer and pelleted using a meat grinder equipped with a (1.2–1.9 mm) die following the method previously described by (Dawood *et al.*, 2015a). The pelleted diets were air-dried in a mechanical convection oven and stored at −20 °C in plastic bags until further use.

Table 3.3.1: Formulation and chemical composition of the test diet

	Diets *no* (BG level, g kg^{-1} dry matter)			
	D1 (0)	D2 (250)	D3 (500)	D4 (1000)
Ingredients (g kg^{-1} dry matter)				
Fish meal[1]	280	280	280	280
Casein[2]	280	280	280	280
α-starch[3]	120	120	120	120
Pollack liver oil[4]	80	80	80	80
Vitamin mixture[5]	30	30	30	30
Mineral mixture[6]	30	30	30	30
Stay-C[7]	0.8	0.8	0.8	0.8
Activated gluten[8]	50	50	50	50
β-glucan (BG)	0	0.25	0.5	1
α-Cellulose	129.2	128.95	128.7	128.2
Proximate composition (g kg^{-1} dry matter)				
Crude protein	511.4	501.8	507.8	510.9
Total lipid	97.2	95.2	92.2	90.1
Ash	112.2	110.9	109.7	114.5
Gross energy (KJ g^{-1})[9]	15.91	15.6	15.63	15.62

[1] Nihon Suisan Co. Ltd (Tokyo, Japan),[2] Wako Pure Chemicals Industries, Inc. (Osaka, Japan),[3] Asahi Chemicals (Wakayama, Japan),[4] Riken Vitamin, Tokyo, Japan,[5] Vitamin mixture (g kg^{-1} diet): β-carotene, 0.10; Vitamin D_3, 0.01;Menadione $NaHSO_3.3H_2O$ (K_3), 0.05; DL-α-Tochopherol Acetate (E), 0.38; Thiamine-Nitrate (B_1), 0.06; Riboflavin (B_2), 0.19; Pyridoxine-HCl (B_6), 0.05; Cyanocobalamin (B_{12}), 0.0001; Biotin, 0.01; Inositol, 3.85; Niacine (Nicotic acid), 0.77; Ca Panthothenate, 0.27; Folic acid, 0.01; Choline choloride, 7.87; ρ-Aminobenzoic acid, 0.38; cellulose, 1.92,[6] Mineral mixture (g kg^{-1} diet): $MgSO_4$, 5.07; Na_2HPO_4, 3.23; K_2HPO_4, 8.87; Fe citrate, 1.10; Ca lactate,12.09; Al $(OH)_3$, 0.01; $ZnSO_4$, 0.13; $CuSO_4$, 0.004; $MnSO_4$, 0.03; Ca $(IO_3)_2$, 0.01; $CoSO_4$, 0.04,[7] L-Ascrobil-2-phosphate-Mg,[8] Glico Nutrition Company Ltd. Osaka, Japan. Commercial name "A-glu SS", [9] Calculated using combustion values for protein, lipid and carbohydrate of 23.6, 39.5 and 17.2 kJ g^{-1}, respectively. Carbohydrate was calculated by the difference: 100− (protein+lipid+ash+moisture).

3.3.3.3 *The feeding trial and sample collection*

Juvenile red sea bream was obtained from Akahoshi farm, Kumamoto Prefecture, Japan, and transported alive to the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. Prior to the feeding trial, all fish were acclimated to the indoor rearing conditions for 2 weeks and fed a commercial pelleted fish feed (50% crude protein, Higashimaru, Kagoshima, Japan). A total of 180 healthy fish (initial body weight 2.85±0.02g) were divided into 12 one hundred-liter round tanks (100 L) with through ambient temperature filtered seawater at the same density (15 fish $tank^{-1}$) with triplicate tanks. The water flow to the tanks was 1.5 L min^{-1}, artificial aeration and natural light/dark regime were applied in the trial. During the experimental period, the monitored water quality parameters (mean±S.D.) were: water temperature 22.3±1.6°C, pH 8±0.5, salinity 33.3±0.5ppt and dissolved oxygen 6.1±0.5 mg l^{-1}. Fish were fed the respective test diets to the satiation level by hand twice a day, 7 days per week for 56 days. A pooled sample of 25 fish at the beginning was stored at −20 °C for initial whole body analysis. At the end of the feeding trial, all experimental fish were fasted for 24 h for final sampling (Dawood *et al.*, 2015a, b). The total number, individual body weight and length of fish from each tank were measured; the growth parameters were calculated accordingly. Six fish per treatment (two fish per tank) were randomly collected, washed with distilled water and then stored at −20 °C for the whole body proximate analysis. Similarly, nine fish per treatment (three fish per tank) were randomly selected and their surfaces were washed with distilled water individually, and skin mucus was collected from the body surface by using a small piece of sterilized cotton and immediately suspended in 1 ml of phosphate-buffered saline (PBS, pH=7.4) according to (Dawood *et al.*, 2015a, b). Blood was collected using heparinized (1600 IU mL^{-1}), needles (25 G × 1”) and syringes (1 mL) form 3 fish per tank and

pooled into 1 tube as a blood sample. Part of heparinized whole blood was used to analyze the hematocrit while plasma was obtained by centrifugation at 3000×*g* for 15min under 4°C. In addition, non-heparinized disposable syringes were used to collect blood for serum analysis. Serum samples were obtained by centrifugation at 3000×*g* for 15 min at 4°C to collect serum. Viscera and liver were removed from the above-mentioned fish and weighted individually to get viscerasomatic index (VSI) and hepatosomatic index (HSI) respectively. The obtained mucus, plasma and serum samples were stored at -80°C until the analysis.

3.3.3.4 *Biochemical analysis*

Experimental diets and fish whole body were analyzed for moisture, crude protein, total lipid and ash, in triplicate, using standard methods (Association of Official Analysis Chemists (AOAC) 1998), including moisture by oven-drying at 110 °C to constant weight, crude protein by the Kjeldahl method, crude lipid by the Soxhlet extraction method and ash by combustion in Muffle furnace at 550°C for 4h.

The micro hematocrit method was used for the determination of hematocrit level (Dawood *et al.*, 2015a, b). Plasma chemical parameters were measured spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan) (Tatsumi *et al.*, 2000). Biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were also measured spectrophotometrically from blood plasma with an automated analyzer FRAS4, Diacron International s.r.l., Grosseto, Italy by following (Morganti *et al.*, 2002; Dawood *et al.*, 2015a, b).

3.3.3.5 *Digestibility assessment*

A digestibility trial was conducted at the end of the feeding period by pooling the remaining fish from the same treatments and distributing randomly into triplicate tanks.

The fish were fed a diet containing chromium oxide (Wako Pure Chemical Industries, Ltd) as the inert marker at a level of 0.5% (Cr_2O_3, 5 g kg^{-1}) to the previous formulation and fed to the fish under the same condition as the feeding experiment apart from the difference in stocking density. After 5-day adaptation of the diets, feces were collected at 3-h intervals between the morning and afternoon feeds using a siphon. Feces of red sea bream very rapidly settled to the bottom of the tank and did not easily break up in the water, this enabled minimal nutrient and marker losses. Feces collection continued for two weeks until a sufficient amount of feces had been collected for analysis. Feces were freeze-dried immediately and kept at −20 ˚C until analysis. Concentration of chromium oxide in diets and feces was determined according to (Furukawa & Tsukahara 1966).

3.3.3.6 *Evaluation of non-specific immune responses*

3.3.3.6.1 *Lysozyme activity of Serum and Mucus*

Lysozyme activity of serum or mucus was determined with turbidometric assays (Lygren *et al.*, 1999). Ten microliters of serum or mucus samples were put into well of microplate, then added 190 µl of substrate (0.2 mg *Micrococcus lysodeikticus*, lyophilized cell, Sigma, USA) ml^{-1} PBS, pH 7.4. Their absorbance was measured after 1 and 5 min, which were incubated with gentle shaking at constant room temperature, at 450 nm with ImmunoMini NJ-2300 (System Instruments, Tokyo, Japan). A unit of enzyme activity was defined as the amount of enzyme that causes a decrease in absorbance of 0.001 min^{-1}.

3.3.3.6.2 *Serum and Mucus bactericidal activity*

The serum or mucus bactericidal activity was measured according to Iida (1989). Samples were diluted 3, 4 and 5 times with a Tris buffer (pH 7.5). The dilutions were mixed with a bacterial suspension (0.001 g ml^{-1}, *Escherichia coli*, IAM1239 cell line,

Kagoshima, Japan) and incubated at 25 ˚C for 24 h by micro tube rotator (MTR-103, AS ONE, Osaka, Japan). The solutions were incubated on TSA (Trypto-Soya agar, Nissui Pharmaceutical Co. Ltd., Japan) at 25 ˚C for 24 h. Colony forming unit (CFU) were counted by the plate counting method as described by (Ren *et al.,* 2007).

3.3.3.6.3 *Serum peroxidase activity*

The total peroxidase content in serum was measured according to Salinas *et al.* (2008), with some modifications. Briefly, 15 µl of serum were diluted with 35 µl of Hank's Buffered salt solution (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. Then, 50 µl of peroxidase substrate (3, 30, 5, 50-tetramethylbenzidine hydrochloride) (TMB; Thermo Scientific Inc., USA) was added. The serum mixture was incubated for 15 min. The colour-developing reaction in serum samples was stopped by adding 50 µl of 2 M sulphuric acid and the OD (450 nm) was measured in a plate reader. PBS was used as a blank instead of serum.

3.3.3.7 *Low salinity stress test*

Tolerance against exposure to low salinity seawater was examined. After the feeding trial, four fish from each rearing tank (total 12 fish per treatment) were randomly selected and transferred into a 100-L black tank containing low-salinity water (0.2%). The city tap water was de-chlorinated by strongly aerating for at least 24 h and mixed with seawater, and then used as low salinity water. The tanks for stress test were equipped with continuous aeration and kept under ambient temperature during the stress test. The test was conducted until half of the test population died. Prior to, and after low salinity seawater stress, fish were sampled from each tank. Mucus samples of surviving fish were collected in each test group with a constant area on the body surface (200 mm^2 on dorsal side) by a small piece of sterilized cotton, then immediately suspended in 1 ml of phosphate buffered saline (PBS, pH = 7.4) and centrifuged (3000×*g* for 5

min under 4 °C) and kept at -80°C until the analysis. Amounts of secreted mucus were quantitated based on the method described by Kakuta *et al.* (1996) and Dawood *et al.* (2015b). Total protein in the mucus supernatant was determined by the method of Lowry *et al.* (1951). Amounts of secreted mucus were expressed as mean relative values on the basis of total protein in the mucus sample of BG free group. Number of dead fish in each test tank was recorded every 20 min. The passing of time to reach 50% death was calculated according to (Dawood *et al.*, 2015a; Ren *et al.*, 2007).

3.3.3.8 *Statistical analysis*

All data were subjected to statistical verification using package super ANOVA 1.11, Abacus Concepts, Berkeley, California, USA. Probabilities of $P<0.05$ were considered significant. Significance differences between means were evaluated using the Turkey Kramer test.

3.3.4 Results

3.3.4.1 *Growth parameters and nutrient utilization*

Growth performance, nutrient utilization and survival of red sea bream juveniles fed test diets for 56 days are shown in Table 3.3.2. All treatments showed high survival rates between 93.3% and 97.8% and there were no significant differences among all groups ($P>0.05$). Growth performance and nutrient utilization of fish in all groups increased during the trial. The final body weight (Fn wt), weight gain (%WG), specific growth rate (SGR), and feed intake (FI) were significantly higher in fish fed diets supplemented with BG than in fish fed the control diet ($P < 0.05$). Additionally, the highest values of Fn wt, %WG, and SGR were obtained in fish fed a diet containing 1000 mg kg^{-1} BG (D4) when compared with other groups. But the Fn wt value was not significantly different from those of fish fed a diet containing 500 mg kg^{-1} BG (D3) (*P*

> 0.05). Feed efficiency ratio (FER) was significantly enhanced in the case of fish group D4 when compared with D1 group (P<0.05). However, no significant differences were observed among D2, D3, and D4 groups (P>0.05). Protein gain (PG) values were significantly higher in case of D2 and D4 groups when compared with D1 group (P<0.05). Protein efficiency ratio (PER) was not significantly affected by BG supplementation. The poorest growth performances and nutrient utilization were obtained in the control group.

3.3.4.2 *Whole body proximate analysis*

The proximate composition of the whole body of juvenile red sea bream is shown in Table 3.3.3. All the fish showed a change in the analyzed parameters compared to those of the initial values. In comparison with the control, dietary treatments had no significant influence on moisture, total lipid and crude ash contents at the end of the feeding trial except for D2 group, which recorded lower moisture and crude ash contents than the control group (D1). Crude protein content was significantly (P<0.05) increased in fish fed BG supplemented diets when compared with the control group. No difference (P>0.05) was also detected in CF, HSI and VSI of fish among treatments (Table 3.3.3).

3.3.4.3 *Blood parameters and oxidative status*

Table 3.3.4 represents the blood parameters of red sea bream after 56 days of feeding trial. Overall, dietary treatments had no effect on blood chemical parameters of fish except for hematocrit, plasma total protein, and glutamyl oxaloacetic transaminase (GOT). Hematocrit level increased significantly in fish fed diet supplemented with 0.5 g kg^{-1} BG (D3) compared with other experimental groups (P<0.05). Plasma total protein was significantly higher in D3 group than in control group (D1) (P<0.05), however no significant differences were detected between other experimental groups.

GOT of fish fed control diet was significantly higher than that of fish fed other experimental diets ($P<0.05$).

Oxidative status of fish was analyzed from plasma (Table 3.3.4; Fig. 3.3.1). Reactive oxygen metabolites (d-ROMs) were affected by the dietary supplementation, where all supplemented groups recorded a significantly lower value than the control group ($P<0.05$). On the other hand, no difference ($P>0.05$) was also detected in biological antioxidant potential (BAP) of fish among treatments (Table 3.3.4). Figure 3.3.1 shows the pattern of combined effects of d-ROMs and BAP. The D3 group was located in zone A, D2 and D4 groups in zone C and D1 group in zone D.

3.3.4.4 *Digestibility coefficients*

The apparent digestibility coefficients of protein ($ADC_{Protein}$) and lipid (ADC_{Lipid}) were found to be significantly different ($P<0.05$) with highest being in D4 group compared with control group (89.1% for protein and 84.9% for lipid) (Table 3.3.5). Moreover, ADC of lipid in D2 group showed a higher significant difference than control group. However, no significant difference was recorded in fish fed D4 diet compared with fish fed other supplemented groups, with the exception of the control group. Furthermore, no difference ($P>0.05$) was detected in ADC of dry matter of fish among treatments.

3.3.4.5 *Non-specific immune responses*

Figures 3.3.2, 3.3.3, and 3.3.4 show non-specific immune parameters. Serum and mucus lysozyme activity increased significantly ($P<0.05$) in the fish fed with different dietary doses of β-glucan compared to the fish fed without β-glucan (Fig. 3.3.2). With regards to both serum bactericidal activity (Fig. 3.3.3) and peroxidase content (Fig. 3.3.4), showed that those of the fish group D4 were significantly higher than control group ($P<0.05$), and there was no significant difference in serum bactericidal activity and peroxidase content of fish fed diet D4 and other supplemented diets (D2 and D3

groups). However, mucus bactericidal activity was not enhanced significantly with BG supplementation (Fig. 3.3.3).

3.3.4.6 *Tolerance to stress*

Prior to low salinity seawater stress, the secreted mucus was not significantly different among all the experimental groups (Fig. 3.3.5). Following low salinity stress test, significantly higher amounts of secreted mucus were observed in all supplemented groups than that in the control group. Although the highest concentrations were found in fish fed D2 and D4 diets, the value was not significantly different from that of fish fed D3 diet ($P>0.05$), nonetheless that value was significantly higher than that of fish fed the control diet ($P<0.05$) (Fig. 3.3.5).

Figure 3.3.6 shows the results of low salinity stress test. Fish groups that received diets supplemented with BG (D2, D3, and D4) showed significantly higher tolerance against low-salinity stress than the control group ($P<0.05$).

Table 3.3.2: Growth parameters and nutrient utilization in red sea bream fed test diets for 56 days

Parameters	Diets *no* (BG level, g kg^{-1} dry matter)			
	D1 (0)	D2 (250)	D3 (500)	D4 (1000)
In wt[1]	2.84±0.01	2.86±0.01	2.87±0.02	2.82±0.02
Fn wt[2]	15.24±0.19[a]	18.11±0.45[b]	18.9±0.24[bc]	20.14±0.14[c]
WG[3]	437.49±3.93[a]	532.36±16.11[b]	558.1±6.6[b]	614.06±7.46[c]
SGR[4]	3.01±0.01[a]	3.29±0.05[b]	3.36±0.02[b]	3.51±0.02[c]
FI[5]	12.11±0.28[a]	14.5±0.39[b]	15.09±0.35[b]	14.69±0.31[b]
FER[6]	1.03±0.04[a]	1.05±0.05[ab]	1.06±0.01[ab]	1.18±0.03[b]
PER[7]	2.01±0.04	2.11±0.15	2.1±0.07	2.31±0.02
PG[8]	166.51±1.53[a]	188.39±1.32[b]	178.83±0.26[ab]	180.5±5.29[b]
Sur[9]	93.33	97.78	97.78	97.78

[1] In wt: initial weight (g),[2] Fn wt: final weight (g),[3] WG: weight gain (%) = (final weight – initial weight) ×100/initial weight,[4] SGR: specific growth rate (% day^{-1}),[5] FI: feed intake (g dry diet $fish^{-1}$ 56 $days^{-1}$),[6] FER: feed efficiency ratio,[7] PER: protein efficiency ratio,[8] PG: protein gain (g kg body weight $gain^{-1}$),[9] Sur: survival (%).

Table 3.3.3: Whole body proximate analysis (g kg^{-1} wet weight) and somatic parameters in juvenile red sea bream fed test diets for 56 days

Parameters	Initial[1]	Diets *no* (BG level, g kg^{-1} dry matter)			
		D1 (0)	D2 (250)	D3 (500)	D4 (1000)
Moisture	813.4	720.4±4[b]	710.1±1.4[a]	716.6±0.1[ab]	713±1[ab]
Crude protein	108.6	155.7±1.3[a]	175.8±0.9[b]	168.2±0.3[b]	170.5±4.6[b]
Total lipid	23.7	75.2±0. 9	75.1±0.5	74.3±1.2	75.3±0.3
Crude ash	45.4	47.3±1[b]	39.9±1[a]	41.2±1.9[ab]	41.9±2.3[ab]
CF[2]	_	1.65±0.25	1.87±0.09	1.87±0.09	1.74±0.06
HSI[3]	_	2.1±0.08	2.2±0.17	1.96±0.13	2.34±0.2
VSI[4]	_	5.34±0.44	5.66±0.45	5.58±0.2	5.49±0.44

[1] Initial values are not included in the statistical analysis,[2] CF: condition factor,[3] HSI: hepatosomatic index,[4] VSI: viscerasomatic index (%).

Table 3.3.4: Blood parameters in juvenile red sea bream fed test diets for 56 days

Parameters	Diets *no* (BG level, g kg^{-1} dry matter)			
	D1 (0)	D2 (250)	D3 (500)	D4 (1000)
Hematocrit (%)	33±0.56[a]	33.33±0.33[a]	41.67±0.88[b]	34±1.16[a]
Total protein (g dl^{-1})	2.93±0.2[a]	3.4±0.21[ab]	3.73±0.1[b]	3.4±0.06[ab]
Total bilirubin (mg dl^{-1})	1.07±0.12	0.83±0.2	0.8±0.06	0.93±0.07
BUN (mg dl^{-1})[1]	7.33±0.67	5.67±0.67	5.33±0.33	5.67±0.33
GOT (IU l^{-1})[2]	159±9.5[b]	102.67±5.78[a]	121±10.69[ab]	137±10.02[ab]
GPT (IU l^{-1})[3]	45±2.52	41.33±3.28	52±3.22	48.33±3.38
Glucose (mg dl^{-1})	68±4	52.33±5.7	67.67±2.73	58.33±3.18
TG (mg dl^{-1})[4]	130.67±4.66	135.67±6.01	133.67±3.39	126.67±4.41
T-Cho (mg dl^{-1})[5]	140±6.66	136.33±4.33	146±4.58	123±5.03
d-ROMs[6]	167.33±14.52[b]	62.33±3.28[a]	62±14.5[a]	74±16.09[a]
BAP[7]	2625±99.32	2699.67±317.2	3051.67±80.05	2805±68.9

Abbreviation used: [1]BUN: blood urea nitrogen, [2]GOT: glutamyl oxaloacetic transaminase, [3]GPT: glutamic-pyruvate transaminase, [4]TG: triglyceride, [5]T-Cho: total cholesterol, [6]d-ROMs: reactive oxygen metabolites, [7]BAP: biological antioxidant potential.

Table 3.3.5: Apparent digestibility coefficients (ADC[1]) in red sea bream fed test diets

Parameters	Diets *no* (BG level, g kg^{-1} dry matter)			
	D1 (0)	D2 (250)	D3 (500)	D4 (1000)
ADC_{DM}	51.79±1.13	59.09±2.4	59.12±4.26	55.53±0.92
$ADC_{Protein}$	81.6±0.63[a]	85.53±0.3[ab]	85.23±0.93[ab]	89.11±1.91[b]
ADC_{Lipid}	75.84±1.43[a]	82.2±1.1[b]	79.08±0.27[ab]	84.89±2.01[b]

[1]ADC: Apparent digestibility coefficient of nutrient (ADC, %) =100-[(%Cr_2O_3 in diet/% Cr_2O_3in feces) × (%nutrient in feces/% nutrient in diet)].

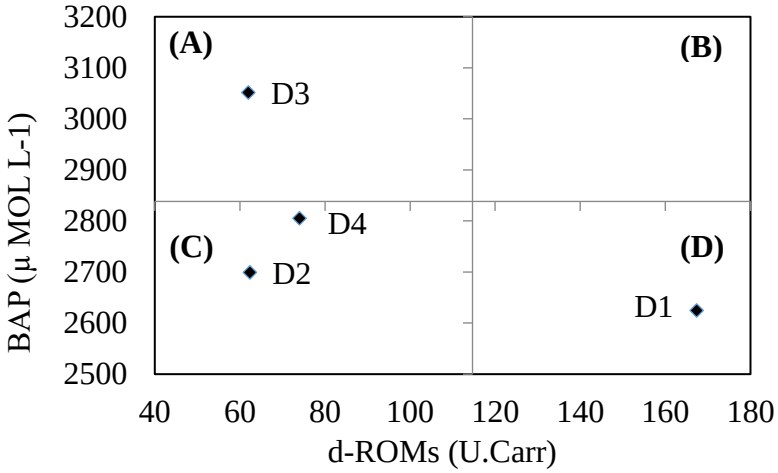


Fig. 3.3.1: Oxidative stress parameters in red sea bream fed test diets for 56 days. Central axis based on mean values of d-ROMs and BAP from each treatment. Zone (A): high antioxidant potential and low reactive oxygen metabolites (good condition); Zone (B): high antioxidant potential and high reactive oxygen metabolites (acceptable condition); Zone (C): low antioxidant potential and low reactive oxygen metabolites (acceptable condition); Zone (D): low antioxidant potential and high reactive oxygen metabolites (stressed condition).

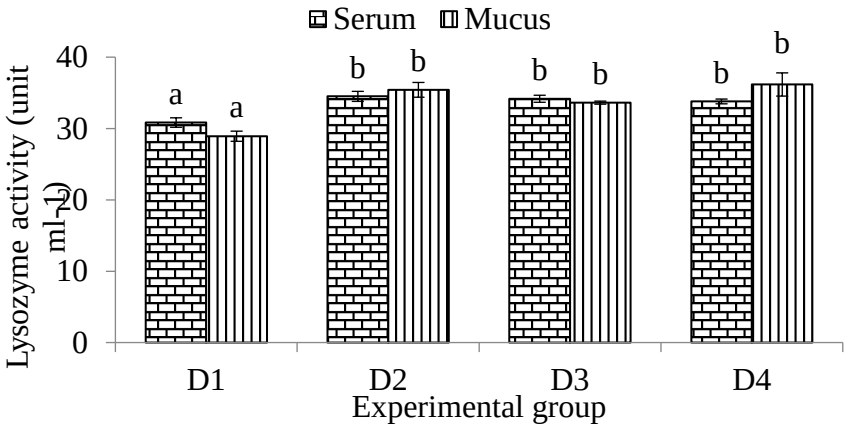


Fig. 3.3.2: Serum and mucus lysozyme activity of red sea bream fed experimental diets for 56 days. Values with the same letter are not significantly different ($P > 0.05$). Means with different alphabet are significantly different ($P < 0.05$). Absence of letters indicates no significant difference between treatments.

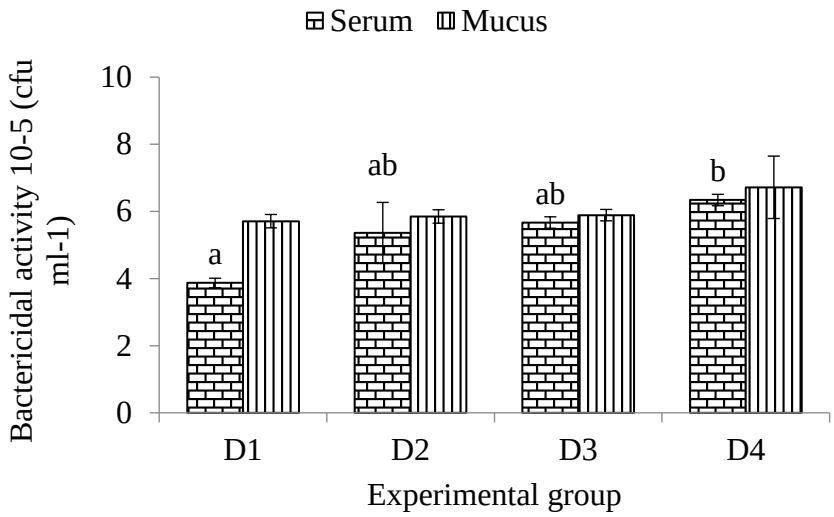


Fig. 3.3.3: Serum and mucus bactericidal activity of red sea bream fed experimental diets for 56 days. Values with the same letter are not significantly different ($P > 0.05$). Means with different alphabet are significantly different ($P < 0.05$). Absence of letters indicates no significant difference between treatments.

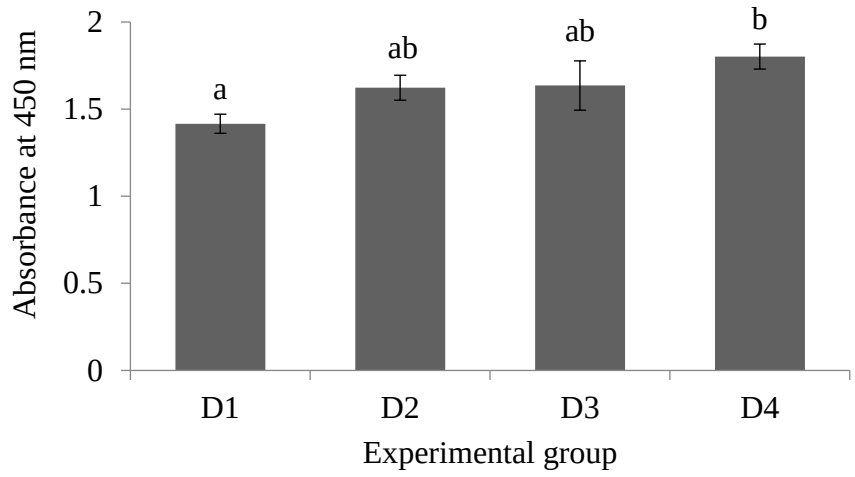


Fig. 3.3.4: Serum peroxidase activity levels of red sea bream juvenile fed experimental diets for 56 days. Values with the same letter are not significantly different ($P > 0.05$). Means with different alphabet are significantly different ($P < 0.05$).

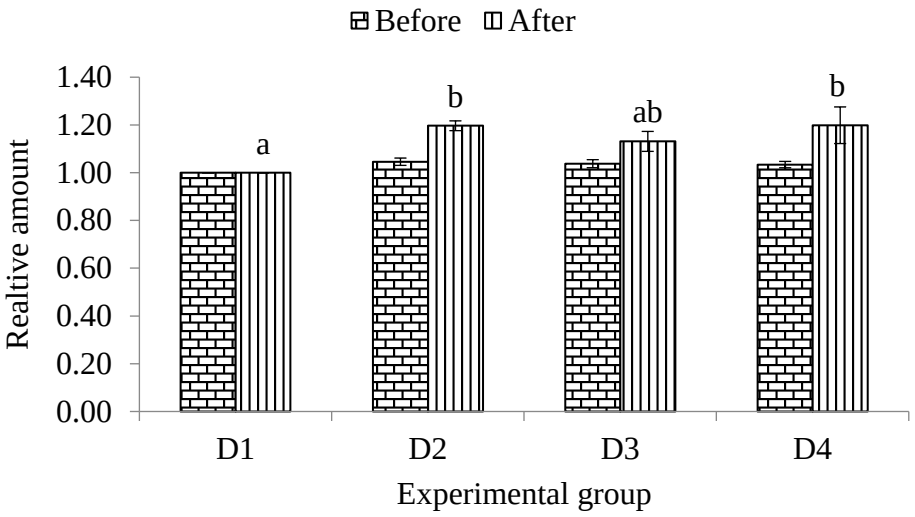


Fig. 3.3.5: Relative amounts of mucus secretion on body surface of red sea bream juvenile. Values are expressed as mean relative means ± pooled SEM (*n*=9 fish) on the basis of amount of mucus in fish group fed control diet. Values with the same letter are not significantly different ($P > 0.05$). Means with different alphabet are significantly different ($P < 0.05$). Absence of letters indicates no significant difference between treatments.

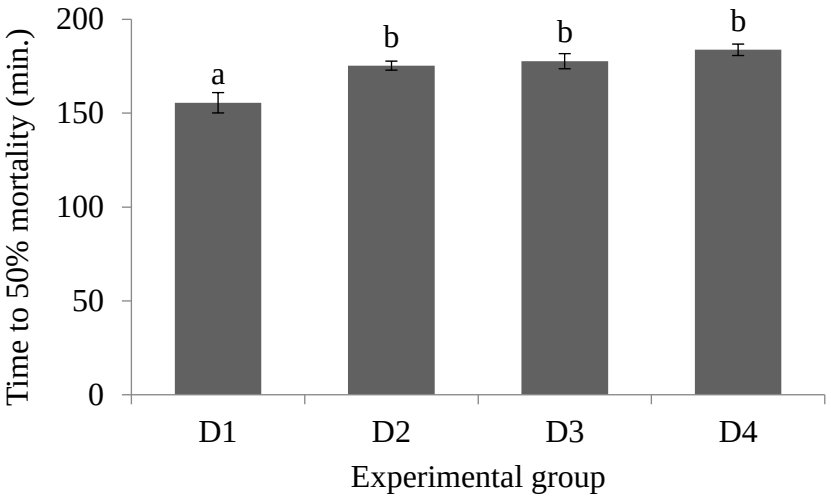


Fig. 3.3.6: Time to 50 % mortality (min.) after low salinity stress test when red sea bream fed experimental diets for 56 days. Values are means ± pooled SEM. Values with the same letter are not significantly different ($P > 0.05$). Means with different alphabet are significantly different ($P < 0.05$).

3.3.5 Discussion

Recently, aquaculture research has focused on probiotics, prebiotics, and β-glucans, in order to improve the health status and growth performance of fish (Dawood *et al.*, 2015a, b; Kühlwein *et al.*, 2014). In the present study, a higher growth performance and a noticeable increase in nutrient utilization of diets were observed in the experimental groups (D2, D3 and D4) compared with the control group (D1) after 56 days of feeding, especially in the case of D4 group. These results are in accord with previous studies which demonstrated that the application of β-glucan improves feed utilization and growth rates of snapper (Cook *et al.,* 2003), mirror carp (Kühlwein *et al.,* 2014), koi carp (Lin *et al.,* 2011), cyprinid rohu (Misra *et al.*, 2006), and large yellow croaker (*Pseudosciaena crocea*) (Ai *et al.,* 2007). Other studies have not observed growth enhancing properties when feeding β-glucan to Nile tilapia (*Oreochromis niloticus*) (Shelby *et al.,* 2009), channel catfish (*Ictalurus punctatus*) (Welker *et al.,* 2007), or hybrid striped bass (*Morone chrysops* X *Morone saxatilis*) (Li *et al.*, 2009). Up to date, it is not clear what causes the improvements in growth observed with dietary β-glucan in previous studies and it is not clear why growth enhancing effects of β-glucan occur in some aquatic species and not in others (Kühlwein *et al.,* 2014; Dawood *et al.,* 2015a). However, Dalmo & Bøgwald (2008) suggested that the effects may depend on the concentration of the β-glucan in the diet, its solubility, the fish species, the water temperature and length of the feeding period. They also hypothesized that when growth enhancing effects occur that the β-glucans induce a localized intestinal immune response that in turn leads to resistance against pathogens which otherwise would cause reduced weight gain and possibly diseases (Dalmo & Bøgwald 2008). The improved digestion and absorption, as shown by feed efficiency ratio and protein gain results in fish fed diet supplemented with β-glucan in this study might be a possible reason for

the higher performances of fish in these groups. Probably β-glucan was degraded in the digestive gland by glucanases to produce energy, permitting the use of more proteins for feed utilization and growth (López *et al.,* 2003).

Whole body compositions and protein gain values affected by the inclusion of dietary β-glucan indicated that enhanced growth performance might be because of a higher retention of these nutrients. In the present study, the apparent digestibility coefficients increased in the case of β-glucan supplemented diets, especially in the D4 group. Therefore, the higher growth performance and feed utilization of the fish fed the β-glucan diets might be partly due to its improving digestibility coefficients. Similar observations were also reported in red sea bream fed diets supplemented with β-glucan combined with dead bacterial cells (Dawood *et al.,* 2015a).

Certain blood parameters served as reliable indicators of fish health, which varied with nutritional status (Dawood *et al.,* 2015a, b). Blood parameters obtained in the present experiment are considered to be within the normal range for juvenile red sea beam, compared with those of the previous findings (Dawood *et al.,* 2015a, b). The blood hematocrit values increased in the case of D3 group, indicating improved health status. Increased hematocrit levels have also been observed also in mirror carp fed β-glucan at a level of 1 to 2% (Kühlwein *et al.,* 2014), Channel catfish (*Ictalurus punctatus*) (Welker *et al.,* 2007), and Nile tilapia (Abdel-Tawwab *et al.,* 2008) fed diets supplemented with β-glucan. Other studies did not observe any changes in hematocrit levels, for example, in spotted rose snapper (*Lutjanus guttatus*) (Del Rio-Zaragoza *et al.,* 2011), or rainbow trout (Siwicki *et al.,* 1994). Furthermore, the plasma total proteins increased significantly in fish fed with diet D3 when compared with fish fed D1. Similar observations were reported in common carp (*Cyprinus carpio* L.) fed diets supplemented with β-1.3/1.6-D-glucan (Dobšíková *et al.,* 2013). A decreased glutamyl

oxaloacetic transaminase (GOT) in this study demonstrated positive effects of β-glucan in which fish fed diets containing these additives showed clinical healthy signs and release of GOT enzyme into blood was at a minimal level. After the feeding trial, the glucose content never increased which indirectly indicates that prolonged dietary administration of β-glucan has no stress effect on the fishes. Similar results have been observed in *Labeo rohita* fed diets supplemented with β-glucan (Misra *et al.,* 2006). Oxidative stress was determined by measuring reactive oxygen metabolites (d-ROM) and biological antioxidant potential (BAP) in plasma samples (Dawood *et al.*, 2015a, b). Oxidative stress is believed to contribute to diseases in fish (Martinez-Alvarez *et al.*, 2005). It occurs when oxidant activity exceeds the neutralizing capacity of antioxidants (Celi *et al.,* 2010). Using these parameters our study showed that fish fed diets D2, D3, and D4 were more tolerant of oxidative stress indicating a higher health status. To date there remains a lack of explanation about how these additives work to effect on these parameters, so more studies are needed.

β-glucans showed better immune activity compared to the other immunostimulants (Cook *et al.,* 2003; Dobšíková *et al.,* 2013; Dawood *et al.,* 2015a; Falco *et al.*, 2014; Kühlwein *et al.,* 2014; Misra *et al.,* 2006). Lysozyme is an important defense molecule in the innate immune system of fish (Saurabh & Sahoo 2008). It is released by leukocytes and has antibiotic properties which enable it to damage bacterial cell walls of gram-positive and some gram-negative bacteria (Grinde 1989). In the present study, serum and mucus lysozyme activity (LA) which were shown to increase when fish were fed β-glucan supplemented diets (D2, D3, and D4). Other studies have also reported an increase in LA after β-glucan supplementation (Misra *et al.,* 2006; Li *et al.,* 2009; Lin *et al.*, 2011; Dalmo & Bøgwald 2008). Such enhancement in the lysozyme could be correlated with enhanced phagocytic activity. The in vivo activation of phagocytic cells

by β-glucan might have also induced other antimicrobial mechanisms, which include release of lysosomal enzymes, cationic peptides and production of reactive oxygen species (Kwak *et al.,* 2003). Serum bactericidal activity (BA) is one of the most important factors in host resistance against pathogenic bacteria. Interestingly, in this study, serum BA in D4 group was higher than in the control group. Increased BA was also reported also by (Misra *et al.,* 2006; Das *et al.,* 2009), following β-glucan immunostimulation. Similarly, higher level of peroxidase was observed in the case of D4 group than in the control group, confirming other results obtained by Kumari & Sahoo (2006). On the contrary, feeding with β-glucan resulted in no increase in serum peroxidase content in gilthead seabream (*Sparus aurata* L.) (Guzmán-Villanueva *et al.,* 2014). This was probably because of different species, fish size, and environmental conditions.

Skin mucus is the first line of defense against stressors (Fast *et al.,* 2002), providing both a physical barrier and enzyme systems (Hjelmeland *et al.,* 1983). Under the stressful conditions fish excrete mucus, and protein concentration in the skin mucus is an indicator for amounts of skin mucus secreted (Dawood *et al.,* 2015a, b; Kakuta *et al.,* 1996; Taoka *et al.,* 2006b). In the present study, following low salinity stress test higher amounts of mucus secretion were observed in all β-glucan supplemented groups compared to the control group indicating the potential of β-glucan for improving red sea bream tolerance to stress. This was also observed in red sea bream fed diets supplemented with β-glucan combined with heat killed probionts (Dawood *et al.,* 2015a). There remains a lack of explanation as to how these additives work to enhance skin mucus secretion.

The lethal time of 50 % mortality (LT_{50}) in low salinity water is used to assess the healthy status of the fish (Dawood *et al.,* 2015b). It is well known that stress affects the

survival and growth of fish, since stress responses tend to increase the energy demand at the expense of anabolic processes (Kubilay & Ulukoy 2002). Our results indicated that the β-glucan supplemented groups showed higher tolerance against stress than control group. Together with high feed intake; as a result, energy and other nutrients will be available to synthesize adrenal steroids to face the physical stressors. This mechanism may have contributed to the higher tolerance against stress in this study.

In connection with the previous results, future studies are recommended to assess the gastrointestinal microbiota, gut histology, and disease challenge study which could be affected directly by the supplementation of BG. Future research is also needed in order to investigate the underlying reasons for the growth promoting effects and the improved health features found in the present study.

In conclusion, it is clear that intake of β-glucan enhanced growth, feed utilization, and health condition of red sea bream juveniles through improvement of various immunological parameters such as lysozyme activity, bactericidal activity, peroxidase content, mucus secretion and it also induced higher resistance to the stress. Feeding of β-glucan at a medium dose of 250 to 1000 mg kg^{-1} is ideal for optimum enhancement of the immune system.

Chapter IV:

Using feed additives to increase soybean meal's (SBM's) inclusion rates in soy-sensitive species

Experiment I: Effects of partial substitution of fish meal by soybean meal with or without heat killed *Lactobacillus plantarum* (LP20) on growth performance, digestibility and immune response of amberjack, *Seriola dumerili* juveniles

Effects of partial substitution of fish meal by soybean meal with or without heat killed *Lactobacillus plantarum* (LP20) on growth performance, digestibility and immune response of amberjack, *Seriola dumerili* juveniles

4.1 Abstract

A 56-days feeding trial was conducted to evaluate the effects of supplemented diets with heat-killed *Lactobacillus plantarum* (HK-LP) with graded levels of soybean meal (SBM) on growth, digestibility, blood parameters, and immune response of *Seriola dumerili* (initial weight, 25.05±0.1g). Seven isonitrogenous and isolipidic practical diets were formulated to contain 0%, 15%, 30% and 45% SBM, and each SBM level was supplemented with HK-LP at 0.0 and 0.1%. Fish fed diet contains 30% SBM with HK-LP grew significantly faster than the other groups with notable feed intake (FI) and protein retention (PR). Further, protein gain (PG), whole body protein content, protease activity, protein and lipid digestibility were significantly increased for all fish groups except for fish fed diet contains 45% SBM with or without HK-LP. Interestingly, lysozyme activity was significantly enhanced in fish fed diets contain 15% and 30% SBM with HK-LP. Hematocrit level, bactericidal and total peroxidase activities revealed a significant increase in 30% SBM with HK-LP group. In addition, fish fed diets contain 0% and 30% SBM with HK-LP showed higher tolerance against low-salinity stress compared with other groups. In conclusion, the addition of HK-LP to amberjack diets appeared to improve SBM utilization, immune response and stress resistance.

Keywords: Soybean meal, *Seriola dumerili*, Growth performance, Blood parameters, Immune response

4.2 Introduction

Fish meal (FM) represents an ideal nutritional source of dietary protein for fish. Increasing demand, unstable supply and high prices of FM along with the continuous expansion of aquaculture are reasons for many nutritionists to realize that soon they will no longer be able to afford it as a major protein source in aquafeeds. Currently one of the challenges that fish nutritionists face is the need to partially or totally replace FM with less expensive, non-traditional animal or plant protein sources (Lunger *et al.*, 2007; Peng *et al.*, 2013).

Soybean proteins have been recognized as one of the most appropriate alternative protein sources for FM in aquafeed because of their consistent nutritional composition, comparatively balanced amino acid profile, availability and reasonable price (Storebakken *et al.*, 2000). Soybean meal (SBM) has proven to be well accepted by yellowtail (Shimeno *et al.*, 1993; Viyakarn *et al.*, 1992; Watanabe *et al.*, 1992). Tomas *et al.* (2005) investigated the possible use of SBM as a substitute for FM in the diets of yellowtail by progressively increasing its inclusion level. The authors found a decrease in final weights as the SBM content increased starting from 30% protein substitution rate.

Methods for increasing SBM's inclusion rates in soy-sensitive species such as amberjack are required, and one of the methods is to apply dietary supplementation of functional compounds. Non-viable microbes exhibit beneficial effects due to their function as immunostimulants. Using live bacteria may cause a potential risk to wild aquatic organisms considering the fact the bacteria may escape into the environment. Therefore, the use of inactivated bacteria clearly solves such safety-related issues since they can no longer interact with other aquatic organisms (Rosales *et al.*, 2006). Besides that, inactivated bacteria are considered one of the most practical candidates of feed

additives. This is due to its high tolerance against temperatures which are produced during preparation of fish diets especially in the course of mincing and pelleting, without affecting its functional activity. This makes it more efficient than other candidates to achieve success in fish farming (Rosales *et al.*, 2006; Salinas *et al.*, 2008; Rodriguez-Estrada *et al.*, 2013).

Heat-killed *Lactobacillus plantarum* (HK-LP) is a potential candidate as one of the functional additives for fish. Recently, effects of HK-LP have been investigated as immunostimulants (Hirose *et al.*, 2006; Hirose *et al.*, 2009; Tung *et al.*, 2010; Khonyoung and Yamauchi, 2012). Khonyoung and Yamauchi (2012) reported that the diet supplemented with HK-LP (L-137) might activate intestinal function by increasing segmented filamentous bacteria, while inducing a better body weight gain in broilers. Oral administration of HK-LP has enhanced growth performance and immune responses of larval and post-larval Kuruma shrimp, *Marsupenaeus japonicus* bate (Tung *et al.*, 2010, 2009). Oral administration of inactivated *Lactobacillus delbrueckii* subsp. *lactis* and *Bacillus subtilis* appear to cause good immune stimulatory properties of gilthead seabream (*Sparus aurata* L.) (Rosales *et al.*, 2006; Salinas *et al.*, 2008). Oral administration of heat-killed *Enterococcus faecalis* enhanced growth performance and immune responses of rainbow trout (Rodriguez-Estrada *et al.*, 2013). Furthermore, heat-killed bacteria were also compared with live form in tilapia (Taoka *et al.*, 2006a). According to these observations, it was hypothesized that HK-LP may also be effective in responses of growth and nonspecific immune systems of amberjack, *Seriola dumerili*.

The amberjack is one of the most important cultured species in Japan because of its delicacy and comparatively higher market value. It is distributed throughout the tropical and subtropical seas except the Pacific Ocean (Laroche *et al.*, 1984; Kader *et al.*, 2013).

There have been no studies about dietary SBM and the effect of HK-LP have been undertaken on amberjack to date, the trial reported here was conducted to determine the effects of the partial substitution of FM by SBM with or without HK-LP on growth, digestibility, blood chemistry, immune responses and stress resistance of amberjack juveniles.

4.3 Materials and methods

4.3.1 *Test diets*

Tables 4.1 and 4.2 show the composition and chemical analysis of the experimental diets. All the dietary components were obtained commercially, except for HK-LP preparation which was provided by House Wellness Foods Corp. (Itami, Japan), and it contains 20% HK-LP and 80% dextrin in dried-weight basis. HK-LP Prep (LP20) was prepared based on the method previously described by Murosaki *et al.* (1998). The product was stored at −20 ˚C until use. Using brown fish meal and soybean meal as main protein sources, Pollack liver oil and soybean lecithin as main lipid source, seven isonitrogenous (50.5% crude protein) and isolipidic (12.3% crude lipid), practical diets were formulated to contain 0%, 15%, 30% and 45% soybean meal, and two levels of HK-LP (0.0 and 0.1%) [SBM0, SBM15, SBM15(0.1), SBM30, SBM30(0.1), SBM45, and SBM45(0.1)]. Moreover, crystalline amino acid (CAA) mixture of lysine, methionine, betaine, glycine and alanine were supplemented to meet essential amino acid (EAA) requirements of juvenile amberjack. Wheat flour was supplied as the carbohydrate or nitrogen-free extract source, activated gluten was used as a binder to produce pellet diet, and cellulose powder was used to adjust to 100% total proportion. The diets were prepared by thoroughly mixing all the dry ingredients in a food mixer for 15 minutes. Pollack liver oil, soybean lecithin and HK-LP Prep were premixed with

a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan), added to the dry ingredients and mixed for another 15 min. Water (35–40% of the dry ingredients) was then added to the premixed ingredients and mixed for an additional 15 min. The pH of the diets was adjusted to the range of 7.0–7.5 with 4 N sodium hydroxide. The mixture was then passed through a meat grinder with an appropriate diameter (2.2–3.1 mm) to prepare pellets, which were then dried in a dry-air mechanical convection oven (DK 400, Yamato Scientific, Tokyo, Japan) at 50 ˚C for about 120 min. to approximately 10-11% moisture. The test diets were stored in a freezer at −20 ˚C until use.

Table 4.1: Formulation of the experimental diets (% dry matter)

Ingredient	Soybean meal (SBM) replacement, % (HK-LP Prep., %)						
	SBM0	SBM15	SBM15(0.1)	SBM30	SBM30(0.1)	SBM45	SBM45(0.1)
Brown fish meal	61	51	51	40	40	30.5	30.5
soybean meal[1]	0	15	15	30	30	45	45
Wheat flour	10	8	8	5	5	1	1
Soybean lecithin	3	3	3	3	3	3	3
Pollack liver oil	5	5	5	5	5	5	5
Vitamin mixture	3	3	3	3	3	3	3
Mineral mixture	3	3	3	3	3	3	3
Stay-C	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Activated gluten	5	5	5	5	5	5	5
α-Cellulose	9	5.63	5.53	4.17	4.07	2.2	2.1
Amino acid premix[2]	0.9	1.27	1.27	1.73	1.73	2.2	2.2
HK- LP Prep	0	0	0.1	0	0.1	0	0.1
Total	100	100	100	100	100	100	100

[1] J. Oil Mills, Japan, [2]Amino acid premix (g 100 g^{-1} diet) at soybean meal replacement level of 15%, the mixed amino acids just as follows: lysine, 0.20; methionine, 0.17; Alanine, 0.30; Betaine, 0.30; Glycine, 0.30. Amino acid premix (g 100 g^{-1}diet) at fish meal replacement level of 30%, the mixed amino acids just as follows: lysine, 0.46; methionine, 0.38; Alanine, 0.30; Betaine, 0.30; Glycine, 0.30. Amino acid premix (g 100 g^{-1}diet) at fish meal replacement level of 45%, the mixed amino acids just as follows: lysine, 0.72; methionine, 0.58; Alanine, 0.30; Betaine, 0.30; Glycine, 0.30.

Table 4.2: Chemical analysis of the experimental diets

Ingredient	Soybean meal (SBM) replacement, % (HK-LP Prep., %)						
	SBM0	SBM15	SBM15(0.1)	SBM30	SBM30(0.1)	SBM45	SBM45(0.1)
Proximate composition (%, dry matter basis)							
Crude protein	50.81	50.47	50.34	50.15	50.74	51.38	50.82
Total lipid	11.85	12.29	11.93	12.74	12.71	12.66	12.30
Ash	11.10	10.78	11.41	11.50	11.54	11.37	11.82
Gross energy (KJ g^{-1})	19.75	19.92	19.63	19.84	19.91	19.95	19.76
Amino acid profiles (AA g 100 g^{-1} diet, dry matter basis)							
Arginine	2.81	2.94	2.86	3.02	2.93	3.17	3.01
Histidine	1.42	1.23	1.35	1.36	1.47	1.54	1.39
Isoleucine	2.26	2.24	2.04	2.38	2.18	2.27	2.47
Leucine	3.97	3.74	3.94	3.64	3.84	3.45	3.85
Lysine	3.91	3.81	3.61	3.75	3.66	3.89	3.59
Methionine	1.77	1.60	1.54	1.39	1.59	1.54	1.24
Phenylalanine	2.01	2.07	2.17	2.10	2.21	2.18	2.18
Therionine	2.18	2.26	2.26	2.11	2.11	2.01	2.29
Valine	2.55	2.76	2.66	2.52	2.62	2.46	2.36
Σ IDAA[1]	22.86	22.64	22.42	22.27	22.61	22.50	22.37

[1] Σ IDAA: total indispensable amino acid contents.

4.3.2 *Experimental fish and feeding protocol*

Juvenile amberjack (*Seriola dumerili*), with mean initial body weight of 25.05±0.1g (mean±S.E.), were purchased from Kagoshima prefecture seed production center, Kagoshima Prefecture, Japan and transferred to the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. The fish were acclimatized for two weeks in laboratory conditions and reared in a 500-L tank with flow-through system. During this period, a commercial diet (50% crude protein; Higashimaru, Japan) was supplied to the fish. Stocking was done at twenty fish per tank with the triplicate tanks per treatment in 200-L polycarbonate tanks (filled with 180L of water) in a flow-through sea water system where each tank was equipped with an inlet, outlet, and continuous aeration. The tanks were maintained under natural light/dark regime. All fish were fed the respective test diets to satiation level by hand twice a day at 9.00 and 16.00 h., 7 days per week for 56 days. Any uneaten feed left was removed after feeding and dried using a freeze drier then subtracted from the total feed intake. The seawater was pumped from the deep basin of Kagoshima Bay, Japan. It was gravel filtered and supplied into the system. A flow rate of 1.5L min^{-1} was maintained throughout the experimental period. During the experimental period, the monitored water quality parameters (mean±S.D.) were as follows, water temperature 25.2±1.3°C, pH 8±0.5, salinity 33.3±0.5ppt and dissolved oxygen 6.1±0.5 mg L^{-1}. These ranges were considered within optimal values for juvenile amberjack.

4.3.3 *Sample collection and biochemical analysis*

At the beginning, a pooled sample of 10 fish was stored at −20°C for initial whole body analysis. While at the end of the feeding trial, all fish were fasted for 24h prior to final sampling. All the fish were anaesthetized with Eugenol (4-allylmethoxyphenol, Wako Pure Chemical Ind., Osaka, Japan) at 50 mg L^{-1}.Then the total number; individual body

weight and length of fish from each tank were measured. Three fish from each replicate tank were randomly collected and stored at −20 °C for final whole body analysis. Blood was taken from the caudal vein of five fish in each replicate tank using heparinized disposable syringes. A small fraction of the heparinized blood was used to analyze the hematocrit and hemoglobin levels. Hematocrit was determined using the micro hematocrit technique. Plasma samples were obtained by centrifugation at 3000×g for 15 min at 4 °C using a high-speed refrigerated micro centrifuge (MX-160; Tomy Tech USA Inc., Tokyo, Japan) and kept at −80 °C. In addition, non-heparinized disposable syringes were used to collect blood for serum analysis. Serum samples were obtained by centrifugation at 3000×g for 15 min at 4°C to collect serum. Three other fish were randomly sampled from each dietary tank and used for collection of liver and viscera. Viscera and liver were removed then weighed to get viscerasomatic index and hepatosomatic index respectively. Digestive tracts were separated, cut into small pieces, washed with pure water, pooled together and stored at −80 °C. Hemoglobin, plasma chemical parameters and total serum protein (TSP) were measured spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan) (Tatsumi *et al.*, 2000). Biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were also measured spectrophotometrically from blood plasma with an automated analyzer (FRAS4, Diacron International s.r.l., Grosseto, Italy) by following (Morganti *et al.*, 2002; Kader *et al.*, 2012). Plasma cortisol was measured Using commercial kits (Cortisol EIA Kit, product number EA65, Oxford Biomedical Research Inc., Oxford, MI) according to the procedure outlined by the manufacturer. Protease activity (PA) was analyzed using digestive organ samples according to Kader *et al.* (2010). The ingredients, diets and fish whole body were analyzed for moisture, crude protein, total lipid and ash, in

triplicate, using standard methods (AOAC, 1998). This entailed moisture analysis by oven-drying at 110 °C to constant weight, crude protein analysis by the Kjeldahl method, crude lipid analysis by the Soxhlet extraction method and ash content analysis by combustion in Muffle furnace at 550°C for 4h. The amino acid profiles of the experimental diets were analyzed by high performance liquid chromatography (HPLC, Shimadzu Corp. Kyoto, Japan) according to the previous studies (Kader *et al.*, 2012; Han *et al.*, 2013).

4.3.4 *Low-salinity stress test*

Tolerance against exposure to low-salinity seawater was examined. After the feeding trial, five fish from each rearing tank (total of 15 fish per treatment) were randomly selected and transferred into a 100-L black tank containing low-salinity water (0.2%). The tanks for stress test were equipped with continuous aeration and kept under ambient temperature during the stress test. The number of dead fish in each test tank was recorded every 20 min. The passing of time to reach 50% death was calculated using the method previously described by (Moe *et al.*, 2004; Ren *et al.*, 2007).

5.3.5 *Evaluation of non-specific immune responses*

Lysozyme activity of serum was determined with turbidometric assays (Lygren *et al.*, 1999) at 450 nm with ImmunoMini NJ-2300 (System Instruments, Tokyo, Japan). A unit of enzyme activity was defined as the amount of enzyme that causes a decrease in absorbance of 0.001/min.

The serum bactericidal activity was measured according to Iida *et al.* (1989). Serum was diluted 3, 4 and 5 times with a Tris buffer (pH 7.5). The dilutions were mixed with a bacterial suspension (0.001g/ml, *Escherichia coli*, IAM1239 cell line, Kagoshima, Japan) and incubated at 25°C for 24 h by micro tube rotator (MTR-103, AS ONE, Osaka, Japan). The solutions were incubated on TSA (Trypto-Soya agar, Nissui

Phatmaceutical Co. Ltd., Japan) at 25°C for 24 h. Colony forming unit (CFU) were counted by the plate counting method as described by Ren et al. (Ren *et al.*, 2007). The total peroxidase content in serum was measured according to Salinas *et al.* (2008), with some modifications. Briefly, 15 µl of serum were diluted with 35 µl of Hank's Buffered salt solution (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. Then, 50 µl of peroxidase substrate (3, 30, 5, 50-tetramethylbenzidine hydrochloride) (TMB; Thermo Scientific Inc., USA) was added. The serum mixture was incubated for 15 min. The colour-developing reaction in serum samples was stopped by adding 50 µl of 2 M sulphuric acid and the OD (450 nm) was measured in a plate reader. PBS was used as a blank instead of serum.

4.3.6 *Digestibility assessment*

Digestibility of each diet was measured after the growth trial. The fish were fed a diet containing chromium oxide (Wako Pure Chemical Industries, Ltd) as the inert marker at a level of 0.5% (Cr_2O_3, 5g/kg) was added. Fish were acclimated to the diet containing chromic oxide for five days. In the morning of the 6^{th} day, fish were fed each diet to apparent satiation twice daily. Six hours after feeding, feces were collected by putting pressure from belly to anus. Feces collection continued for ten days until a sufficient amount of feces had been collected for analysis. Pooled fecal samples were immediately ground after freeze-drying and kept at −20°C until analysis. Concentration of chromium oxide in diets and feces was determined according to Furukawa and Tsukahara (1966).

5.3.7 *Evaluation of growth performance parameters*

The following variables were evaluated:

Weight gain (%) = (final weight – initial weight) ×100/initial weight; Specific growth rate (day^{-1}) = {(Ln (final weight) –Ln (initial weight)) / duration} ×100; Survival (%) =100× (final no. of fish/ initial no. of fish); Feed intake = (dry diet given−dry remaining

diet recovered)/ no. of fish; Feed efficiency ratio =live weight gain (g) /dry feed intake (g); Protein efficiency ratio =live weight gain (g)/dry protein intake (g); Protein gain (g kg weight gain^{-1}) = {(final weight (g) ×final whole body protein content (%)/100) − (initial weight (g) ×initial whole body protein content (%)/100)}/ (weight gain (g)) ×1000; Protein retention (% of intake) = [protein gain (g kg weight gain^{-1}) ×100]/ protein intake (g kg weight gain^{-1}); Condition factor =weight of fish (g)/ (length of fish) ^{3} (cm) ^{3}×100; Hepatosomatic index (%) =weight of liver /weight of fish×100; Viscerasomatic index (%) =weight of viscera/weight of fish×100; Apparent digestibility coefficient (%) =100-[(%Cr_2O_3 in diet/% Cr_2O_3in feces) × (%nutrient in feces/% nutrient in diet)].

5.3.8 *Statistical analysis*

All data were subjected to statistical verification using package super ANOVA 1.11, Abacus Concepts, Berkeley, California, USA. Probabilities of $P<0.05$ were considered significant. Differences in significance between means were evaluated using the Turkey Kramer test.

4.4 Results

4.4.1 *Growth parameters and nutrient utilization*

Growth performance and feed utilization of the fish are given in Table 4.3. Survival (%) of fish did not differ significantly ($P>0.05$) among treatments. Final weight, weight gain (%), specific growth rate (SGR), and protein gain (PG) of fish fed SBM30(0.1) diet was significantly higher than those fed the other diets. On the other hand, the growth parameters of fish fed SBM15, SBM15(0.1), and SBM30 were not significantly different from those of fish fed FM (SBM0). The poorest growth performance was found in fish fed SBM45 and SBM45(0.1). However, SGR was significantly ($P<0.05$)

higher in fish fed SBM45(0.1) than SBM45. Similarly, PG and protein retention (PR) were also significantly decreased in fish fed SBM45 and SBM45(0.1) while no difference was detected between FM (SBM0) and the remaining treatments. However, no difference was detected in feed efficiency ratio (FER) and protein efficiency ratio (PER) between FM (SBM0) and other dietary groups. Dietary treatments significantly affected (P<0.05) feed intake (FI) of fish. FI was markedly improved by supplementing CAA and HK-LP Prep. Significantly higher FI was found in fish fed SBM30(0.1) compared to other diet groups. However, there were no significant differences in FI of fish fed SBM15, SBM15(0.1), SBM30 and FM (SBM0). On the other hand, SBM45 and SBM45(0.1) diets were not well accepted by the fish, and the value was significantly lower (P<0.05) than other test diets.

4.4.2 *Whole body proximate analysis*

The proximate composition of the whole body of juvenile amberjack is shown in Table 4. 4. In comparison with the control, dietary treatments had no significant influence on the total lipid and crude ash contents at the end of the feeding trial. However, whole body crude protein contents in all experimental groups were significantly higher than SBM45 and SBM45(0.1) groups. Moreover, moisture content was significantly (P<0.05) decreased in fish fed SBM0 and SBM15(0.1) groups. No difference (P>0.05) was also detected in CF, HSI and VSI of fish among treatments (Table 4.4).

4.4.3 *Blood parameters and responses against stress*

Table 4.5 represents the blood parameters of amberjack after 56 days of feeding trial. Overall, dietary treatments had no effect on blood chemical parameters of fish except for the case of hematocrit, hemoglobin, glucose, and triglyceride (TG). Hematocrit level was significantly (P<0.05) higher in fish fed SBM30(0.1) than those fed SBM30 diet while no significant (P>0.05) differences were detected among other groups. Similarly,

hemoglobin was significantly increased in fish fed SBM0 when compared with the SBM45 group while no significant differences were detected among other groups. Plasma glucose content was significantly (P<0.05) more decreased in the SBM0 group than in other groups. On the other hand, TG was significantly (P<0.05) higher in SBM0 than SBM30(0.1) group while no significant (P>0.05) differences were detected among other groups. Experimental diets had no significant effect on the relative value (%) of plasma cortisol levels among all treatments. Oxidative status of fish was analyzed from plasma (Table 4.5). The lowest values of reactive oxygen molecules (d-ROMs) were detected in fish fed SBM0, SBM15 and SBM30(0.1) diets. On the other hand, biological antioxidant potential (BAP) was found highest in the SBM30(0.1) group. Figure 4.2 shows the pattern of combined effects of d-ROMs and BAP. The SBM0, SBM30, and SBM30(0.1) groups were located in zone (A), SBM15(0.1) group in zone (B), SBM15 and SBM45(0.1) in zone (C); and SBM45 group in zone (D), respectively. Figure 5.1 shows the results of the low-salinity stress test. The fish that received SBM0 and SBM30(0.1) diets clearly showed significantly (P<0.05) higher tolerance against low-salinity stress than those of other groups. However, time to 50% mortality was found significantly lowest in SBM45 group.

4.4.4 *Non-specific immune responses*

Serum lysozyme activity was significantly (P<0.05) increased in the SBM15(0.1) group when compared with other groups while no significant difference was detected between the SBM15(0.1) and SBM30(0.1) groups (4.3). Fish fed the SBM30 diet supplemented with 1g kg^{-1} HK-LP showed significantly higher serum bactericidal activity than the other groups (Fig. 4.4). Similarly, serum peroxidase activity recorded the highest significant values (P<0.05) in the SBM15 and SBM30(0.1) groups (Fig. 4.5) while no significant differences were detected among other groups. Although not statistically

significant, the comparatively higher total serum protein values were found in SBM0, SBM15, and SBM15(0.1) groups (Fig. 4.6).

4.4.5 *Protease activity and digestibility coefficients*

Protease activity (PA, unit mg^{-1} protein) in the digestive tract of amberjack recorded no significant differences between SBM0, SBM15, SBM15(0.1), SBM30, and SBM30(0.1) groups. However, the SBM45 group recorded the poorest PA value among other experimental groups; moreover, the PA was significantly higher in the SBM45(0.1) group than the SBM45 group. The apparent digestibility coefficient (ADC) of protein was significantly (P<0.05) higher in fish fed SBM0, SBM15, SBM15(0.1), SBM30, and SBM30(0.1) groups than SBM45 and SBM45(0.1) groups. ADC of lipid was found to be significantly different (P<0.05) with being higher in the SBM15 and SBM30(0.1) groups than the other experimental groups (Table 5.6).

Table 4.3: Growth parameters and nutrient utilization in amberjack fed test diets for 56 days*

Parameters	Soybean meal (SBM) replacement, % (HK-LP Prep., %)						
	SBM0	SBM15	SBM15(0.1)	SBM30	SBM30(0.1)	SBM45	SBM45(0.1)
Fn wt[1]	136.6±2.8^{b}	136±3.06^{b}	136.7±2.78^{b}	136.9±3.11^{b}	149.2±2.36^{c}	101.7±1.31^{a}	105.8±1.29^{a}
WG[2]	445.1±11.5^{b}	443.8±11.94^{b}	446.4±11.21^{b}	446.8±12.72^{b}	494.1±8.52^{c}	304.8±5.78^{a}	320.95±6.06^{a}
SGR[3]	3.03±0.04^{c}	3.02±0.04^{c}	3.03±0.04^{c}	3.04±0.04^{c}	3.18±0.03^{d}	2.49±0.03^{a}	2.57±0.02^{b}
FI[4]	120.62±3.06^{b}	118.68±3.19^{b}	120.63±1.56^{b}	119.58±2.39^{b}	130.27±2.6^{c}	88.2±4.24^{a}	90.33±2.68^{a}
FER[5]	0.93±0.03	0.94±0.04	0.93±0.02	0.94±0.02	0.95±0.03	0.87±0.03	0.89±0.03
PER[6]	1.82±0.06	1.86±0.08	1.83±0.05	1.85±0.04	1.87±0.06	1.7±0.06	1.76±0.05
PG[7]	203.65±1.88^{b}	204.38±5.08^{b}	207.05±3.69^{b}	200.92±1.22^{b}	206.85±2.01^{b}	179.9±1.64^{a}	183.17±0.2^{a}
PR[8]	124.76±2.57^{b}	122.67±6.1^{b}	126.49±2.19^{b}	121.12±3.55^{b}	137.17±1.93^{c}	81.32±3.26^{a}	84.07±2.29^{a}
Sur[9]	100	95	100	100	100	90	93.33

*Values are means of triplicate groups ±S.E.M. Within a row, means with different letters are significantly different (P<0.05), means with the same letters are not significantly different (P>0.05). Absence of letters indicates no significant difference between treatments. Average initial body weight, means ± S.E.M., 25.05 ± 0.1 g.

[1] Fn wt: final weight (g), [2] WG: percent weight gain (%), [3] SGR: specific growth rate (% day^{-1}), [4] FI: feed intake (g dry diet fish^{-1} 56 days^{-1}), [5] FER: feed efficiency ratio, [6] PER: protein efficiency ratio, [7] PG: protein gain (g kg body weight gain^{-1}), [8] PR: protein retention (% of intake), [9] Sur: survival (%).

Table 4.4: Whole body proximate analysis (%) and somatic parameters in juvenile amberjack fed test diets for 56 days *

Parameters	Initial [1]	Soybean meal (SBM) replacement, % (HK-LP Prep., %)						
		SBM0	SBM15	SBM15(0.1)	SBM30	SBM30(0.1)	SBM45	SBM45(0.1)
Moisture	72.84	71.07±0.2[a]	71.17±0.31[ab]	71.02±0.09 [a]	71.18±0.55 [ab]	71.2±0.2[ab]	72.44±0.05 [b]	72.44±0.05 [b]
Crude protein	19.58	20.22±0.15[b]	20.27±0.41[b]	20.5±0.3[b]	20±0.1[b]	20.5±0.16[b]	18.38±0.13 [a]	18.62±0.01[a]
Total lipid	3.42	4.48±0.08	4.17±0.14	4.35±0.15	4.43±0.15	4.26±0.05	4.49±0.01	4.33±0.14
Crude ash	4.05	4.1±0.09	3.95±0.09	3.78±0.02	3.96±0.01	3.99±0.05	3.75±0.36	3.72±0.17
CF [2]	_	1.41±0.02	1.39±0.04	1.37±0.03	1.45±0.03	1.4±0.05	1.53±0.04	1.39±0.04
HSI [3]	_	1.1±0.06	1.13±0.12	1.08±0.1	1.13±0.06	1.04±0.09	1.35±0.01	1.13±0.06
VSI [4]	_	3.75±0.21	3.67±0.12	3.65±0.27	3.84±0.07	4.04±0.28	3.84±0.05	3.78±0.1

* Values are means of triplicate groups±S.E.M. Within a row, means with different letters are significantly different (P<0.05), means with the same letters are not significantly different (P>0.05). Absence of letters indicates no significant difference between treatments. Crude protein, crude lipid and ash are expressed on a wet weight basis.

[1] Initial values are not included in the statistical analysis.

[2] CF: condition factor (%), [3] HSI: hepatosomatic index (%), [4] VSI: viscerasomatic index.

Table 4.5: Blood parameters in juvenile amberjack fed test diets for 56 days

Parameters	Soybean meal (SBM) replacement, % (HK-LP Prep., %)						
	SBM0	SBM15	SBM15(0.1)	SBM30	SBM30(0.1)	SBM45	SBM45(0.1)
Hematocrit (%)	48.7±1.2^{ab}	46.7±0.7^{ab}	47.3±0.3^{ab}	44±1^{a}	49.7±1.7^{b}	44.7±0.9^{ab}	46.7±1.2^{ab}
Hemoglobin (g/dl)	12.5±0.00^{b}	12±0.3^{ab}	12.3±0.2^{ab}	11.2±0.8^{ab}	12.2±0.2^{ab}	10.7±0.2^{a}	11.8±0.1^{ab}
Total protein (g/dl)	4±0.1	3.6±0.3	3.9±0.03	4.1±0.2	4.1±0.3	3.6±0.1	3.6±0.1
Total bilirubin (mg/dl)	0.5±0.1	0.4±0.1	0.4±0.1	0.7±0.1	0.4±0.1	0.4±0.1	0.6±0.2
Glucose (mg/dl)	68±3.1^{a}	93.3±3.3^{bc}	97.3±1.2^{c}	90.7±3.2^{bc}	89±3.2b^{c}	79±4.6^{ab}	85.7±4.4^{bc}
GOT (IU/l)[1]	35±12.3	44±8.7	39.7±3.8	55.3±7.8	40.3±5.5	31.3±3.7	53±4.9
GPT (IU/l)[2]	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00	<1.00
BUN (mg/dl)[3]	9±0.6	8.7±0.9	11.7±0.9	9±1.5	13.3±1.5	11±2	10.3±0.3
TG (mg/dl)[4]	158±9^{b}	108±5^{ab}	145.3±10.7^{ab}	143.7±22.9^{ab}	93±4.4^{a}	123.3±17.6^{ab}	121±6.5^{ab}
T-Cho (mg/dl)[5]	238±3.6	242.3±25.8	290±4	298.3±29.7	255.3±15.4	251.7±20.5	229.3±14.3
CORT (%)[6]	103.1±3.1	101.3±2.4	100.6±1.4	102.2±1.1	100±2	110±2.5	106.9±4.9
d-ROMs [7]	32.7±5.2^{a}	33.7±4.8^{a}	51.7±8.8^{b}	38.3±3.8^{ab}	32±2.3^{a}	42.7±3^{ab}	33.7±5.4^{ab}
BAP[8]	3369.3±84^{ab}	3267.7±169.9^{ab}	3429.3±335.6^{ab}	3570.7±16.9^{ab}	3848±92.5^{b}	2803±186.6^{a}	3202.3±108.3^{ab}

Abbreviation used: [1] GOT: glutamyl oxaloacetic transaminase, [2] GPT: glutamic-pyruvate transaminase, [3] BUN: blood urea nitrogen, [4] TG: triglyceride, [5] T-Cho: total cholesterol, [6] CORT (%): relative value of cortisol, [7] d-ROMs: reactive oxygen metabolites, [8] BAP: biological antioxidant potential.

Table 4.6: Protease activity (PA, unit mg^{-1} protein) in the digestive tract and apparent digestibility coefficients (ADC) in amberjack fed test diets*

Parameters	Soybean meal (SBM) replacement, % (HK-LP Prep., %)						
	SBM0	SBM15	SBM15(0.1)	SBM30	SBM30(0.1)	SBM45	SBM45(0.1)
PA (unit mg^{-1} protein)[1]	0.039 ± 0.001^{c}	0.04 ± 0.002^{c}	0.041 ± 0.001^{c}	0.04 ± 0.001^{c}	0.043 ± 0.003^{c}	0.029 ± 0.001^{a}	0.035 ± 0.001^{b}
ADC $_{Protein}$[2]	90.01 ± 0.17^{b}	90.77 ± 0.48^{b}	89.68 ± 0.6^{b}	90.11 ± 0.42^{b}	91.34 ± 0.94^{b}	86.93 ± 0.69^{a}	87.59 ± 0.69^{a}
ADC $_{Lipid}$[3]	85.39 ± 0.63^{b}	89.51 ± 0.23^{c}	85.78 ± 0.63^{b}	86.76 ± 0.85^{b}	88.78 ± 0.5^{c}	82.32 ± 0.49^{a}	83.26 ± 0.55^{a}

*Values are means of triplicate groups ±S.E.M. Within a row, means with different letters are significantly different ($P<0.05$), means with the same letters are not significantly different ($P>0.05$).

[1] Protease activity (PA, unit mg^{-1} protein) in the digestive tract, apparent digestibility coefficients (ADC %) [2] for crude protein, [3] for lipid, respectively.

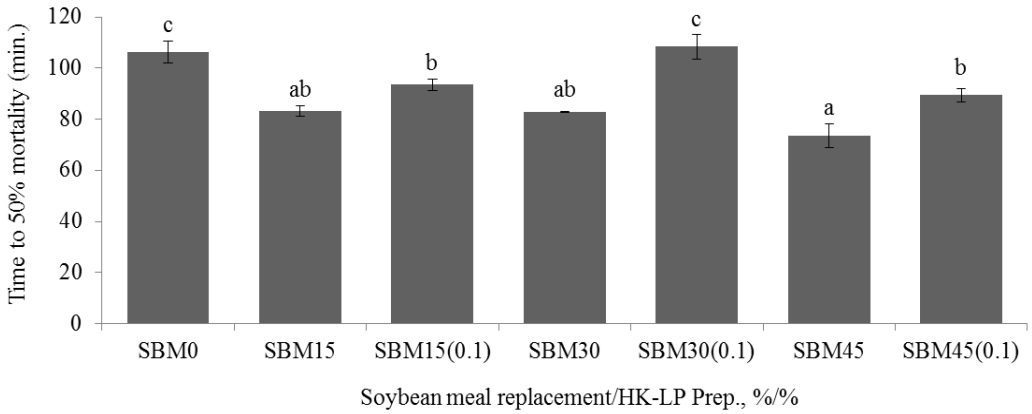


Fig. 4.1: Time to 50 % mortality (min.) after low salinity stress test when amberjack fed increasing levels of SBM with or without the inclusion of HK-LP for 56 days. Means with different alphabet are significantly different (P<0.05).

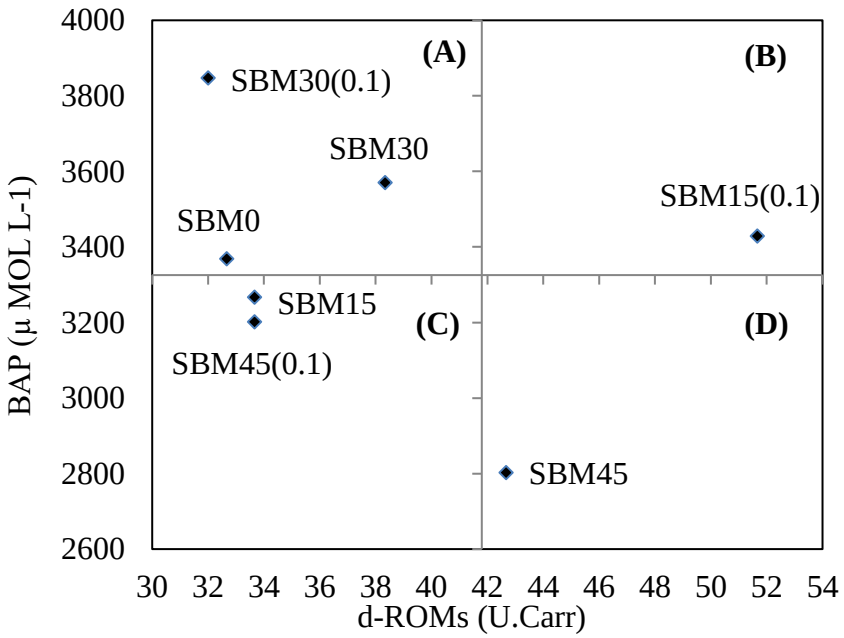


Fig. 4.2: Oxidative stress parameters in amberjack fed test diets for 56 days. Central axis based on mean values of d-ROMs and BAP from each treatment. Zone (A): high antioxidant potential and low reactive oxygen metabolites (good condition); Zone (B): high antioxidant potential and high reactive oxygen metabolites (acceptable condition); Zone (C): low antioxidant potential and low reactive oxygen metabolites (acceptable condition); Zone (D): low antioxidant potential and high reactive oxygen metabolites (stressed condition).

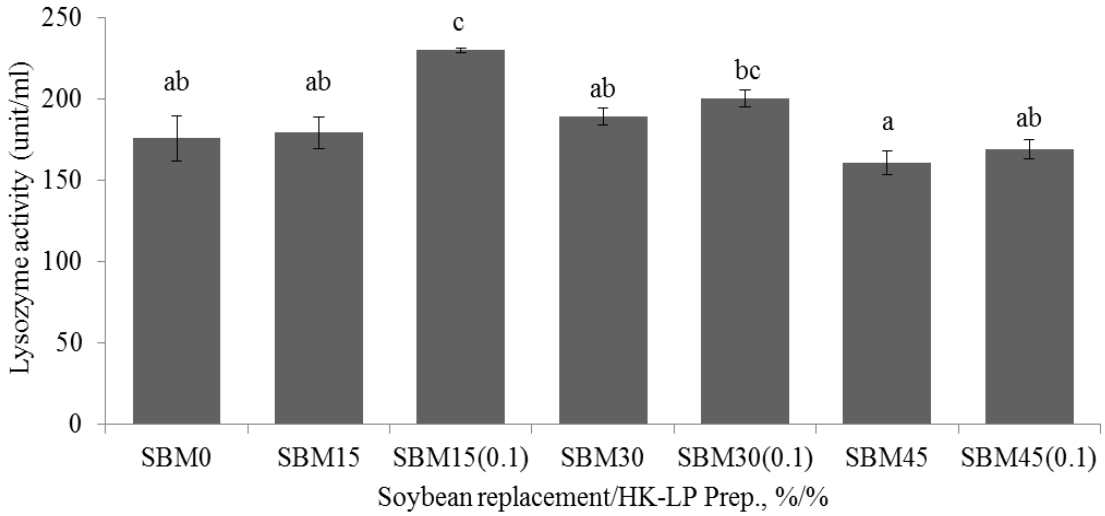


Fig. 4.3: Serum lysozyme activity (unit/ml, *n*=3) of amberjack juveniles fed diets containing increasing levels of SBM with or without the inclusion of HK-LP for 56 days. Data represent means±SE. Values with the same letter are not significantly different (*P*>0.05). Means with different alphabet are significantly different (*P*<0.05).

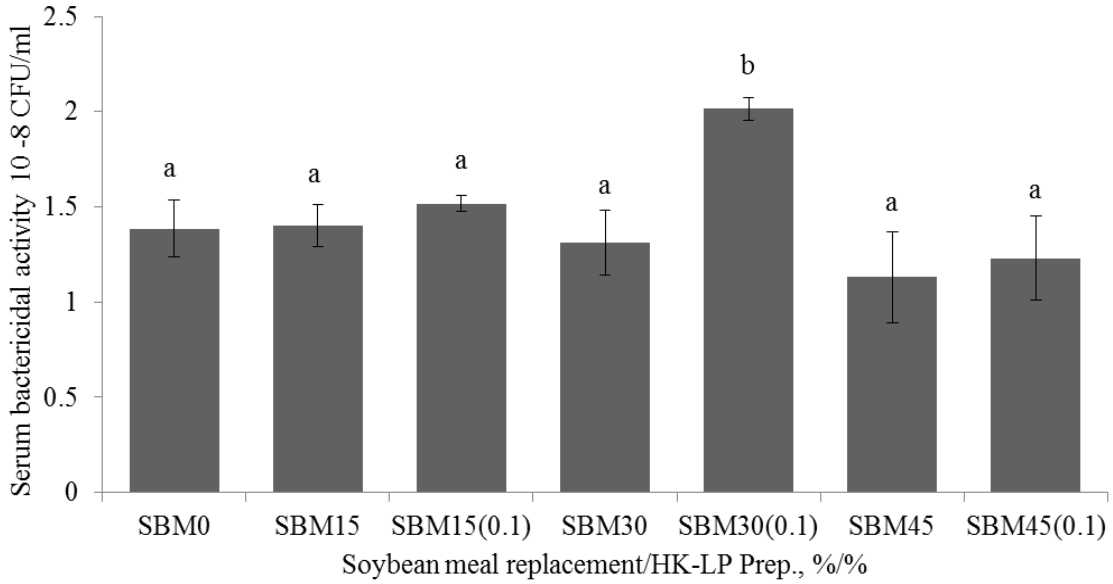


Fig. 4.4: Serum bactericidal activity (10^8 cfu/ml, *n*=3) of amberjack juveniles fed diets containing increasing levels of SBM with or without the inclusion of HK-LP for 56 days. Data represent means±SE. Values with the same letter are not significantly different (*P*>0.05). Means with different alphabet are significantly different (*P*<0.05).

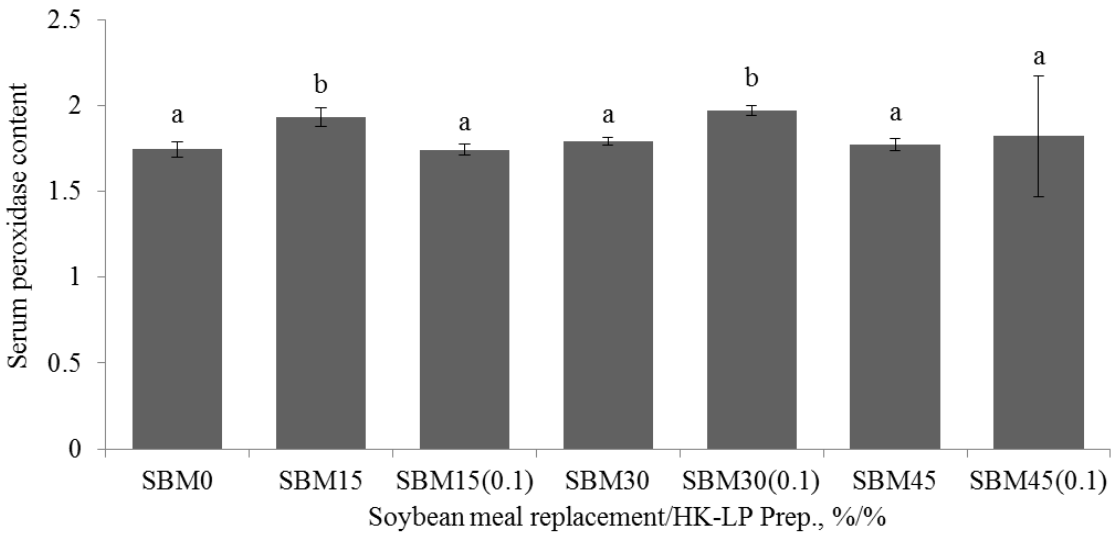


Fig. 4.5: Serum peroxidase activity (*n*=9) of amberjack juveniles fed diets containing increasing levels of SBM with or without the inclusion of HK-LP for 56 days. Data represent means±SE. Values with the same letter are not significantly different (*P*>0.05). Means with different alphabet are significantly different (*P*<0.05).

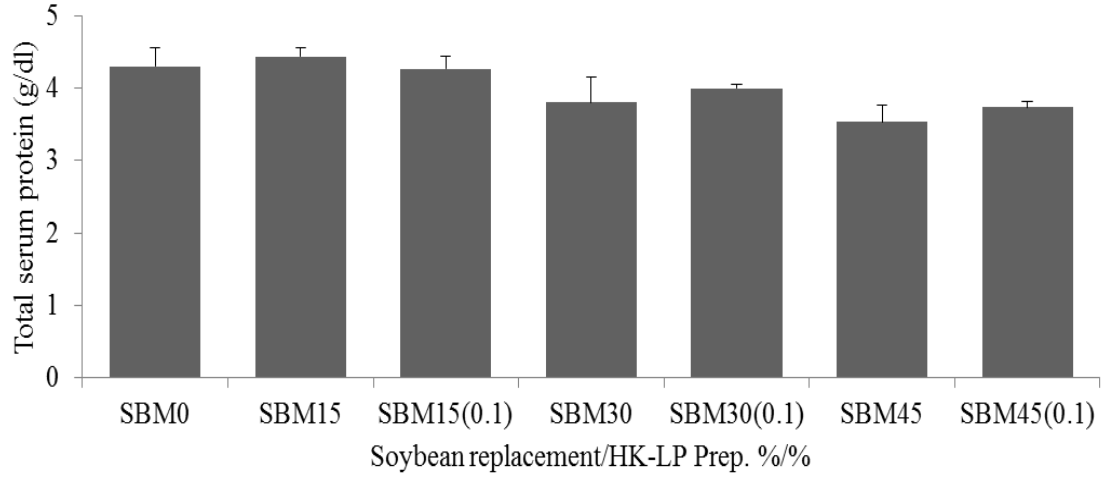


Fig. 4.6: Total serum protein (g/dl, *n*=3) of amberjack juveniles fed diets containing increasing levels of SBM with or without the inclusion of HK-LP for 56 days. Data represent means±SE. Absence of letters indicates no significant difference between treatments.

4.5 Discussion

Usually, lower feed intake could be the main reason for reduced growth performance when fish meal was replaced by soybean meal (SBM) (Chen *et al.*, 2011). However, the practical application of heat-killed *Lactobacillus plantarum* (HK-LP) to improve SBM utilization in amberjack diets represents a novel HK-LP application in the present study. Up to 30% SBM replacement level with amino acid mixture did not significantly reduce growth and feed utilization of amberjack. This was consistent with the findings of other previous studies in yellowtail (Shimeno *et al.*, 1993; Viyakarn *et al.*, 1992; Watanabe *et al.*, 1992; Tomas *et al.*, 2005). Moreover, SBM30 diet supplemented with 1g kg^{-1} HK-LP recorded the highest growth performance compared with other experimental diets. The beneficial effects of HK-LP supplementation on final body weight, weight gain and specific growth rate of fish fed SBM30(0.1) diet were also found in the study of Tung et al. (Tung *et al.*, 2010), who reported improved growth performance of kuruma shrimp *Marsupenaeus japonicus* fed with HK-LP. Growth promoting activity has been noted also in rainbow trout fed diet supplemented with heat-killed *Enterococcus faecalis* (Rodriguez-Estrada *et al.*, 2013). Significantly higher protein gains and protein retention in fish fed diet SBM30(0.1) would be a possible reason for the higher performances of fish in this group. These results suggest that the tested fish utilized experimental diets effectively by HK-LP supplementation resulting in increased feed intake in SBM30(0.1) group. Several authors have reported that the dietary administration of different bacterial forms enhanced the secretion of intestinal enzymes and characterization of these enzymes provides some information regarding the digestive capacity of fish to hydrolyze carbohydrate, protein and lipid of feed ingredients, leading to better growth performance and feed efficiency (Tovar-Ramirez *et al.*, 2002; Mohapatra *et al.*, 2012; Ray *et al.*, 2012; Lemieux *et al.*, 1999).

Khonyoung and Yamauchi (2012) reported that the intestine is the direct organ for digestion, absorption and immunity, as the gut microflora is continuously exposed to other strain of HK-LP (L-137). The latter was also thought to affect the production of extracellular enzymes by the microflora within the gastrointestinal (GI) tract of fish. The bacterial flora in the GI tract of fish shows very broad and variable enzymatic potential, and these enzymatic masses may positively affect the digestive process of fish (Ray *et al.*, 2012; Das *et al.*, 2014). All together, the relatively enhanced growth performance and feed efficiency in the amberjack fingerlings fed the HK-LP supplemented diets could be related to the improvement of intestinal microbiota. The protease activity (PA) of the digestive tract could provide further insight into the possible effects of different diets on fish performance (Kader *et al.*, 2010). In this study, PA was significantly enhanced in the SBM0, SBM15, SBM15(0.1), SBM30 and SBM30(0.1) groups compared to other experimental groups, while PA was significantly higher in fish fed SBM45(0.1) diet than that in fish fed SBM45 without HK-LP diet, indicating the positive effect of HK-LP. Similarly, it has been reported that the secretion of proteases was enhanced by supplementing immunostimulants in yellowtail, *Seriola quinqueradiata* diet (Kofuji *et al.*, 2006). Watanabe *et al.* (1992) and Tomas *et al.* (2005) reported that ADC of dry matter, protein, and lipid were high due to the process used for preparing diets in which pellets heating might have inactivated the trypsin inhibitor. Previous studies have also demonstrated that growth-promoting additives resulted in the improved digestibility of nutrients (Rodriguez-Estrada *et al.*, 2013; Ringø and Gatesoupe, 1998). Comparatively low digestibility values recorded here were likely due to the quality of raw material or to the method of feces collection (Regost *et al.*, 1999). Blood parameters are important tools for indication of physiological stress response, general health conditions and welfare of fish towards

nutritional and environmental changes (Maita *et al.*, 2002). Blood parameters obtained in the present experiment are considered to be within the normal range for juvenile amberjack, compared to those of the previous findings (Ruchimat *et al.*, 1997; Kader *et al.*, 2013). Results of the present study showed that the hematocrit values increased in the case of SBM30(0.1) group, implying improved health status. High hematocrit values indicate HK-LP efficiency, wherein iron is evenly distributed without any reduction in the synthesis of hemoglobin. Similarly, Rodriguez-Estrada *et al.* (2013) reported that hematocrit level was enhanced by the supplementation of inactivated *Enterococcus faecalis* in Rainbow Trout diets. Moreover, lower triglyceride and cholesterol contents in fish fed SBM30(0.1) diet showed that the optimum availability of HK-LP in fish diets maintains low level of plasma triglycerides and cholesterol in fish. Oxidative stress was measured using the free radical analytical system assessing the derivatives of oxidative stress by measuring reactive oxygen metabolites (d-ROMs test) and biological antioxidant potential (BAP test) in plasma samples. It is the consequence of an imbalance between oxidants and antioxidants in which oxidant activity exceeds the neutralizing capacity of antioxidants (Celi *et al.*, 2010). Recently, d-ROMs and BAP were reported to be reliable parameters for determining the oxidative stress conditions of fish (Gao *et al.*, 2012). It would be concluded that fish fed diets SBM0, SBM30, and SBM30(0.1) were in less oxidative stress conditions compared to the SBM45 group in this study. The lethal stress test is used to assess the healthy status by measuring the lethal time of 50 % mortality (LT_{50}) in fresh water of the fish (Han *et al.*, 2013). It is well known that stress affects the survival and growth of fish, since stress responses tend to increase the energy demand at the expense of anabolic processes (Kubilay and Ulukoy, 2002). The higher value of LT_{50} in the SBM0 and SBM30(0.1) groups indicated a higher tolerance of the amberjack against low-salinity

stress. Fish antioxidant status is strongly related to immune system, contributing to enhance resistance towards different stressors (Tovar-Ramírez *et al.*, 2010). In the light of the previous findings, results of the current study confirmed a higher tolerance against low-salinity stress in fish in less oxidative stress conditions. -Lysozyme is an important defense molecule of fish innate immune system (Saurabh and Sahoo, 2008). Lysozyme activity has been used to evaluate the non-specific defense ability in many fish species, such as Japanese eel, *Anguilla japonica* (Ren *et al.*, 2007), yellowtail kingfish, *Seriola lalandi* (Le *et al.*, 2014), Japanese flounder, *Paralichthys olivaceus* (Zhou *et al.*, 2006), and so on. The lowest lysozyme activity was found in SBM45 group, which together with other parameters (bactericidal activity, peroxidase activity and total serum protein), implied a less-healthy condition of the fish fed with this diet. The increasing trends in serum lysozyme activity in this study might have contributed to the enhancement in the non-specific defense mechanisms (Engstad *et al.*, 1992). Serum bactericidal activity is one of the most important factors in host resistance against pathogenic bacteria (Kawakami *et al.*, 2000). In this study, the highest serum bactericidal activity was found in SBM30(0.1) group. Similarly, the highest levels of peroxidase were observed in the case of SBM15 and SBM30(0.1) confirming other results obtained by Salinas *et al.* (2008). From the mentioned results, it could be concluded that the non-specific immune response was enhanced by HK-LP supplementation. Similarly, Irianto and Austin (2003) illustrated that dietary supplementation of inactivated bacteria also stimulated the innate immune parameters of rainbow trout, *Oncorhynchus mykiss*. The potentials for reducing stress and enhancement of immunity and stress resistance by manipulation of nutritional factors and use of feed additives (such as HK-LP) were demonstrated in this study. However, very little work in this area has been conducted in fish. Thus, the effects of dietary

functional feed additives and their interactions need to be assessed to develop economically viable feeds and feeding practices to optimize growth, improve stress resistance, immune response and disease resistance, and improve product quality of aquaculture species.

In conclusion, the present study shows that up to 30% SBM substitution level with essential amino acid supplementation did not significantly reduce growth, feed utilization and immune response of amberjack. Furthermore, the addition of HK-LP to diets appeared to improve SBM utilization by amberjack. However, further studies are needed in order to evaluate the effects of HK-LP on amberjack health with attention to the intestinal microbiota and histology.

Chapter V:

Efficiency of probiotics, prebiotics and synbiotics for cultured fish species

Experiment I: Interaction effects of dietary supplementation of heat-killed *Lactobacillus plantarum* and β-glucan on growth performance, digestibility and immune response of juvenile red sea bream, *Pagrus major*

Interaction effects of dietary supplementation of heat-killed *Lactobacillus plantarum* and β-glucan on growth performance, digestibility and immune response of juvenile red sea bream, *Pagrus major*

5.1 Abstract

Both heat-killed *Lactobacillus plantarum* (HK-LP) and β-glucan (BG) play important roles in growth performance, feed utilization and health status of fish. Therefore, a feeding trial was conducted to determine the interactive effects of dietary HK-LP and BG on growth performance, digestibility, oxidative status and immune response of red sea bream for 56 days. A significant interaction was found between HK-LP and BG on final body weight, total plasma protein, glucose, serum bactericidal activity (BA), total serum protein, serum alternative complement pathway (ACP) activity, protein and dry matter digestibility coefficients ($P < 0.05$). In addition, body weight gain, specific growth rate, feed intake, protein efficiency ratio as well as serum lysozyme activity, ACP activity and mucus secretion were significantly affected by either HK-LP or BG ($P < 0.05$). Further, feeding 0.025% HK-LP combined with 0.1% BG significantly increased serum peroxidase activity compared with the other groups ($P < 0.05$). However, protein body content, somatic parameters, total bilirubin, blood urea nitrogen, glutamyl oxaloacetic transaminase (GOT), glutamic-pyruvate transaminase (GPT), triglycerides and mucus BA were not significantly altered by supplementations ($P > 0.05$). Interestingly, fish fed with both HK-LP at (0.025 and 0.1%) in combination with BG at (0 and 0.1%) showed higher oxidative stress resistance. Under the experimental conditions, dietary HK-LP and BG had a significant interaction on enhancing the growth, digestibility and immune responses of red sea bream.

Keywords: Red sea bream, heat-killed *Lactobacillus plantarum*, β-glucan, Growth, Immunity

5.2 Introduction

The red sea bream is one of the important culture species in the world because of its high market value and demand. Especially in recent years, red sea bream aquaculture has developed rapidly and widely in China, Japan and Korea. As intensive aquaculture expanded, diseases occurred more frequently. Moreover, the application of antibiotics and chemotherapeutics to control these diseases caused many other problems such as the spread of drug resistant pathogens, suppression of aquatic animal's immune system, environmental hazards and food safety problems (Bachère, 2003). Increasing with the demand for environment friendly aquaculture, works on finding out the alternatives for the antibiotics are urgently needed (Miranda and Zemelman, 2002).

Recently, the use of dietary components that enhance the immune response of fish has grown significantly (Merrifield *et al.*, 2010). Among these additives, the most effective and widely used are probiotics and prebiotics, which have various health promoting properties used as immunostimulants (Zhang *et al.*, 2014). Bacteria with probiotic properties elicit positive effects when supplemented in either a viable (Butprom *et al.*, 2013; Giri *et al.*, 2014; Son *et al.*, 2009) or nonviable form (Biswas *et al.*, 2013a; Choi and Yoon, 2008; Dı´az-Rosales *et al.*, 2006; Panigrahi *et al.*, 2010; Rodriguez-Estrada *et al.*, 2013; Salinas *et al.*, 2008). Inactivated probiotic preparations, appear as an interesting alternative to live probiotics, which function as immunostimulants for modification of enzyme activity or microflora in gastrointestinal tracts that have beneficial effects on host health (Naidu *et al.*, 1999; Salminen *et al.*, 1999). Heat killed *Lactobacillus plantarum* (HK-LP) is a lactic acid bacterium which is resistant to high temperature and high pressure in the pelleting has been supplemented extensively in farmed animal diets especially in recent five years (Tung *et al.*, 2009, 2010; Khonyoung and Yamauchi, 2012). HK-LP is a commercial preparation (LP20) was prepared based

on the method previously described by Murosaki *et al.* (1998). Immune stimulation and adjuvant benefits have been reported when animal diets were supplemented with HK-LP (Biswas *et al.*, 2013a; Tung *et al.*, 2010; Khonyoung and Yamauchi, 2012).

Prebiotics, on the other hand, are indigestible substances that allow specific changes in the composition and/or activity of gastrointestinal microbiota, which has a positive effect on the nutrition and health status of the host (Song *et al.*, 2014). Previous research efforts have demonstrated the benefits of prebiotics on growth, physiological status and immune responses (Merrifield *et al.*, 2010; Song *et al.*, 2014). β-glucans (BG) as one of prebiotics has been used extensively in different fish species such as Atlantic Salmon (*S. salar* L.) (Paulsen *et al.*, 2001), Rainbow trout (*Oncorhynchus mykiss*) (Lauridsen and Buchmann, 2010), rohu (*Labeo rohita*) (Misra *et al.*, 2006), koi (*Cyprinus carpio* koi) (Lin *et al.*, 2011), and mirror carp (*Cyprinus carpio* L.) (Kühlwein *et al.*, 2014).

In recent years, aquaculture research has focused on heat killed probionts and prebiotics, in order to improve health status and growth performance. However, information regarding the effects of HK-LP and/or BG for red sea bream (*Pagrus major*) is scarce. Hence, the present study was conducted to determine the interaction effects of dietary supplementation of HK-LP and BG on the growth, blood chemistry, and innate immunity of red sea bream, *Pagrus major*.

5.3 Materials and methods

5.3.1 *HK-LP and BG preparations*

HK-LP Prep used in this study was kindly provided by House Wellness Foods Corp. (Itami, Japan), and it contains 20% HK-LP and 80% dextrin in dried-weight basis. The concentration of HK-LP in the dry product is 2×10^{11} cfu/g. The product was stored at

−20 ˚C until use. BG Prep used is a commercial product containing 85%BG produced by Daigon do (Tokyo, Japan).

5.3.2 *Test diets*

Tables 5.1 and 5.2 show the composition and chemical analysis of the experimental diets. All the dietary components were obtained commercially, using brown fish meal and casein as main protein sources, Pollack liver oil as main lipid source, α-Starch was supplied as the carbohydrate source, activated gluten was used as a binder to produce pellet diet, and cellulose powder was used to adjust to 100% total proportion. Six isonitrogenous (50.5% crude protein) and isolipidic (12.3% crude lipid) diets were formulated to contain three levels of HK-LP (0.025, 0.05 and 0.1% of dry diet) combined with two levels of BG (0 and 0.1% of dry diet) according to a 3×2 factorial design. A diet without HK-LP and BG supplementation was used as a negative control. HK-LP Prep and BG were thoroughly mixed with Pollack liver oil before adding other ingredients using a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan), then added to the dry ingredients and mixed for another 15 min in a food mixer. The dry ingredients were mixed with water (30-35%), and then mixed for another 15 min. The pH of the diets was adjusted to the range of 7.0–7.5 with 4 N sodium hydroxide. The mixture was then passed through a meat grinder with an appropriate diameter (1.6–2.1 mm) to prepare pellets, which were then dried in a dry-air mechanical convection oven (DK 400, Yamato Scientific, Tokyo, Japan) at 50 ˚C for about 120 min. to approximately 11% moisture, the test diets were stored at −20 ˚C until use.

Table 5.1: Ingredients of the basal diets

Ingredients	(%)
Brown Fish meal[1]	28
Casein[2]	28
α-starch[3]	12
Pollack liver oil[4]	8
Vitamin mixture[5]	3
Mineral mixture[6]	3
Stay-C[7]	0.08
Activated gluten[8]	5
α-Cellulose+HK-LP Prep[9]+BG[10]	12.92

[1] Nihon Suisan Co. Ltd (Tokyo, Japan).

[2] Wako Pure Chemicals Industries, Inc. (Osaka, Japan).

[3] Asahi Chemicals (Wakayama, Japan).

[4] Riken Vitamin, Tokyo, Japan.

[5] Vitamin mixture (g kg^{-1} diet): β-carotene, 0.10; Vitamin D_3, 0.01; Menadione $NaHSO_3.3H_2O$ (K_3), 0.05; DL-α-Tochopherol Acetate (E), 0.38; Thiamine-Nitrate (B_1), 0.06; Riboflavin (B_2), 0.19; Pyridoxine-HCl (B_6), 0.05; Cyanocobalamin (B_{12}), 0.0001; Biotin, 0.01; Inositol, 3.85; Niacine (Nicotic acid), 0.77; Ca Panthothenate, 0.27; Folic acid, 0.01; Choline choloride, 7.87; ρ-Aminobenzoic acid, 0.38; cellulose, 1.92.

[6] Mineral mixture (g kg^{-1} diet): $MgSO_4$, 5.07; Na_2HPO_4, 3.23; K_2HPO_4, 8.87; Fe citrate, 1.10; Ca lactate,12.09; $Al(OH)_3$, 0.01; $ZnSO_4$, 0.13; $CuSO_4$, 0.004; $MnSO_4$, 0.03; $Ca(IO_3)_2$, 0.01; $CoSO_4$, 0.04.

[7] L-Ascrobil-2-phosphate-Mg.

[8] Glico Nutrition Company Ltd. Osaka, Japan. Commercial name "A-glu SS".

[9] HK-LP Prep: preparation of heat-killed *Lactobacillus plantarum* made by House Wellness Foods Corp. (Itami, Japan).

[10] β-glucan (BG) preparation was commercial forms produced by Daigon do (Tokyo, Japan).

Table 5.2: Chemical analysis of the experimental diets (% dry matter basis)

Diet NO. (HK-LP/BG gkg^{-1} diet)	HK-LP (gkg^{-1} diet)	BG (gkg^{-1} diet)	Crude protein	Total lipid	Ash	Gross energy (KJ g^{-1}) *
Negative control (0/0)	0	0	51.7	9.6	11.4	19.1
Diet 2 (0.25/0)	0.25	0	51.9	9.6	11.3	19.02
Diet 3 (0.5/0)	0.5	0	51.9	10.1	11.1	19.2
Diet 4 (1/0)	1	0	51.7	9.5	11.1	19.1
Diet 5 (0.25/1)	0.25	1	51.4	10.1	11.3	19.1
Diet 6 (0.5/1)	0.5	1	51.9	10.4	11.2	19.3
Diet 7 (1/1)	1	1	51.9	9.7	11.6	19.02

* Calculated using combustion values for protein, lipid and carbohydrate of 23.6, 39.5 and 17.2 kJ g^{-1}, respectively. Carbohydrate was calculated by the difference: 100− (protein+lipid+ash+moisture).

5.3.3 *Experimental fish and feeding protocol*

Juvenile red sea bream (*Pagrus major*), with mean initial body weight of 3.42±0.02g (mean±S.E.), were purchased from Akahoshi farm, Kumamoto Prefecture, Japan and transferred to the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. The seawater was pumped from the deep basin of Kagoshima bay, Japan; gravel filtered and supplied to the system. A flow rate of 1.5 L min^{-1} was maintained throughout the experimental period. During the experimental period, the monitored water quality parameters (mean±S.D.) were: water temperature 21.4±1.6°C, pH 8±0.5, salinity 33.3±0.5 ppt and dissolved oxygen 6.1±0.5 mg L^{-1}. These ranges were considered within optimal values for juvenile red sea bream. 315 healthy fish were acclimatized to the laboratory conditions and reared in a 500-L tank in a flow-thru system. During this period, a commercial diet (50% crude protein; Higashimaru, Japan) was supplied to the fish twice a day at 7 to 8% of body weight. Fifteen fish per tank with the triplicate tanks per treatment were stocked in 100-L polycarbonate tanks (filled with 80 L of water) in a flow through sea water system where each tank was equipped with an inlet, outlet, and continuous aeration. The tanks were maintained under natural light/dark regime. All fish were fed the respective test diets to the satiation level by hand twice daily, at 9.00 and 16.00 h., for 56 days. Any uneaten feed left was removed one hour after feeding and dried using a freeze drier then subtracted from total feed intake. All fish were weighed in bulk at 2 weeks' interval to determine growth and visually check their health condition. At the end of the feeding trial, all experimental fish were fasted for 24 h. The total number, individual body weight and length of fish from each tank were measured. Survival rate and growth parameters (as following) were calculated using the following equations:

Body weight gain (%) = (final weight – initial weight) ×100/initial weight; Specific growth rate = {(Ln (final weight) –Ln (initial weight)) / duration} ×100; Survival =100× (final no. of fish/ initial no. of fish); Feed intake = (dry diet given−dry remaining diet recovered)/ no. of fish; Feed efficiency ratio =live weight gain (g) /dry feed intake (g); Protein efficiency ratio =live weight gain (g)/dry protein intake (g); Condition factor =weight of fish (g)/ (length of fish) ^{3} (cm) ^{3}×100

5.3.4 *Sample collection and biochemical analysis*

A pooled sample of 25 fish before the feeding trial were randomly collected, washed with distilled water and then stored at −20 ˚C for initial whole body analysis. Nine fish per treatment (three fish per each tank) were randomly collected, washed with distilled water and then stored at −20 ˚C for the whole body proximate analysis. Similarly, nine fish were randomly selected and their surfaces were washed with distilled water individually, and skin mucus was collected from body surface by using a small piece of sterilized cotton and immediately suspended in 1 ml of phosphate-buffered saline (PBS, pH=7.4). Sample was then put into a handmade set of two centrifugal tubes, the upper tube had a small filter, with which the mucus in the cotton will be collected in the lower tube while centrifuged. The sets of the double-tube (1.5 ml centrifugal tube) were centrifuged at 3000×g for 5 min under 4 ˚C (MX-160, Tomy Seiko Co., LTD, Tokyo, Japan), and the supernatant in the under tube was transferred into anther centrifugal tube (510-GRD, QSP, San Diego, CA, USA) and kept at -80˚C until the analysis (Han et al., 2014). Viscera and Liver was dissected out from the fish above, weight individually to get viscerasomatic index (VSI) and hepatosomatic index (HSI) using the following equations:

VSI (%) =weight of viscera/weight of fish×100; HSI (%) =weight of liver /weight of fish×100

Heparinized (1600 UI/ml, Nacalai Tesque, Kyoto, Japan) syringes were used to collect blood from the caudal vein of five fish in each replicate tank (fifteen fish per treatment) and pooled. Partial heparinized whole blood was used to analyze the hematocrit while plasma was obtained by centrifugation at 3000×g for 15min under 4°C, and then stored at -80°C until the analysis. In addition, non-heparinized disposable syringes were used to collect blood for serum analysis. Serum samples were obtained by centrifugation at 3000×g for 15 min at 4°C to collect serum. Hematocrit was determined using the micro hematocrit technique. Commercial reagent kits (Arkray, Inc. Kyoto, Japan) were used to be the color reaction reagents to measure plasma chemical parameters and total serum protein (TSP) spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan) (Tatsumi *et al.*, 2000). After the fresh samples were injected into the machine, an analysis procedure will be finished automatically. Biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were also measured spectrophotometrically from blood plasma with an automated analyzer FRAS4, Diacron International s.r.l., Grosseto, Italy by following (Morganti *et al.*, 2002; Kader *et al.*, 2012). Briefly, for BAP measurement, 50 µl of R2 reagent (ferric salt) was transferred to R1 reagent (a particular thiocyanate derivative), mixed gently and read on a photometer at a wavelength of 505 nm. Then, 10 µl plasma sample was added to the cuvette, mixed gently and read on the previous photometer. In case of d-ROMs, 20 µl plasma was added to 1 ml R2 reagent (a buffer, pH 4.8) and mixed gently. Then, 10 µl R1 reagent (an alkylamine) was added and incubated for 1 min at 37 °C. The resulting deep red coloring was photometrically detected at a wavelength of 505 nm. Free radicals were expressed in Carratelli Units (U. Carr). One Carratelli Unit is equal to a hydrogen peroxide concentration of 0.08% mg. The test diets and fish whole body were analyzed for moisture, crude protein, total lipid and ash,

in triplicate, using standard methods (AOAC, 1990), including moisture by oven-drying at 110 °C to constant weight, crude protein by the Kjeldahl method, crude lipid by the Soxhlet extraction method and ash by combustion in Muffle furnace at 550°C for 4h.

5.3.5 *Evaluation of immune parameters*

Lysozyme activities of serum and mucus were determined with turbidimetric assay (Lygren *et al.*, 1999). Ten microliters of samples were put into well of microplate, then added 190 µl of substrate (0.2 mg *Micrococcus lysodeikticus*, lyophilized cell, Sigma, USA)/ml PBS, pH 7.4, their absorbance were measured after 1 and 5 min, which were incubated with gentle shaking at constant room temperature, at 450 nm with ImmunoMini NJ-2300 (System Instruments, Tokyo, Japan). A unit of enzyme activity was defined as the amount of enzyme that causes a decrease in absorbance of 0.001/min. Serum and mucus bactericidal activities were measured according to Iida *et al.* (1989). Samples were diluted 3, 4 and 5 times with a Tris buffer (pH 7.5). The dilutions were mixed with a bacterial suspension (0.001 g/ml, *Escherichia coli*, IAM1239 cell line, Kagoshima, Japan) and incubated at 25 °C for 24 h in micro tube rotator (MTR-103, AS ONE, Osaka, Japan). 50 µl of the reaction solutions were incubated on TSA (Trypto-Soya agar, Nissui Pharmaceutical Co. Ltd., Japan) at 25 °C for 24 h (on X-plate). Colony forming unit (CFU) was counted by the plate counting method as described by Ren *et al.* (2007). The total peroxidase content in serum was measured according to Salinas *et al.* (2008), with some modifications. Briefly, 15 µl of serum were diluted with 35 µl of Hank's Buffered salt solution (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. Then, 50 µl of peroxidase substrate (3, 30, 5, 50-tetramethylbenzidine hydrochloride) (TMB; Thermo Scientific Inc., USA) was added. The serum mixture was incubated for 15 min. The colour-developing reaction in serum samples was stopped by adding 50 µl of 2 M sulphuric acid and the OD (450 nm) was

measured in a plate reader. PBS was used as a blank instead of serum. Serum alternative complement pathway (ACP) activity was assayed according to Yano (1992). Briefly, a series of volumes of the diluted serum ranging from 0.1 to 0.25 ml were dispensed into test tubes and the total volume made up to 0.25 ml with barbitone buffer in the presence of ethyleneglycol-bis (2-aminoethoxy)-tetraacetic acid (EGTA) and Mg^{2+}, then 0.1 ml of rabbit red blood cells (RaRBC) was added to each tube. After incubation for 90 min at 20 °C, 3.15 ml 0.9% NaCl was added. Following this, the sample was centrifuged at 1600 ×g for 10 min at 4 °C to eliminate unlysed RaRBC. The optical density of the supernatant was measured at 414 nm. A lysis curve was obtained by plotting the percentage of haemolysis against the volume of serum added. The volume yielding 50 % haemolysis was determined and in turn used for assaying the complement activity of the sample (ACH_{50} value = units/ml) for each experimental group.

5.3.6 *Amount of secreted mucus*

After 56 days feeding trial. Amounts of secreted mucus were quantitated by the method described in Kakuta *et al.* (1996). Secreted mucus from 3 fish in each test group with a constant area on the body surface (200 mm^2 on dorsal side) was collected by a small piece of sterilized cotton, and immediately suspended in 1 ml of phosphate buffered saline (PBS, pH = 7.4) and centrifuged (3000×g for 5 min under 4 $^{\circ}$C). Total protein in the mucus supernatant was determined by the method of Lowry *et al.* (1951). Amounts of secreted mucus were expressed as mean relative values on the basis of total protein in the mucus sample of HK-LP or BG free group (Yokoyama *et al.*, 2006).

5.3.7 *Digestibility assessment*

A digestibility trial was conducted at the end of the feeding period by pooling the remaining fish from the same treatments and distributing randomly into triplicate tanks. The fish were fed a diet containing chromium oxide (Wako Pure Chemical Industries,

Ltd) as the inert marker at a level of 0.5% (Cr_2O_3, 5 g/kg) to the previous formulation and fed to the fish under the same condition as the feeding experiment apart from the difference in stocking density. After 5-day adaptation of the diets, feces were collected at 3-h intervals between the morning and afternoon feeds using a siphon for two weeks. Feces were freeze-dried immediately and kept at − 20 ˚C until analysis. Concentration of chromium oxide in diets and feces was determined according to Furukawa and Tsukahara (1966) using the following equation:

Apparent digestibility coefficient (ADC, %) =100-[(%Cr_2O_3 in diet/% Cr_2O_3in feces) × (%nutrient in feces/% nutrient in diet)].

5.3.8 *Statistical analysis*

All data were presented as means values ± standard error of mean (S.E.M., *n*=3). Data from each group were compared to negative control group using Tukey's test (one-way ANOVA). Differences between them were considered significant when $P < 0.05$. Two-way ANOVA was employed to test the effects of dietary HK-LP levels and BG levels, as well as their interactions excluding the control diet.

5.4 Results

5.4.1 *Growth parameters, feed intake and nutrient utilizations of red sea bream*

A two-way ANOVA test showed that the FBW was significantly affected by both HK-LP and BG ($P < 0.05$; Table 5.3), and a significant interaction was found between HK-LP and BG ($P<0.05$; Table 5.3). Similar trends were found in BWG and SGR, however, no interaction was found between the parameters ($P > 0.05$; Table 5.3). Fish fed with diet 7 (HK-LP at dose of 1 gkg^{-1} diet in combination with 0.1% BG level) produced the highest FBW, BWG, SGR and were significantly higher than the group fed with the negative control ($P < 0.05$; Table 5.3). FI of red sea bream were increased by both

dietary levels of HK-LP and BG, and fish fed with diet 7 got higher FI than fish fed with negative control, diet 2, diet 3 or diet 6 ($P < 0.05$; Table 5.3). Only BG affected on FER, meanwhile all treatments showed significant higher FER compared with the negative control ($P < 0.05$). Both HK-LP and BG levels positively affected the PER, statistically significant difference was observed between the groups fed with 0.25, 0.5 or 1 g HK-LP kg^{-1} diet at 0.1% BG level and control diet ($P < 0.05$). However, no significant difference was observed between the other groups ($P > 0.05$; Table 5.3). During the whole experimental period, the survival rates of fish fed with graded levels of dietary HK-LP and BG ranged from 93.3 to 100%. No significant difference in survival rate was observed among dietary treatments ($P > 0.05$; Table 5.3).

5.4.2 *Whole body proximate analysis*

In comparison with the negative control, dietary treatments had no significant influence on the crude protein content at the end of the feeding trial. Dietary BG affected on whole body total lipid and ash contents, the level of total lipid content in fish fed with diet 7 was significantly higher than those in negative control, diet 2 and diet 3 groups. Moreover, moisture content was significantly ($P < 0.05$; Table 5.4) decreased in fish fed diet 7. In two-way ANOVA, no interactive effect was found on HSI and VSI, meanwhile all those parameters were not significantly affected by either HK-LP or BG ($P > 0.05$; Table 5.4).

5.4.3 *Digestibility Coefficients*

A two-way ANOVA test showed that the apparent digestibility coefficient of protein ($ADC_{Protein}$) was significantly affected by both HK-LP and BG ($P < 0.05$; Table 5.5), and a significant interaction was found between HK-LP and BG ($P < 0.05$). Fish fed diets supplemented with HK-LP and BG produced higher $ADC_{Protein}$ and ADC_{Lipid} than the group fed with the negative control ($P < 0.05$; Table 5.5). On the other hand, the

apparent digestibility coefficient of dry matter (ADC_{DM}) was not affected with BG supplementation, meanwhile fish fed diets 3, 5, 6 and 7 were significantly higher than those of fish fed with negative control diet ($P < 0.05$; Table 5.5).

5.4.4 *Blood parameters and responses against stress*

No significant effect of both factors was found on total bilirubin, blood urea nitrogen, GOT, GPT and triglycerides of test fish. Dietary HK-LP significantly affected hematocrit and glucose of red sea bream meanwhile dietary BG altered the glucose of the test fish. Interactions were observed on total plasma protein and glucose ($P < 0.05$; Table 5.6). Hematocrit of fish fed with diets 4, 5 and 7 was significantly higher than that of the negative control group. Fish fed with diets 3, 5 and 7 got higher total plasma protein than those of fish fed negative control and diet 2 ($P < 0.05$; Table 5.6). Interestingly, glucose content was significantly ($P < 0.05$; Table 5.6) decreased in fish fed diet 7 (HK-LP at dose of 0.1 gkg^{-1} diet in combination with 0.1% BG level). Oxidative status, the combined effects pattern of d-ROMs and BAP, of red sea bream fed with test diets were illustrated in Fig. 5.1. Fish fed with diets 2, 4, 5 and 7 were located in zone A, meanwhile Fish fed with diets 3 and 6 were in zone B. Zone D was occupied by fish fed with negative control diet.

5.4.5 *Immune parameters*

Both dietary HK-LP and BG levels were significant factors on serum lysozyme activity (LA), serum bactericidal activity (BA), serum alternative complement pathway (ACP) activity and total serum protein (TSP). Otherwise, only BG was a significant factor on mucus LA. Neither dietary HK-LP nor BG was a significant factor on mucus BA. Interactions were observed on serum BA, ACP activity and TSP ($P < 0.05$; Table 5.7). Fish fed with the negative control diet, diet 2 and diet 3 produced the lowest serum LA, and were significantly different from those fed with diet 4 (0.1% HK-LP), diet 5

(0.025% HK-LP in combination with 0.1% BG), diet 6 (0.05 % HK-LP in combination with 0.1% BG) and diet 7 (0.1% HK-LP in combination with 0.1% BG) ($P < 0.05$; Table 5.7). Fish fed with diets 4, 5, 6 and 7 exhibited higher serum BA than the other groups. Similarly, TSP and mucus LA recorded higher values in case of fish fed diets 3, 4, 5, 6 and 7 than the other groups. However, there was no significant difference in mucus BA among all treatment groups. The serum peroxidase activity of fish treated by HK-LP and/or BG is illustrated in Fig. 5.2. Fish fed diet 5 (0.025% HK-LP in combination with 0.1% BG) was significantly higher than the other groups ($P < 0.05$), however no significant difference was observed among other groups.

5.4.6 *Amounts of secreted mucus*

The amount of mucus secretion in fish fed on 0.1% HK-LP in combination with 0.1% BG (diet 7) was higher than that in the negative control group ($P < 0.05$). Although the highest concentration was found in fish fed diet 7, the value was not significantly different from the other groups ($P > 0.05$; Fig. 5.3).

Table 5.3: Growth parameters and nutrient utilizations of red sea bream fed diets containing different levels of heat killed *Lactobacillus plantarum* (HK-LP) and β-glucan (BG) *

Diet NO. (HK-LP/BG gkg^{-1} diet)	HK-LP (gkg^{-1} diet)	BG (gkg^{-1} diet)	FBW[1]	BWG[2]	SGR[3]	FI[4]	FER[5]	PER[6]	Sur[7]
Negative control (0/0)	0	0	14.5±0.18^{a}	323.5±5.6^{a}	2.58±0.02^{a}	12.6±0.3^{a}	0.88±0.01^{a}	1.71±0.05^{a}	93.3±3.9
Diet 2 (0.25/0)	0.25	0	16±0.3^{b}	372.4±7.1^{b}	2.77±0.03^{b}	14.1±0.4^{b}	0.9±0.02^{ab}	1.73±0.01^{a}	97.8±2.2
Diet 3 (0.5/0)	0.5	0	17.1±0.1^{bc}	397±2.9^{bc}	2.86±0.01^{bc}	14.1±0.2^{b}	0.97±0.02^{bc}	1.87±0.03^{ab}	97.8±2.2
Diet 4 (1/0)	1	0	17.9±0.2^{c}	422±6.8^{c}	2.95±0.02^{c}	15.1±0.2^{bc}	0.96±0.02^{bc}	1.86±0.03^{ab}	93.3±3.9
Diet 5 (0.25/1)	0.25	1	18.3±0.3^{c}	429.5±13.4^{c}	2.98±0.05^{c}	14.8±0.3^{bc}	1.01±0.01^{cd}	1.95±0.03^{b}	100±0.00
Diet 6 (0.5/1)	0.5	1	18.1±0.2^{c}	425.6±6.9^{c}	2.96±0.02^{c}	14.4±0.3^{b}	1.02±0.01^{cd}	1.97±0.04^{b}	95.6±2.2
Diet 7 (1/1)	1	1	20.2±0.4^{d}	494.3±10.8^{d}	3.18±0.03^{d}	15.9±0.2^{c}	1.06±0.02^{d}	2.04±0.06^{b}	100±0.00
Two-way ANOVA (*P*-value)									
HK-LP			0.0001	0.0001	0.0001	0.0017	NS	0.038	NS
BG			0.0001	0.0001	0.0001	0.0215	0.0001	0.0002	NS
HK-LP×BG			0.0415	NS[8]	NS	NS	NS	NS	NS

* Average initial body weight=3.42±0.02g.

[1] FBW: final weight (g), [2] BWG: percent weight gain (%), [3] SGR: specific growth rate (% day^{-1}), [4] FI: feed intake (g dry diet fish^{-1} 56 days^{-1}), [5] FER: feed efficiency ratio, [6] PER: protein efficiency ratio, [7] Sur: survival (%), [8] No significant difference.

Table 5.4: Whole body proximate analysis (%) and somatic parameters of red sea bream fed diets containing different levels of heat killed *Lactobacillus plantarum* (HK-LP) and β-glucan (BG)*

Diet NO. (HK-LP/BG gkg^{-1} diet)	HK-LP (gkg^{-1} diet)	BG (gkg^{-1} diet)	Moisture	Crude protein	Total lipid	Crude ash	CF[1]	HSI[2]	VSI[3]
Negative control (0/0)	0	0	72.04±0.4^{cd}	15.57±0.1	7.52±0.1^{a}	4.73±0.1^{b}	1.68±0.04	2.35±0.2	4.66±0.3
Diet 2 (0.25/0)	0.25	0	72.35±0.1^{d}	15.72±0.1	7.63±0.1^{a}	4.29±0.1^{a}	1.7±0.1	2.35±0.1	5.12±0.3
Diet 3 (0.5/0)	0.5	0	71.67±0.01^{bcd}	16.26±0.6	7.54±0.2^{a}	4.39±0.1^{a}	1.82±0.1	2.11±0.01	5.23±0.1
Diet 4 (1/0)	1	0	71.21±0.1^{abcd}	16.28±0.3	7.83±0.1^{ab}	4.47±0.04^{ab}	1.72±0.04	2.22±0.1	5.07±0.2
Diet 5 (0.25/1)	0.25	1	70.56±0.1^{ab}	15.65±0.4	8.34±0.2^{ab}	4.52±0.1^{ab}	1.8±0.1	2.35±0.1	5.36±0.4
Diet 6 (0.5/1)	0.5	1	71.12±0.4^{abc}	15.98±0.3	8.28±0.4^{ab}	4.59±0.1^{ab}	1.76±0.1	2.11±0.1	4.61±1.1
Diet 7 (1/1)	1	1	70.1±0.3^{a}	16.57±0.3	8.71±0.1^{b}	4.49±0.1^{b}	1.81±0.1	2.27±0.1	5.68±0.4
Two-way ANOVA (*P*-value)									
HK-LP			0.0048	NS[4]	NS	NS	NS	NS	NS
BG			0.0001	NS	0.0012	0.0102	NS	NS	NS
HK-LP×BG			0.0452	NS	NS	NS	NS	NS	NS

* Values are means of triplicate groups±S.E.M. Within a column, means with different letters are significantly different ($P<0.05$), means with the same letters are not significantly different ($P > 0.05$). Absence of letters indicates no significant difference between treatments. Crude protein, crude lipid and ash are expressed on a wet weight basis, [1] CF: condition factor (%), [2] HSI: hepatosomatic index (%), [3] VSI: viscerasomatic index, [4] No significant difference.

Table 5.5: Apparent digestibility coefficients (ADC) of red sea bream fed diets containing different levels of heat killed *Lactobacillus plantarum* (HK-LP) and β-glucan (BG)*

Diet NO. (HK-LP/BG gkg^{-1} diet)	HK-LP (gkg^{-1} diet)	BG (gkg^{-1} diet)	ADC $_{DM}$[1]	ADC $_{Protein}$	ADC $_{Lipid}$
Negative control (0/0)	0	0	51.7±1.2^{a}	81.1±0.7^{a}	56.1±1.7^{a}
Diet 2 (0.25/0)	0.25	0	55.3±0.2^{abc}	86.5±0.3^{cd}	69.4±1.1^{b}
Diet 3 (0.5/0)	0.5	0	61.5±0.8^{d}	86.1±0.2^{c}	69.3±1.5^{b}
Diet 4 (1/0)	1	0	53.9±1.1^{ab}	84.4±0.1^{b}	66.7±0.5^{b}
Diet 5 (0.25/1)	0.25	1	58.9±0.5^{cd}	87.96±0.3^{d}	70.3±0.65^{b}
Diet 6 (0.5/1)	0.5	1	58.2±0.1^{cd}	86.9±0.3^{cd}	71.6±1.5^{b}
Diet 7 (1/1)	1	1	56.7±0.5^{bc}	87.9±0.2^{d}	69.7±1^{b}
Two-way ANOVA (*P*-value)					
HK-LP			0.0001	0.0002	0.1426
BG			NS[2]	0.0001	0.0385
HK-LP×BG			0.0004	0.0003	0.6337

* Values are means of triplicate groups ±S.E.M. Within a column, means with different letters are significantly different ($P < 0.05$), means with the same letters are not significantly different ($P > 0.05$), [1]ADC $_{DM}$ Apparent digestibility coefficient of dry matter.

[2] No significant difference.

Table 5.6: Blood parameters of red sea bream fed diets containing different levels of heat killed *Lactobacillus plantarum* (HK-LP) and β-glucan (BG)*

Diet NO. (HK-LP/BG gkg^{-1} diet)	HK-LP (gkg^{-1} diet)	BG (gkg^{-1} diet)	Hematocrit (%)	Total Prot.[1] (g/dl)	Total BIL.[2] (mg/dl)	BUN [3] (mg/dl)	GOT [4] (IU/l)	GPT [5] (IU/l)	TG [6] (mg/dl)	Glucose (mg/dl)
Negative control (0/0)	0	0	34.3±1^{a}	3.27±0.2^{a}	0.9±0.1	6.7±0.3	148.3±2	28.3±1	153.3±8	63.7±6^{abc}
Diet 2 (0.25/0)	0.25	0	40.7±2^{ab}	3.17±0.1^{a}	0.97±0.2	5.7±0.7	139.3±17	31.7±3	125.7±8	76.7±4^{bc}
Diet 3 (0.5/0)	0.5	0	39±1.5^{ab}	3.87±0.1^{b}	0.83±0.2	5±0.00	139.7±14	33.7±3	147.7±9	72±4^{bc}
Diet 4 (1/0)	1	0	41.7±1^{b}	3.33±0.3^{ab}	0.6±0.1	5.3±0.3	131±5.6	32±2.7	142.7±11	77.7±8^{c}
Diet 5 (0.25/1)	0.25	1	42.3±2^{b}	3.83±0.3^{b}	0.63±0.1	5±0.00	135±8.6	33±2.1	134±7.6	50.3±6^{ab}
Diet 6 (0.5/1)	0.5	1	39.3±1^{ab}	3.37±0.3^{ab}	0.73±0.1	5±0.00	122.3±1	34.3±4	127.7±4	76.3±6^{bc}
Diet 7 (1/1)	1	1	41±0.6^{b}	3.87±0.1^{b}	0.63±0.1	5.7±0.7	119.3±7	26±2.1	134±17.1	39.3±3^{a}
Two-way ANOVA (*P*-value)										
HK-LP			0.0219	NS	NS	NS	NS	NS	NS	0.0438
BG			NS[7]	NS	NS	NS	NS	NS	NS	0.0009
HK-LP×BG			NS	0.0072	NS	NS	NS	NS	NS	0.0069

* Values are means of triplicate groups ±S.E.M. Within a column, means with different letters are significantly different ($P < 0.05$), means with the same letters are not significantly different ($P > 0.05$). Absence of letters indicates no significant difference between treatments. Abbreviation used: [1]Total Prot.: plasma total protein, [2]Total BIL: plasma total bilirubin, [3] BUN: blood urea nitrogen, [4]GOT: glutamyl oxaloacetic transaminase, [5] GPT: glutamic-pyruvate transaminase, [6] TG: triglyceride, [7] No significant difference.

Table 5.7: Non-specific immune parameters of red sea bream fed diets containing different levels of heat killed *Lactobacillus plantarum* (HK-LP) and β-glucan (BG)*

Diet NO.	HK-LP	BG	Serum				Mucus	
			LA[1]	BA[2]	TSP[3]	ACP[4] (ACH_{50}	LA[1]	BA[2]
(HK-LP/BG gkg^{-1} diet)	(gkg^{-1} diet)	(gkg^{-1} diet)	(unit/ml)	(10^5 cfu/ml)	(g/dl)	unit ml^{-1})	(unit/ml)	(10^5 cfu/ml)
Negative control (0/0)	0	0	33.5±0.5[a]	3.87±0.1[a]	4.2±0.1[a]	55.5±1.5[a]	31.63±1.4[a]	5.71±0.2
Diet 2 (0.25/0)	0.25	0	33.21±0.3[a]	5.19±0.8[ab]	4.4±0.1[ab]	58.33±1.2[a]	37.04±1.1[ab]	5.69±0.2
Diet 3 (0.5/0)	0.5	0	35.5±0.1[a]	5.34±0.3[ab]	4.6±0.03[bc]	65.33±2[b]	37.88±1.3[b]	5.34±0.3
Diet 4 (1/0)	1	0	38.83±0.6[bc]	6.17±0.3[bc]	4.8±0.1[c]	77±1.16[c]	38.71±1.1[b]	6.67±0.9
Diet 5 (0.25/1)	0.25	1	37.83±0.4[b]	8.52±0.3[d]	5.2±0.1[d]	71.33±0.8[bc]	42.33±1.6[b]	6.19±0.9
Diet 6 (0.5/1)	0.5	1	38.71±0.8[bc]	5.47±0.6[ab]	5.27±0.03[d]	83.67±0.9[d]	42.75±1.6[b]	7.47±1.2
Diet 7 (1/1)	1	1	40.92±0.5[c]	7.87±0.4[cd]	5.31±0.1[d]	89.33±1[d]	40.5±1.4[b]	6.7±0.9
Two-way ANOVA (*P*-value)								
HK-LP			0.0001	0.0131	0.0013	0.0001	NS	NS
BG			0.0001	0.0012	0.0001	0.0001	0.001	NS
HK-LP×BG			NS[5]	0.0242	0.0378	0.04	NS	NS

* Values are means of triplicate groups (serum LA and mucus LA, *n*=6; ACP activity, TSP, serum BA and mucus BA, *n*=3) ±S.E.M. Within a column, means with different letters are significantly different ($P < 0.05$), means with the same letters are not significantly different ($P > 0.05$),[1] LA: lysozyme activity, [2] BA: bactericidal activity, [3] TSP: total serum protein, [4]ACP: alternative complement pathway, [5] NS: no significant difference.

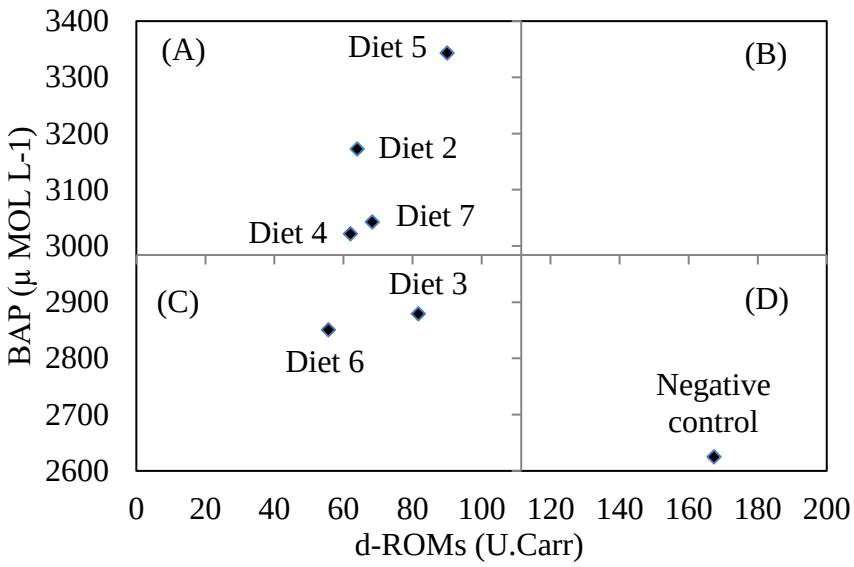


Fig. 5.1: Oxidative stress parameters in red sea bream fed test diets. Values are expressed as mean ± SE (*n*=3). Central axis based on mean values of d-ROMs and BAP from each treatment. Zone (A): high antioxidant potential and low reactive oxygen metabolites (good condition); Zone (B): high antioxidant potential and high reactive oxygen metabolites (acceptable condition); Zone (C): low antioxidant potential and low reactive oxygen metabolites (acceptable condition); Zone (D): low antioxidant potential and high reactive oxygen metabolites (stressed condition). Where negative control (0/0), Diet 2 (0.25/0), Diet 3 (0.5/0), Diet 4 (1/0), Diet 5 (0.25/1), Diet 6 (0.5/1), and Diet 7 (1/1) (HK-LP/BG gkg^{-1} diet), respectively.

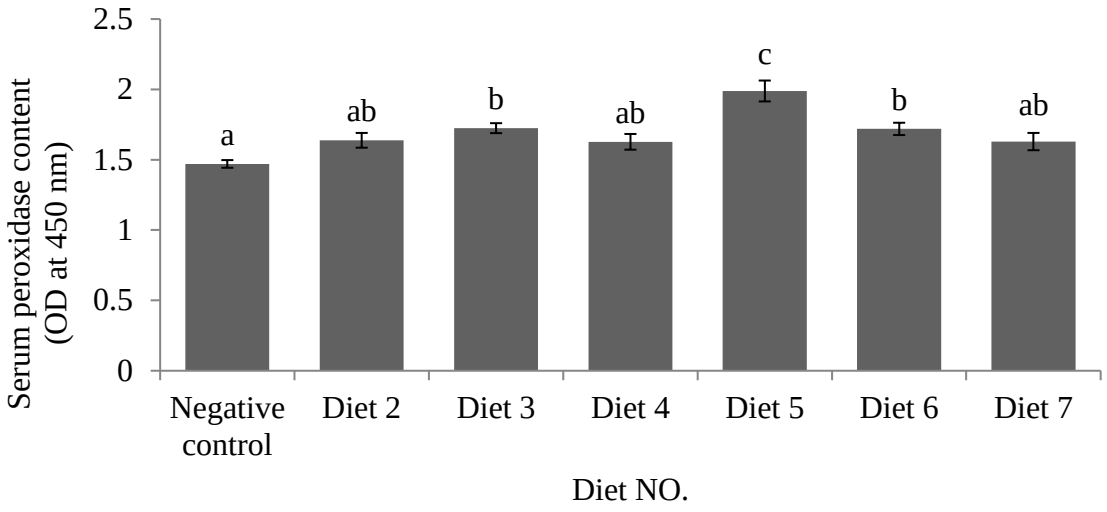


Fig. 5.2: Serum peroxidase activity levels of red sea bream juvenile fed test diets. Data represent means± pooled SEM (*n*=9 fish). Values with different letters are significantly different ($P < 0.05$). Values with the same letter are not significantly different ($P > 0.05$).

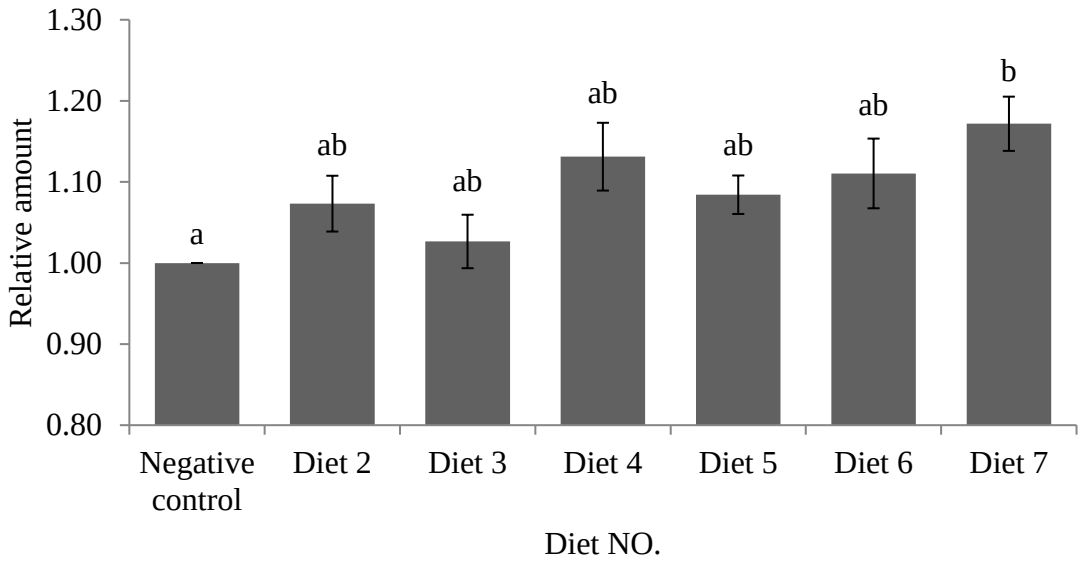


Fig. 5.3: Relative amounts of mucus secretion on body surface of red sea bream juvenile. Values are expressed as mean relative means ± pooled SEM on the basis of amount of mucus in fish group fed negative control diet (*n*=3 fish). Values with the same letter are not significantly different ($P > 0.05$). Means with different alphabet are significantly different ($P < 0.05$).

5.5 Discussion

Beneficial bacteria cells and β-glucans which have various health promoting properties used as immunostimulants, have received increasing scientific and commercial interest in aquaculture (Biswas *et al.*, 2013a; Rodriguez-Estrada *et al.*, 2013; Salinas *et al.*, 2008; Tung *et al.*, 2010; Misra *et al.*, 2006; Kühlwein *et al.*, 2014; Hirose *et al.*, 2006, 2009; Rosales *et al.*, 2006; Taoka *et al.*, 2006b). But their interactions are still unrevealed. Under these circumstances, it is important to investigate the interactions between related functional feed additives.

The results of this study indicated that both of HK-LP and BG preparations exerted a positive effect on growth parameters (FBW, BWG and SGR) of red sea bream. Rodriguez-Estrada *et al.* (2013) reported that enhanced weight gain and feed efficiency were generally observed in rainbow trout fed diets supplemented with heat-killed *Enterococcus faecalis* (at 0.25-0.5%) compared to fish fed basal diets. Tung *et al.* (2009, 2010) also reported dietary HK-LP Prep can alter the growth performance of kuruma shrimp (*Marsupenaeus japonicas*). Several authors have suggested that cultured species are primarily affected by beneficial bacteria through the enhancement of host nutrition due to the stimulation of digestive enzymes resulting in a higher growth and feed efficiency ratio (Suzer *et al.*, 2008). Furthermore, the presence of beneficial bacterial cells in the intestine improves microbial balance, which in turn improves nutrient absorption and utilization (Gatesoupe, 1999; Lara-Flores *et al.*, 2003). Growth enhancing effects of BG on red sea bream have not been reported previously to the authors' knowledge but superior weight gain and specific growth rates were reported recently in mirror carp (*Cyprinus carpio* L.) fed BG at a level of 1 to 2 g kg^{-1} (Kühlwein *et al.*, 2014), and koi carp (*Cyprinus carpio* koi) fed BG

at a level of 0.09% (Lin *et al.*, 2011). On contrary, other studies have not observed growth enhancing properties when feeding BG to Nile tilapia (*Oreochromis niloticus*) (Shelby *et al.*, 2009), channel catfish (*Ictalurus punctatus*) (Welker *et al.*, 2007), or hybrid striped bass (*Morone chrysops* X *Morone saxatilis*) (Li *et al.*, 2009). Up to date, it is not clear what causes the improvements in growth observed with dietary BG in previous studies and it is not clear why growth enhancing effects of BG occur in some aquatic species and not in others (Kühlwein *et al.*, 2014). However, Dalmo and Bøgwald (2008) suggested that the effects may depend on the concentration of the BG in the diet, its solubility, the fish species, and the experimental conditions. Synergistic action between HK-LP and BG has been revealed on FBW of red sea bream in the present study. The improvement in FBW in the present study caused by HK-LP combined with BG can be attributed to the enhancement of the production of extracellular enzymes induced by the gut microflora, as the gut microflora is continuously exposed to HK-LP and BG, which also affect the production of extracellular enzymes by the microflora within the gastrointestinal (GI) tract of fish. The bacterial flora within the GI tract of fish shows very broad and variable enzymatic potential, and these enzymatic masses may positively interfere in the digestive process of fish (Khonyoung and Yamauchi, 2012; Das *et al.*, 2014; Ray *et al.*, 2012). In that instance, the extracellular enzymes might be activated by HK-LP and BG supplementation resulting in high feed utilization followed by high observed growth performance. Nonetheless, more research in this area is needed in the future.

The apparent digestibility coefficient of protein ($ADC_{Protein}$) was significantly affected by both HK-LP and BG, and a significant interaction was found between HK-LP and BG in the current study. Previous studies have demonstrated that growth-promoting additives

result in the improved digestibility of nutrients (Rodriguez-Estrada *et al.*, 2013; Ringø and Gatesoupe, 1998; Sørensen *et al.*, 2011). The enhanced digestibility coefficients of red sea bream might be due to increasing digestive enzyme activity induced by both HK-LP and BG. Several authors have reported that the dietary administration of different bacterial forms enhanced the secretion and activity of digestive pancreatic and intestinal enzymes, leading to better feed utilization (Ray *et al.*, 2012; Mohapatra *et al.*, 2012). Gibson *et al.* (2004) suggested that a prebiotic could be selectively fermented by the intestinal microbiota and stimulate the growth and/or activity of intestinal bacteria. Therefore, the higher growth performance and feed utilization of the fish fed the HK-LP and BG diets might be partly due to it improving digestibility. CF, HSI and VSI of red sea bream were not significantly altered by various levels of HK-LP and/ or BG. However, the results reflect the improvements in body weight together with body length of fish fed HK-LP and/ or BG diets when compared with the fish fed negative control diet. It is also limited to estimate the liver functions only based on HSI of fish. The liver functions of present experiment were further estimated by other blood parameters (GOT and GPT levels).

Blood parameters are important tools for indication of physiological stress response and general health conditions of fish towards nutritional and environmental changes (Kader *et al.*, 2012). The obtained results in the present study are considered to be within the accepted values for juvenile red sea beam, compared to those of the previous findings (Kader *et al.*, 2012; Gao *et al.*, 2012). In the current study, dietary HK-LP Prep significantly affected hematocrit of red sea bream. Similarly, Rodriguez-Estrada *et al.* (2013) reported that hematocrit level was enhanced by the supplementation of inactivated *Enterococcus faecalis* in Rainbow Trout diets. Interestingly, dietary HK-LP and BG significantly affected

glucose of red sea bream. Plasma glucose is commonly considered to be one stress indicator in fish, high glucose level often indicates the higher stress status of fish (Eslamloo *et al.*, 2012). The lower glucose content in fish fed diet 7 indicated that red sea bream fed with HK-LP at dose of 1 g kg^{-1} diet in combination with 0.1% BG level induced an optimal physiological condition for the fish. Total bilirubin, blood urea nitrogen, glutamyl oxaloacetic transaminase (GOT), glutamic-pyruvate transaminase (GPT) and triglyceride were not significantly altered by various treatments. The lower glucose, TG, GPT, GOT, BUN, and BIL content in fish indicated that red sea bream fed with diet 7 induced an ideal physiological condition when compared with the negative control group. Oxidative stress was measured using the free radical analytical system assessing the derivatives of oxidative stress by measuring reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) in plasma samples (Gao *et al.*, 2012). Kader *et al.* (2012) reported that, fish with higher value of d-ROMs indicate a more oxidative condition, therefore, higher BAP value indicate they have stronger tolerance against oxidation. Using these parameters our study showed that fish fed diets 2, 4, 5 and 7 were more tolerant of oxidative stress indicating a higher health status, which indicated high BAP and low d-ROMs values. To date there remains a lack of explanation about how these additives work to effect on these parameters, so more studies are needed.

Although the immune responses among various fish species can be very different, the ranges of determined parameters in the present study were similar to those of the earlier studies on red sea bream (Ji *et al.*, 2007; Rahimnejad and Lee, 2013; Ren *et al.*, 2008). Both dietary HK-LP and BG levels enhanced immune parameters, including serum LA, serum BA, ACP activity and TSP. Moreover, significant interactions between HK-LP and

BG on serum BA, ACP activity and TSP were observed in the present study. The health promoting effects of dietary inactivated bacteria has been reported to stimulate the innate immune responses, such as phagocytic activity of rainbow trout (Rodriguez-Estrada *et al.*, 2013). Salinas *et al.* (2008) reported that Gilthead Seabream *Sparus aurata* fed heat-inactivated *Lactobacillus delbru¨eckii ssp. lactis* and *Bacillus subtilis* obtained an improved immunity by increasing natural complement, serum peroxidase and phagocytic activities. Similarly, Irianto and Austin (2003) illustrated that dietary supplementation of inactivated bacteria also stimulated the innate immune parameters of rainbow trout (*Oncorhynchus mykiss*). The immunomodulating activity of inactivated bacteria may be due to the existence of certain microbial components, such as capsular polysaccharides, peptidoglycans, and lipoteichoic acids, which are potent stimulators of the immune system (Secombes *et al.*, 2001; Nayak, 2010). The exact mechanism by which beneficial microorganism cells as well as their components enhance immunity in fish still remains unclear. It is believed that interaction between beneficial bacterial cells and intestinal epithelial cells plays an important role in host gut mucosal immunity which controls the physical as well as immunological barrier property of intestine through several mechanisms such as tightening the epithelial junction, production of antimicrobial peptides, production of mucosal immunoglobulin and modulating inflammatory reaction, which then triggers a cascade of reactions leading to an immune response stimulation (Thomas and Versalovic, 2010; Wells, 2011; Liu *et al.*, 2013; Shida and Nanno, 2008). Glucans are capable of enhancing innate immunity by activating macrophages, increasing their capacity to kill pathogens (Jorgensen and Robertsen, 1995; Cook *et al.*, 2003). They have also been shown to enhance other non-specific immune factors such as lysozyme and complement activities

(Paulsen, 2001; Misra *et al.*, 2006; Lin *et al.*, 2011; Kühlwein *et al.*, 2014; Dalmo and Bøgwald, 2008).

Bactericidal activity (BA) is one of the most important factors in host resistance against pathogenic bacteria. Our results revealed that, the highest serum BA was found in fish fed diet 7. Similarly, Taoka *et al.* (2006b) reported that dietary compound probiotics significantly increased the BA of tilapia. Total serum protein (TSP) content reflects the nutritional and metabolic status of fish, and indirectly reflects the level of non-specific immunity (Ortuño *et al.*, 2001). The increase of TSP in fish fed the diets containing HK-LP and BG supported the higher growth performance of fish in these groups. The peroxidase is an important enzyme that utilizes oxidative radicals to produce hypochlorous acid to kill pathogens (Nayak, 2010). In the current study, the highest levels of peroxidase were observed in the case of fish fed 0.025% HK-LP combined with 0.1% BG confirming other results obtained by Kumari and Sahoo (2006) and Salinas *et al.* (2008).

Skin mucus is the first line of defense against different pollutants and stressors through environmental water (Fast *et al.*, 2002) providing both a physical barrier and enzyme systems (Hjelmeland *et al.*, 1983). Previous research has indicated the importance of the use of immunostimulants for enhancing mucus production in fish. For example, the capacity of heat inactivated *Enterococcus faecalis* and Mannan Oligosaccharide (MOS) (Rodriguez-Estrada *et al.*, 2013) to modify the epidermal mucus production in fish has been demonstrated in previous studies. Results of the present study revealed that, the amount of mucus secretion in fish fed on 0.1% HK-LP combined with 0.1% BG was higher than that in the negative control group, which together with other parameters (serum LA, BA, ACP activity, TSP and mucus LA), implied an improved health condition of the fish fed with

this diet. Moreover, the increase of mucus secretion in fish fed with HK-LP and/or BG can be possibly explained on the basis of increased immune response. In contrast to our investigation, skin mucus of Tilapia *Oreochromis niloticus* was decreased after probiotic feeding (Taoka *et al.*, 2006b). These differences may be explained by different fish species, bacterial strains in the feed and the length of the studies. However, there remains a lack of explanation about how these additives work to effect on body mucus secretion, so more studies are needed.

Synergistic actions between probiotics and prebiotics have been revealed in studies on rainbow trout (Rodriguez-Estrada *et al.*, 2013), koi (*Cyprinus carpio* koi) (Lin *et al.*, 2012) and ovate pompano (*Trachinotus ovatus*) (Zhang et al., 2014). The similar significant interactions between HK-LP and BG on non-specific immunity responses were observed in the present study. In connection with the previous results, future studies are recommended to assess the gastrointestinal microbiota which could be affected directly by the supplementation of HK-LP and/or BG. Future research is also needed in order to further investigate the relation and mechanism of HK-LP and BG with the significant increase of non-specific immunity and disease resistance in juvenile red sea bream.

In conclusion, under the experimental conditions, dietary HK-LP and BG can significantly improve growth performance and feed utilization of fish. Further, the present work is the first study demonstrated that dietary HK-LP and BG had a significant interaction on enhancing blood chemistry and immunological parameters of red sea bream, *Pagrus major*. This might be helpful to those involved in aquaculture research and the fish farming industry.

Chapter VI:

Effects of individual and combined effects of probiotics on performances of cultured fish species

Experiment I: Effects of dietary supplementation of *Lactobacillus rhamnosus* or/and *Lactococcus lactis* on the growth, gut microbiota and immune responses of red sea bream, *Pagrus major*

Effects of dietary supplementation of *Lactobacillus rhamnosus* or/and *Lactococcus lactis* on the growth, gut microbiota and immune responses of red sea bream, *Pagrus major*

6.1 Abstract

Pagrus major fingerlings (3·29±0·02g) were fed with basal diet (control) supplemented with *Lactobacillus rhamnosus* (LR), *Lactococcus lactis* (LL), and *L. rhamnosus* + *L. lactis* (LR+LL) at 10^6 cell g^{-1} feed for 56 days. Feeding a mixture of LR and LL significantly increased feed utilization (FER and PER), intestine lactic acid bacteria (LAB) count, plasma total protein, alternative complement pathway (ACP), peroxidase, and mucus secretion compared with the other groups ($P < 0.05$). Serum lysozyme activity (LZY) significantly increased in LR+LL when compared with the control group. Additionally, fish fed the LR+LL diet showed a higher growth performance (Fn wt, WG, and SGR) and protein digestibility than the groups fed an individual LR or the control diet. Superoxide dismutase (SOD) significantly increased in LR and LR+LL groups when compared with the other groups. Moreover, the fish fed LR or LL had better improvement ($P < 0.05$) in growth, feed utilization, body protein and lipid contents, digestibility coefficients (dry matter, protein, and lipid), protease activity, total intestine and LAB counts, hematocrit, total plasma protein, biological antioxidant potential, ACP, serum and mucus LZY and bactericidal activities, peroxidase, SOD, and mucus secretion than the control group. Interestingly, fish fed diets with LR+LL showed significantly lower total cholesterol and triglycerides when compared with the other groups ($P < 0.05$). These data strongly suggest that a mixture of LR and LL probiotics may serve as a healthy immunostimulating feed additive in red sea bream aquaculture.

Keywords: Red sea bream; *Lactobacillus rhamnosus*; *Lactococcus lactis*; Growth; Feed utilization; Immunity

6.2 Introduction

Red sea bream is one of the main marine finfish species cultured in Japan. The demand for red sea bream has grown tremendously for the last decade since it is a high-quality sashimi grade fish with high market value (Dawood *et al.*, 2015a; Khosravi *et al.*, 2015). Recently, intensive aquaculture system has been expanded and is emerging as one of the most practical and promising tools to meet the requirements of red sea bream. However, in intensive fish farming, animals are subjected to stress conditions that weaken fish immune systems, leading to increased susceptibility to pathogens (Cerezuela *et al.*, 2012b). These diseases have resulted in production losses and remain as one of the major cause of concern in fish farms (Magnadottir, 2010). In recent years, one of the major limiting factors in intensive fish culture, an environmental friendly alternative approach is the use of probiotics. These natural ingredients enhance the immune response of fish, confer tolerance against different stressors, and minimize the risk associated with the use of chemical products such as: vaccines, antibiotics, and chemotherapeutics (Magnadottir, 2010; Merrifield *et al.*, 2010).

Probiotics are live microorganisms that could improve digestive functions and promote growth and welfare of fish when consumed in adequate amounts (Ringø and Gatesoupe, 1998). Several reports suggested that probiotics supplementation can improve the growth, feed utilization, immune response, and stress resistance of fish (Iwashita *et al.*, 2015; Kesarcodi-Watson *et al.*, 2008). Lactic acid bacteria (LAB) as probiotic supplements have

been widely applied to enhance the immunity and disease resistance of fish, most notably *Lactobacillus spp., Bacillus spp.* and other Gram-negative bacteria (Brunt and Austin, 2005; Pirarat *et al.*, 2006; Vendrell *et al.*, 2008). LAB plays a beneficial role in the host gut environment by producing antibacterial substances such as lactic acid, acetic acid, hydrogen peroxide, bacteriocin and to inhibit the increment of harmful intestinal bacteria that suppress growth of competing bacteria (Calo-Mata *et al.*, 2008; Gatesoupe, 1999; Perdigon *et al.*, 1990). At present, LAB as dietary supplements has been widely applied to enhance the immunity and disease resistance of fish (Dawood *et al.*, 2015b,c; Dimitroglou *et al.*, 2011; Geng *et al.*, 2012).

Dietary administration of *Lactobacillus spp.* enhanced the growth and immunity of *Epinephelus coioides* (Son *et al.*, 2009), *Epinephelus bruneus* (Harikrishnan *et al.*, 2010), *Rachycentron canadum* (Geng *et al.*, 2012), *Oreochromis niloticus* (Aly *et al.*, 2008), *Labeo rohita* (Giri *et al.*, 2014) and more recently *Pagrus major* (Dawood *et al.*, 2015b). *Lactococcus lactis* has demonstrated growth or health benefit to grouper *E. coioides* (Sun *et al.*, 2012) and olive flounder *Paralichthys olivaceus* (Beck *et al.*, 2015; Kim *et al.*, 2013), however, there remains a distinct lack of literature regarding *L. lactis* for red sea bream. Although use of probiotics has greater potential for higher fish production, in most of the cases, probiotic microorganisms were used as a single species at one or more doses (Dawood *et al.*, 2015b,c; Salinas *et al.*, 2008). Limited literatures are available on the use of two or more microorganisms as a probiotic mixture and their effects on growth performances and immunity of fish (Beck *et al.*, 2015; Cerezuela *et al.*, 2012b; Geng *et al.*, 2012; Mohapatra *et al.*, 2011). Some authors have reported that multi-species probiotic supplementation could be more effective than mono-species or mono-strain ones (Nayak,

2010; Timmerman *et al.*, 2004), mainly due to the higher probability of a microorganism *consortium* to survive in a changing environment like the gastrointestinal tract (GIT) and to dominate the associate microbiota (Bezkorovainy, 2001; Verschuere *et al.*, 2000), a potential stimulation of the immune system, given the diverse immune stimulation properties of strain specific properties (Nayak, 2010; Panigrahi *et al.*, 2010; Salinas *et al.*, 2005) and a greater variety of antimicrobial properties associated with mixed formulations (*i.e.* production of organic acids, bacteriocins, hydrogen peroxide, biosurfactants, etc) preventing pathogen colonization and prosper in GIT (Timmerman *et al.*, 2004).

The literature on the use of two probiotic species at the same time in the diet and their effect on growth, nutrient utilization, gut microbial population and immune response of fish are very limited. Therefore, the present investigation was carried out to evaluate the effects of dietary supplementation of *L. rhamnosus* or/and *L. lactis* on the growth, immunity and gut microbiota of red sea bream, *P. major*.

6.3 Materials and methods

6.3.1 *Probiotic bacteria*

Lactobacillus rhamnosus (LR) used in this study was kindly provided by Morinaga Milk Industry CO., LTD., Kanagawa, Japan, and the concentration of LR in the dry product is 1×10^{9} cells g^{-1}. *Lactococcus lactis* (D1813) (LL) was made by Kyushu Medical Company, Fukuoka, Japan, and the concentration of LL in the dry product is 1×10^{8} cells g^{-1}. α-Cellulose powder used to adjust to the required concentrations of LR and LL. The bacteria were stored at −20 ˚C until use.

6.3.2 *Experimental diets and design*

All the dietary components were obtained commercially, using brown fish meal and casein as main protein sources, soybean lecithin and pollack liver oil as main lipid sources, dextrin and α-Starch were supplied as the carbohydrate sources, activated gluten was used as a binder to produce pellet diet, and cellulose powder was used to adjust to 100% total proportion. Four experimental diets were formulated to be isonitrogenous (51.8% crude protein) and isolipidic (11.4 % crude lipid), containing 0 (control), 1×10^6 cells g^{-1} *L. rhamnosus* (LR), 1×10^6 cells g^{-1} *L. lactis* (LL), or both 0.5×10^6 cells g^{-1} *L. rhamnosus* and 0.5×10^6 cells g^{-1} *L. lactis* (LR+LL) (Table 6.1). A lyophilized probiotic in powder form of LR or/and LL were added to the basal diet at the expense of α-Cellulose to obtain the levels required. Powdered dietary ingredients were thoroughly mixed for 15 min in a food mixer, then blended oil and water were added to form a soft dough. The dough was then pelleted (without steam injection) using a Pillet Mill with a (1.6–2.1 mm) diameter die. The experiment feed was dried at room temperature and stored in sealed plastic bags at −20 °C until use. New batches of feed were produced every two weeks to keep up LR and LL viability. The viability of the incorporated bacterial cells into feed was assessed by spreading onto triplicate plates of DeMan, Rogosa and Sharpe agar (MRS, MERCK, Darmastadt, Germany). Diet samples were first powdered well and serially diluted with sterile saline [phosphate-buffered saline (PBS, pH=7.4)]. The agar plate inoculated with each dilution was incubated for 3 to 5 days at 25°C. Colony forming unit (CFU g^{-1}) were determined for viable bacterial populations.

Table 6.1: Formulation and proximate composition of the experimental diets for red sea bream (%)*.

Ingredients[1]	%	Proximate composition	% dry matter basis
Brown fish meal	28	Crude protein	51.83
Casein	28	Total lipid	11.42
Dextrin	7	Ash	10.43
α-starch	6	Gross energy (KJ g^{-1})[4]	19.76
Soybean lecithin	5		
Pollack liver oil	6		
Vitamin mixture	3		
Mineral mixture	3		
Stay-C	0.08		
Activated gluten	5		
α-Cellulose+LR[2] or/and LL[3]	8.92		

*According to Dawood *et al.* (2015b).

[1]According to Dawood *et al.* (2015b).

[2]Preparation of *Lactobacillus rhamnosus* (LR) was obtained from Morinaga Milk Industry (Kanagawa, Japan).

[3]Preparation of *Lactococcus lactis* (D1813) (LL) made by Kyushu Medical Company (Fukuoka, Japan).

[4]Calculated using combustion values for protein, lipid and carbohydrate of 23.6, 39.5 and 17.2 kJ g^{-1}, respectively. Carbohydrate was calculated by the difference: 100−(protein+lipid+ash+moisture).

6.3.3 *Fish and feeding trial*

A total of 240 red sea bream (3·29±0·02g) were obtained from Akahoshi farm, Kumamoto Prefecture, Japan and transferred to the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. The fish were acclimatized for 1 week in the laboratory condition and reared in a 500-L tank. During this period, a commercial diet (50% crude protein; Higashimaru, Japan) was supplied to the fish. Thereafter, fish were randomly allocated to twelve 100-litre tanks (twenty fish per tank and triplicate tanks per treatment) in a flow through sea water system where each tank was equipped with an inlet, outlet, and continuous aeration. The tanks were maintained under natural light/dark regime. During the experimental period, the monitored water quality parameters (mean±S.D.) were: water temperature 21.4±1.6°C, pH 8±0.5, salinity 33.3±0.5 ppt and dissolved oxygen 6.1±0.5 mg L^{-1}. Fish were hand fed to apparent satiation twice a day (09.00 and 16.00 hours) for 56 days. Any uneaten feed left was removed after feeding and dried using a freeze drier then subtracted from total feed intake.

At the end of the feeding trial, all experimental fish were fasted for 24 h. The total number, individual body weight and length of fish from each tank were measured. Survival rate and growth parameters were calculated using the following equations:

Body weight gain (%) = (final weight – initial weight) ×100/initial weight; Specific growth rate = {(Ln (final weight) –Ln (initial weight)) / duration (56 days)} ×100; Survival (%) =100× (final no. of fish/ initial no. of fish); Feed intake = (dry diet given−dry remaining diet recovered)/ no. of fish

Feed efficiency ratio (FER) =live weight gain (g) /dry feed intake (g); Protein efficiency ratio =live weight gain (g)/dry protein intake (g); Protein gain = {(final weight (g) × final

whole body protein content (%)/100) − (initial weight (g) × initial whole body protein content (%)/100)}/ (weight gain (g)) ×1000; Condition factor =weight of fish (g)/ (length of fish)^3 (cm)^3×100

6.3.4 *Sample collection and biochemical analysis*

For the whole body proximate analysis, nine fish per treatment (three fish per each tank) were randomly collected, washed with distilled water and then stored at −20 ˚C. Similarly, nine fish were randomly selected and their surfaces were washed with distilled water individually, and skin mucus was collected from body surface by using a small piece of sterilized cotton and immediately suspended in 1 ml of phosphate-buffered saline (PBS, pH=7.4). Sample was then put into a handmade set of two centrifugal tubes, the upper tube had a small filter, with which the mucus in the cotton will be collected in the lower tube while centrifuged. The sets of the double-tube (1.5 ml centrifugal tube) were centrifuged at 3000×g for 5 min under 4 ˚C (MX-160, Tomy Seiko Co., LTD, Tokyo, Japan), and the supernatant in the under tube was transferred into another centrifugal tube (510-GRD, QSP, San Diego, CA, USA) and kept at -80˚C until the analysis (Dawood *et al.*, 2015b).

Viscera and liver were dissected out from the fish above, weight individually to get viscerasomatic index (VSI) and hepatosomatic index (HSI) using the following equations: VSI (%) =weight of viscera/weight of fish×100; HSI (%) =weight of liver /weight of fish×100

Digestive tracts were separated, cut into small pieces, washed with pure water, pooled together and stored at −80 ˚C for the analysis of protease activity. Heparinized (1600 UI/ml, Nacalai Tesque, Kyoto, Japan) syringes were used to collect blood from the caudal vein of five fish in each replicate tank (fifteen fish per treatment) and pooled. Partial heparinized

whole blood was used to analyze the hematocrit while plasma was obtained by centrifugation at 3000×g for 15min under 4°C, and then stored at -80°C until the analysis. In addition, non-heparinized disposable syringes were used to collect blood for serum analysis. Serum samples were obtained by centrifugation at 3000×g for 15 min at 4°C to collect serum. Hematocrit was determined using the micro hematocrit technique. Commercial reagent kits (Arkray, Inc. Kyoto, Japan) were used to be the color reaction reagents to measure total plasma protein, total cholesterol, and triglyceride spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan) (Dawood *et al.*, 2015b; Tatsumi *et al.*, 2000). Biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were also measured spectrophotometrically from blood plasma with an automated analyzer FRAS4, Diacron International s.r.l., Grosseto, Italy by following (Dawood *et al.*, 2015a; Morganti *et al.*, 2002). Protease activity was measured using commercial kits (QuantiCleave™ Protease Assay Kit, product number 23263, Thermo Fisher Scientific Inc., USA) according to the procedure outlined by the manufacturer. The test diets and fish whole body were analyzed for moisture, crude protein, total lipid and ash, in triplicate, using standard methods (AOAC, 1998) including moisture by oven-drying at 110 °C to constant weight, crude protein by the Kjeldahl method, crude lipid by the Soxhlet extraction method and ash by combustion in Muffle furnace at 550°C for 4h.

6.3.5 *Intestinal bacterial counts*

Another nine fish were sampled in each treatment after cease of feeding for 24 h to enumerate the total intestinal microbiota and lactic acid bacteria (LAB). The fish were transferred from the rearing tank onto a clean bench. The surface of the fish body was

sterilized with 70% ethanol before opening the ventral surface with sterile scissor. Intestinal tract of fish was removed, weighed and washed thoroughly three times with PBS. Samples were serially diluted with PBS and 100 µl of the solution was spread onto triplicate TSA (Trypto-Soya agar, Nissui Pharmaceutical Co. Ltd., Japan) media to determine total bacterial populations. DeMan, Rogosa and Sharpe (MRS, Merck) were also used to detect viable LAB species.The agar plate inoculated with each dilution was incubated for 3 to 5 days at 25°C. Colony forming units (CFU ml^{-1}) were determined for viable bacterial populations (Nikoskelainen *et al.*, 2003).

6.3.6 *Evaluation of immune responses*

Serum alternative complement pathway (ACP) activity was assayed according to Yano (1992). Briefly, a series of volumes of the diluted serum ranging from 0.1 to 0.25 ml were dispensed into test tubes and the total volume made up to 0.25 ml with barbitone buffer in the presence of ethyleneglycol-bis (2-aminoethoxy)-tetraacetic acid (EGTA) and Mg^{2+}, then 0.1 ml of rabbit red blood cells (RaRBC) was added to each tube. After incubation for 90 min at 20 °C, 3.15 ml 0.9% NaCl was added. Following this, the sample was centrifuged at 1600×g for 10 min at 4 °C to eliminate unlysed RaRBC. The optical density of the supernatant was measured at 414 nm. A lysis curve was obtained by plotting the percentage of haemolysis against the volume of serum added. The volume yielding 50 % haemolysis was determined and in turn used for assaying the complement activity of the sample (ACH_{50} value = unit ml^{-1}) for each experimental group. Serum and mucus bactericidal activities were measured according to Iida *et al.* (1989). Samples were diluted 3, 4 and 5 times with a Tris buffer (pH 7.5). The dilutions were mixed with a bacterial suspension (0.001 g ml^{-1}, *Escherichia coli*, IAM1239 cell line, Kagoshima, Japan) and incubated at

25 °C for 24 h in micro tube rotator (MTR-103, AS ONE, Osaka, Japan). 50 µl of the reaction solutions were incubated on TSA at 25 °C for 24 h (on X-plate). Colony forming unit (CFU) was counted by the plate counting method. Lysozyme activities of serum and mucus were determined with turbidimetric assay (Lygren *et al.*, 1999). Ten microliters of samples were put into well of microplate, then added 190 µl of substrate (0.2 mg *Micrococcus lysodeikticus*, lyophilized cell, Sigma, USA)/ml PBS, pH 7.4, their absorbance were measured after 1 and 5 min, which were incubated with gentle shaking at constant room temperature, at 450 nm with ImmunoMini NJ-2300 (System Instruments, Tokyo, Japan). A unit of enzyme activity was defined as the amount of enzyme that causes a decrease in absorbance of 0.001 min^{-1}. The total peroxidase content in serum was measured according to Salinas *et al.* (2008). Briefly, 15 µl of serum were diluted with 35 µl of Hank's Buffered salt solution (HBSS) without Ca^{+2} or Mg^{+2} in flat-bottomed 96-well plates. Then, 50 µl of peroxidase substrate (3, 30, 5, 50-tetramethylbenzidine hydrochloride) (TMB; Thermo Scientific Inc., USA) was added. The serum mixture was incubated for 15 min. The colour-developing reaction in serum samples was stopped by adding 50 µl of 2 M sulphuric acid and the OD (450 nm) was measured in a plate reader. PBS was used as a blank instead of serum. Serum superoxide dismutase (SOD) activity was measured by the percentage reaction inhibition rate of enzyme with WST (Water Soluble Tetrazolium dye) substrate and xanthine oxidase using a SOD Assay Kit (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's instructions. The percent inhibition was normalized by mg protein and presented as SOD activity units. One unit of SOD is defined as the amount of the enzyme in 20 µl of sample solution that inhibits the reduction reaction of WST-1 with superoide anion by 50%.

6.3.7 *Amount of secreted mucus*

Amounts of secreted mucus were quantitated based on the method described by Kakuta *et al.* (1996) and Dawood *et al.* (2015c). Bovine serum albumin (Sigma Aldrich) was used as standard and the soluble protein concentration of mucus samples was determined following the method of Lowry *et al.* (1951). The absorbance at 750 nm was read by spectrophotometer.

6.3.8 *Digestibility assessment*

A digestibility trial was conducted at the end of the feeding period according to Dawood *et al.* (2015a). The fish were fed a diet containing chromium oxide (Wako Pure Chemical Industries, Ltd) as the inert marker at a level of 0.5% (Cr_2O_3, 5 g kg^{-1}) to the previous formulation and fed to the fish under the same condition as the feeding experiment apart from the difference in stocking density. After 5-day adaptation of the diets, feces were collected at 3-h intervals between the morning and afternoon feeds using a siphon for two weeks. Feces collection continued for two weeks until a sufficient amount of feces had been collected for analysis. Feces were freeze-dried immediately and kept at − 20 ˚C until analysis. Concentration of chromium oxide in diets and feces was determined according to Furukawa and Tsukahara (1966) using the following equation:

Apparent digestibility coefficient (ADC, %) =100-[(%Cr_2O_3 in diet/% Cr_2O_3in feces) × (%nutrient in feces/% nutrient in diet)].

6.3.9 *Statistical analysis*

All data were presented as means values ± standard error of mean (S.E.M., n=3). Data were subjected to statistical verification using package super ANOVA 1.11, Abacus Concepts, Berkeley, California, USA. Probabilities of $P < 0.05$ were considered significant.

Significance differences between means were evaluated using the Duncan's multiple range test.

6.4 Results

6.4.1 *Growth parameters, survival, and nutrient utilization*

Compared to the control group, fish fed *L. rhamnosus* (LR) or/and *L. lactis* (LL) showed a significant final body weight (Fn wt), weight gain (WG), and specific growth rate (SGR) ($P < 0.05$; Table 6.2). Additionally, fish fed the mixture of LR and LL showed higher Fn wt, WG, and SGR values significantly than the groups fed an individual LR or the control diet ($P < 0.05$). Although *L. lactis* promoted a greater Fn wt, WG, and SGR than *L. rhamnosus*, the differences did not produce a statistically meaningful increase in Fn wt, WG, and SGR compared to that found in the single *L. rhamnosus*-fed group. Feed intake (FI) of red sea bream were increased by both dietaries of LR and LL, and fish fed with only LL got higher FI than fish fed with control diet ($P < 0.05$; Table 6.2). However, no significant difference was observed between LR or LL groups ($P > 0.05$). The highest values of feed efficiency ratio (FER) and protein efficiency ratio (PER) were obtained in fish fed the diet containing both *L. rhamnosus* and *L. lactis* (LR+LL) ($P < 0.05$). Moreover, fish fed LR or LL probiotics showed higher significant FER and PER values than the control group. Similarly, protein gain was significantly altered by LR or/and LL supplementation when compared with the control group ($P < 0.05$; Table 6.2). All treatments showed high survival rates between 91.7% and 98.3% and there were no significant differences among all groups ($P > 0.05$).

6.4.2 *Whole body proximate analysis and biometric indices*

The whole body proximate compositions of red sea bream are shown in Table 6.3. In comparison with the control group, dietary treatments had no significant influence on the moisture and ash contents at the end of the feeding trial. Dietary probiotic supplements (LR or/and LL) affected on whole body crude protein content, the level of crude protein content in fish fed LR, LL, and LR+LL diets were significantly higher than those in the control group ($P < 0.05$). Total lipid content increased significantly in fish fed LL and LR+LL diets when compared with the control group ($P < 0.05$), however, no significant difference was observed between LR and LL or LR+LL groups ($P > 0.05$). No differences were also detected in CF, HSI, and VSI of fish among treatments (Table 6.3).

6.4.3 *Digestibility coefficients and protease activity*

The apparent digestibility coefficients of dry matter (ADCD), protein (ADCP), lipid (ADCL), and protease activity were found to be significantly different ($P < 0.05$) in LR, LL, and LR+LL groups than the control group (Table 6.4). Additionally, ADCP in fish fed LR+LL diet was significantly higher than the other groups, except for the fish fed LL diet, meanwhile no significant difference was observed between fish fed LR and LL diets ($P > 0.05$).

6.4.4 *Gut microbiota levels*

The levels of total intestine bacteria in the fish fed LR, LL, and LR+LL diets were significantly higher than the control group ($P < 0.05$), however, no significant difference was observed among the fish fed LR or/and LL probiotic supplementations ($P > 0.05$; Fig. 6.1). No cultivable lactic acid bacteria (LAB) was detected in the control group; all other supplemented groups had significantly ($P < 0.05$) higher LAB viable count when compared

with the control group (Fig. 6.1). Furthermore, fish fed LR+LL-supplemented diet was significantly higher than that of other groups ($P < 0.05$).

6.4.5 *Biochemical blood parameters and oxidative status*

Compared to the control group, fish fed LR or/and LL probiotic supplementations showed a significant improved hematocrit and plasma total protein levels ($P > 0.05$; Table 6.5). Moreover, the supplementation of both *L. rhamnosus* and *L. lactis* (LR+LL) recorded a significantly highest total plasma protein ($P < 0.05$). Total cholesterol (T-Cho) and triglycerides (TG) of fish fed LR or/and LL free diet was significantly lower than the control group ($P < 0.05$), with the lowest being in LR+LL group when compared with the other groups. However, TG showed no significant difference between fish fed LR and LL or LR+LL diets ($P > 0.05$; Table 6.5). No significant effect of LR or/and LL probiotic supplementations were found on reactive oxygen metabolites (d-ROMs) of test fish. Biological antioxidant potential (BAP) was affected by the supplementation of LR or/and LL significantly higher than those of the control group ($P < 0.05$). Oxidative status, the combined effects pattern of d-ROMs and BAP, of red sea bream fed with test diets were illustrated in Fig. 6.2. Fish fed with diets LR and LR+LL were located in zone A, meanwhile fish fed with LL diet was in zone B. Zone D was occupied by fish fed with control diet.

6.4.6 *Immune responses and mucus secretion*

When compared with the control group, fish fed LR or/and LL probiotics showed a significant elevation of alternative complement pathway (ACP) (Fig. 6.3), serum bactericidal activity (BA) (Fig. 6.4), mucus BA (Fig. 6.4), mucus lysozyme activity (LA) (Fig. 6.5), peroxidase activity (Fig. 6.6), superoxide dismutase (SOD) (Fig. 6.7).

Additionally, ACP and peroxidase of fish fed both *L. rhamnosus* and *L. lactis* (LR+LL) were significantly higher than that of fish fed other diets. Serum LA was significantly higher in fish fed both LR and LL (LR+LL) when compared with the control group ($P > 0.05$; Fig. 6.5). However, the differences in serum LA between the LR, LL and LR+LL groups were statistically insignificant. SOD in fish fed LR and LR+LL diets showed significantly higher values when compared with the other groups.

Significantly higher amounts of secreted mucus were observed ($P > 0.05$; Fig. 6.8) in all LR or/and LL supplemented groups than that in the control group. Moreover, fish fed LR+LL diet exhibited higher amounts of mucus secretion when compared with the other groups.

Table 6.2: Growth performance of red sea bream fed diets supplemented with *Lactobacillus rhamnosus* (LR) or/and *Lactococcus lactis* (LL) for 56 days

	Experimental diets			
	Control	LR	LL	LR+LL
In wt[1]	3.31±0.01	3.31±0.02	3.28±0.03	3.31±0.02
Fn wt[2]	15.19±0.77^{a}	18.29±0.83^{b}	19.2±0.35^{bc}	21.14±0.17^{c}
WG[3]	360.12±24.99^{a}	454.01±27.5^{b}	486.57±6.31^{bc}	538.73±7.54^{c}
SGR[4]	2.72±0.1^{a}	3.05±0.09^{b}	3.16±0.02^{bc}	3.31±0.02^{c}
FI[5]	11.45±0.34^{a}	12.64±0.5^{ab}	13.34±0.31^{b}	13.61±0.42^{b}
FER[6]	1.04±0.05^{a}	1.19±0.02^{b}	1.19±0.01^{b}	1.31±0.03^{c}
PER[7]	2.03±0.1^{a}	2.29±0.01^{b}	2.25±0.04^{b}	2.53±0.08^{c}
PG[8]	154.29±8^{a}	175.06±2.19^{b}	183.64±3.81^{b}	177.19±5.08^{b}
Sur[9]	91.67±1.67	95±2.89	98.33±1.67	96.67±3.33

[1] In wt: initial weight (g), [2] Fn wt: final weight (g), [3] WG: percent weight gain (%), [4] SGR: specific growth rate (% day^{-1}), [5] FI: feed intake (g dry diet fish^{-1} 56 days^{-1}), [6] FER: feed efficiency ratio, [7] PER: protein efficiency ratio, [8] PG: protein gain, [9] Sur: survival (%).

Table 6.3: Whole body proximate analysis (%) and somatic parameters in juvenile red sea bream fed test diets for 56 days

	Experimental diets			
	Control	LR	LL	LR+LL
Moisture	71.36±1.07	70.04±0.3	68.29±0.65	69.25±0.76
Crude protein	14.59±0.65^{a}	16.43±0.23^{b}	17.2±0.31^{b}	16.76±0.44^{b}
Total lipid	6.9±0.14^{a}	7.65±0.07^{ab}	8.42±0.37^{b}	8.43±0.32^{b}
Crude ash	4.87±0.48	4.69±0.18	4.65±0.16	4.92±0.41
CF[1]	1.85±0.04	1.89±0.04	1.95±0.09	1.98±0.06
HSI[2]	2.07±0.18	2.05±0.06	2.3±0.12	2.26±0.18
VSI[3]	2.58±0.05	2.68±0.08	2.6±0.18	2.51±0.21

[1] CF: condition factor (%), [2] HSI: hepatosomatic index (%), [3] VSI: viscerasomatic index.

Table 6.4: Protease activity (PA, unit mg^{-1} protein) in the digestive tract and apparent digestibility coefficients (ADC) in red sea bream fed test diets for 56 days*.

	Experimental diets			
	Control	LR	LL	LR+LL
ADCD (%)[1]	59.18±0.49[a]	67.52±1.57[b]	70.36±0.8[b]	68.4±1[b]
ADCP (%)[2]	83.14±0.25[a]	88.97±0.74[b]	90.13±0.06[bc]	91.51±0.39[c]
ADCL (%)[3]	76.54±1.4[a]	83.41±1.35[b]	84.03±1.52[b]	84.35±1.33[b]
PA (unit/mg protein)[4]	0.023±0.003[a]	0.028±0.001[b]	0.028±0.002[b]	0.029±0.002[b]

* In the same line, means with different letters are significantly different ($P < 0.05$), means with the same letters are not significantly different ($P > 0.05$). Data represent means± pooled SEM (n=9 fish).

[1]ADCD: apparent digestibility coefficient of dry matter, [2]ADCP: apparent digestibility coefficient of protein, [3]ADCL: apparent digestibility coefficient of lipid, [4]PA: protease activity (unit/mg protein).

Table 6.5: Blood parameters in juvenile red sea bream fed test diets for 56 days*.

	Experimental diets			
	Control	LR	LL	LR+LL
Hematocrit (%)	34.33±0.9[a]	41±0.58[b]	40.33±0.9[b]	42.67±1.2[b]
Total protein (g/dl)	3.07±0.09[a]	3.47±0.09[b]	3.5±0.1[b]	3.8±0.06[c]
T-Cho (mg/dl)[1]	261±2.52[c]	235±5.86[b]	236.67±4.26[b]	218.67±2.19[a]
TG (mg/dl)[2]	176.67±4.41[c]	154.33±4.41[ab]	159±2.89[b]	144±3.22[a]
d-ROMs (U. Carr)[3]	50.67±3.28	44.67±2.33	68±10.6	50.33±2.6
BAP (µ MOL/L)[4]	2205.67±57.21[a]	2727.33±140.79[b]	2560.67±31.53[b]	2731±11.52[b]

* In the same line, means with different letters are significantly different ($P < 0.05$), means with the same letters are not significantly different ($P > 0.05$). Absence of letters indicates no significant difference between treatments. Data represent means± pooled SEM (n=15 fish).

[1] T-Cho: total cholesterol, [2] TG: triglyceride, [3] d-ROMs: reactive oxygen metabolites, [4] BAP: biological antioxidant potential.

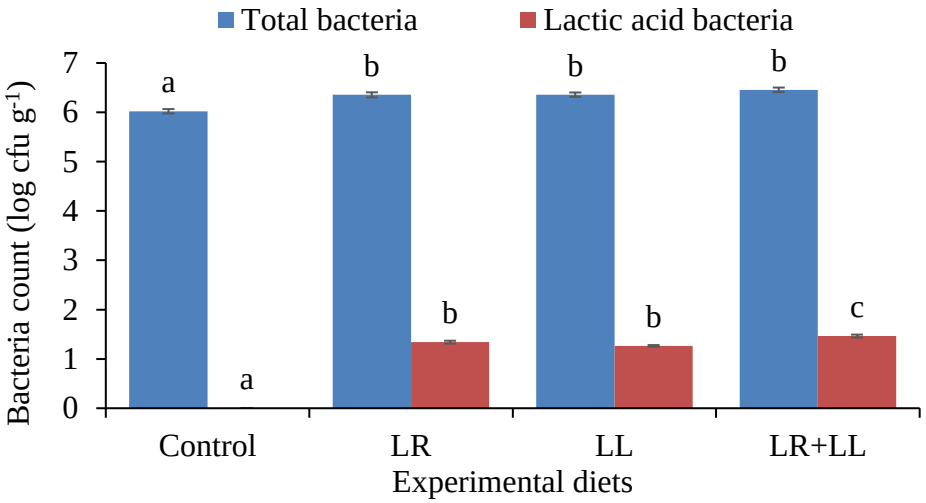


Fig. 6.1: Total culturable bacteria and LAB levels (log CFU g^{-1} intestine) of red sea bream fed with diets containing LR or/and LL for 56 days. Data represent means± pooled SEM (*n*=9 fish). Values with different letters are significantly different ($P < 0.05$). Values with the same letter are not significantly different ($P > 0.05$).

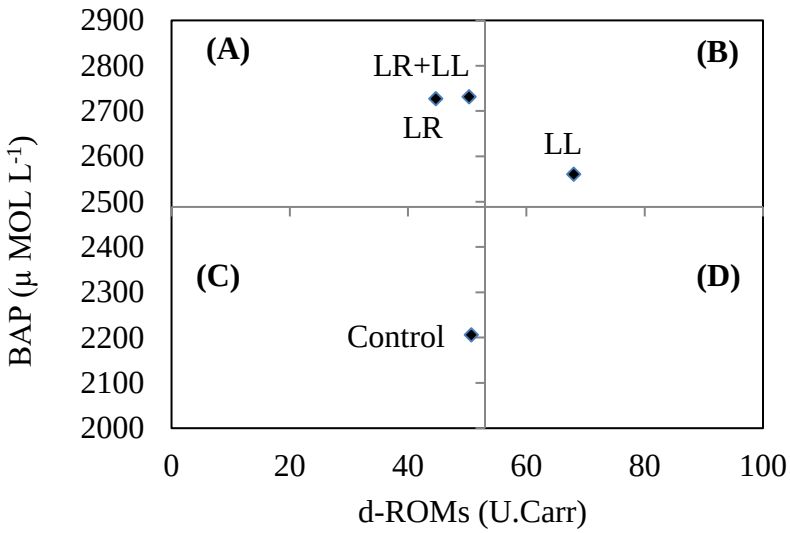


Fig. 6.2: Oxidative stress parameters in red sea bream fed test diets. Central axis based on mean values of d-ROMs and BAP from each treatment. Zone (A): high antioxidant potential and low reactive oxygen metabolites (good condition); Zone (B): high antioxidant potential and high reactive oxygen metabolites (acceptable condition); Zone (C): low antioxidant potential and low reactive oxygen metabolites (acceptable condition); Zone (D): low antioxidant potential and high reactive oxygen metabolites (stressed condition).

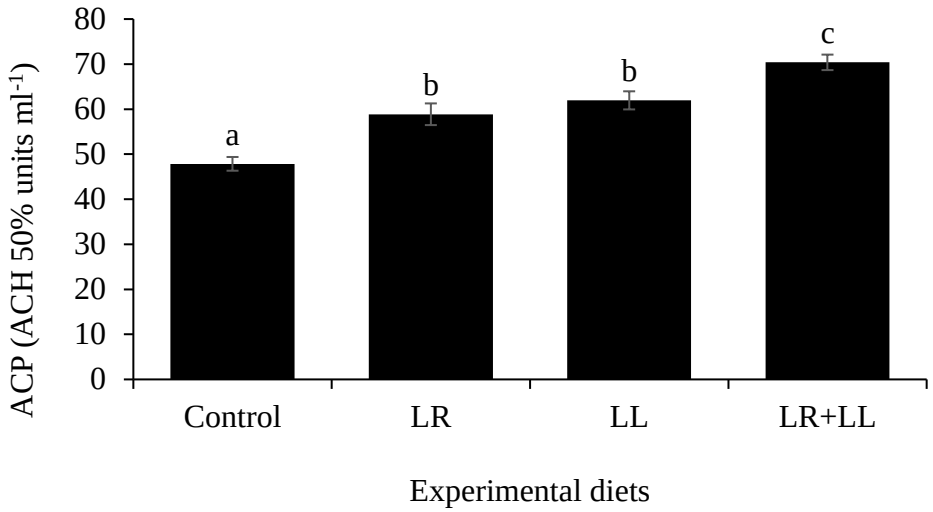


Fig. 6.3: Alternative complement pathway (ACP) of red sea bream fed test diets. Data represent means± pooled SEM (*n*=9). Values with different letters are significantly different ($P < 0.05$). Values with the same letter are not significantly different ($P > 0.05$).

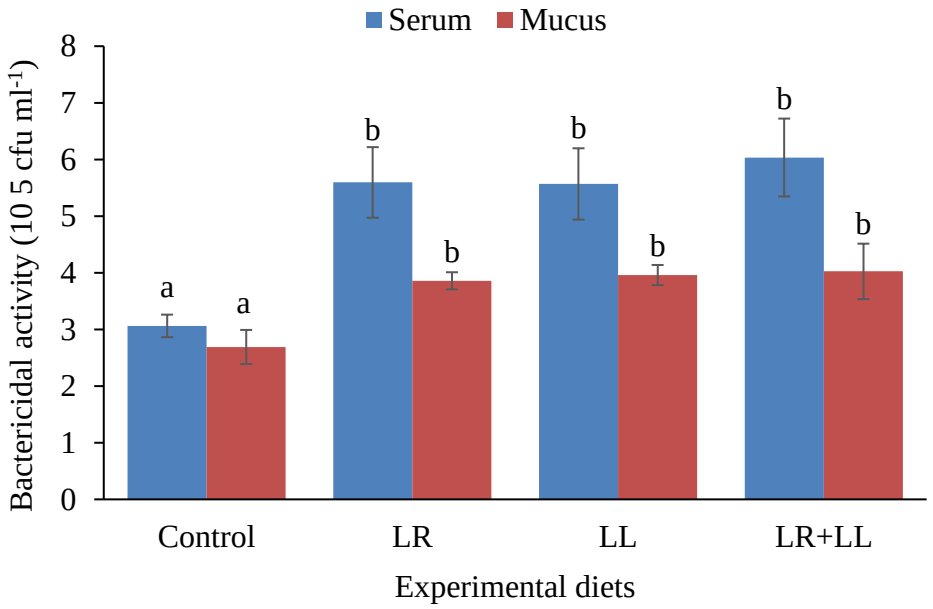


Fig. 6.4: Serum and mucus bactericidal activity of red sea bream fed test diets. Data represent means± pooled SEM (*n*=3). Values with different letters are significantly different ($P < 0.05$). Values with the same letter are not significantly different ($P > 0.05$).

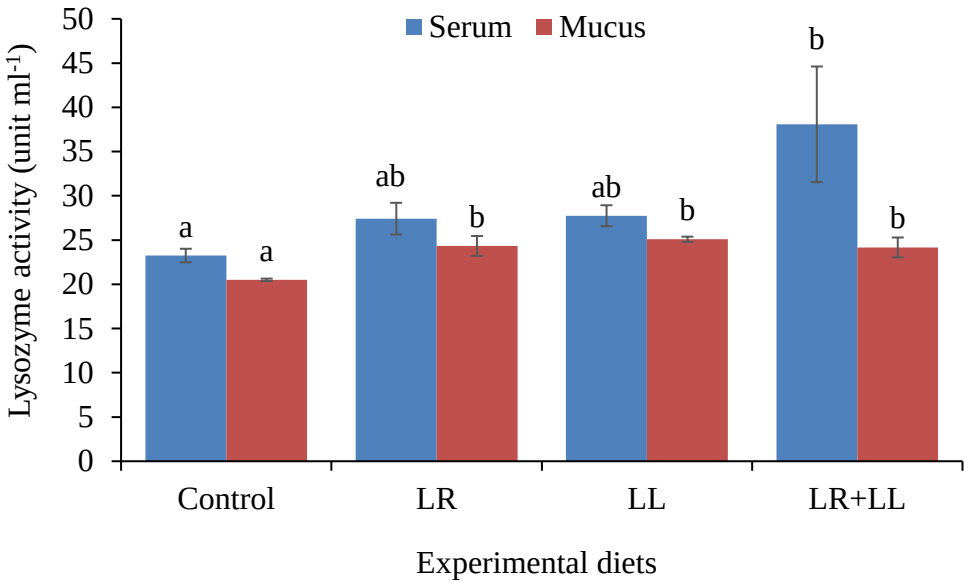


Fig. 6.5: Serum and mucus lysozyme activity of red sea bream fed test diets. Data represent means± pooled SEM (*n*=9). Values with different letters are significantly different ($P < 0.05$). Values with the same letter are not significantly different ($P > 0.05$).

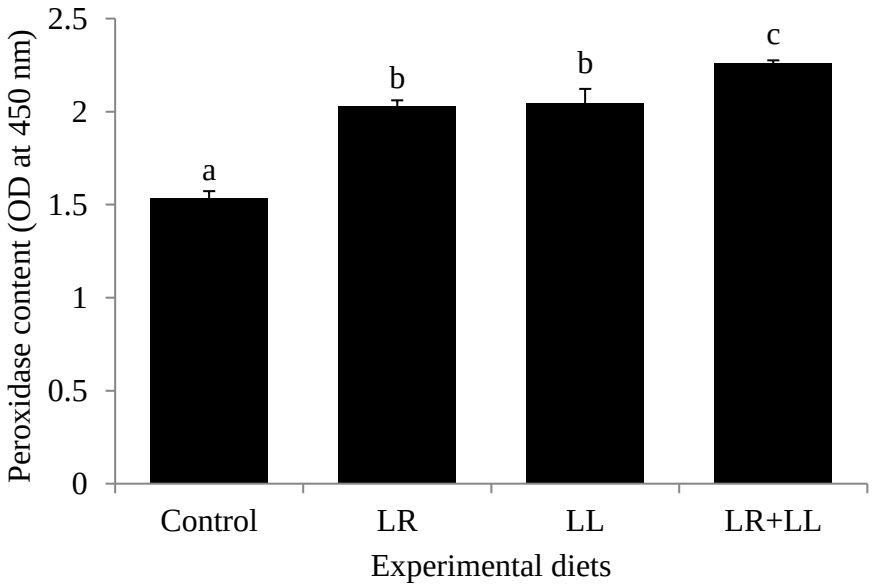


Fig. 6.6: Serum peroxidase activity of red sea bream fed test diets. Data represent means± pooled SEM (*n*=9). Values with different letters are significantly different ($P < 0.05$). Values with the same letter are not significantly different ($P > 0.05$).

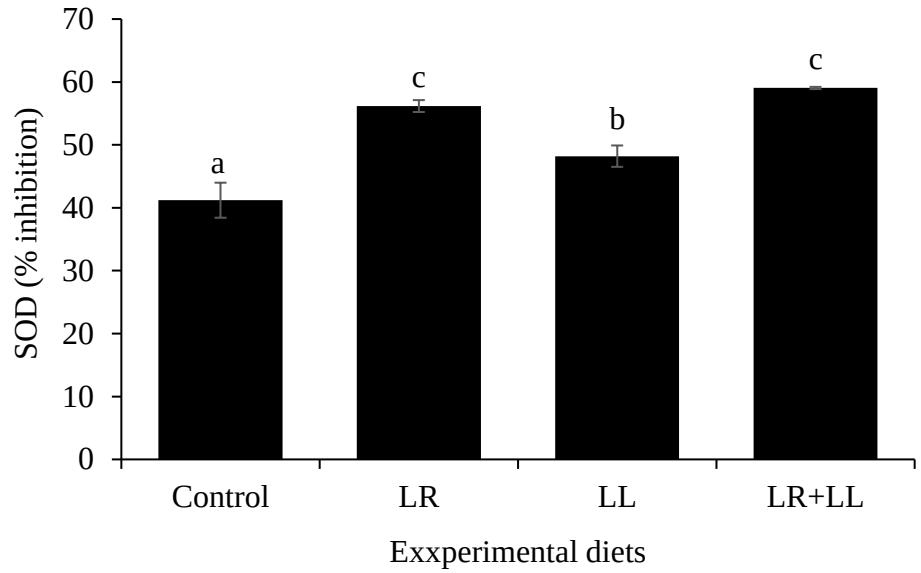


Fig. 6.7: Superoxide dismutase (% inhibition, SOD) of red sea bream fed test diets. Data represent means± pooled SEM (*n*=3). Values with different letters are significantly different ($P < 0.05$). Values with the same letter are not significantly different ($P > 0.05$).

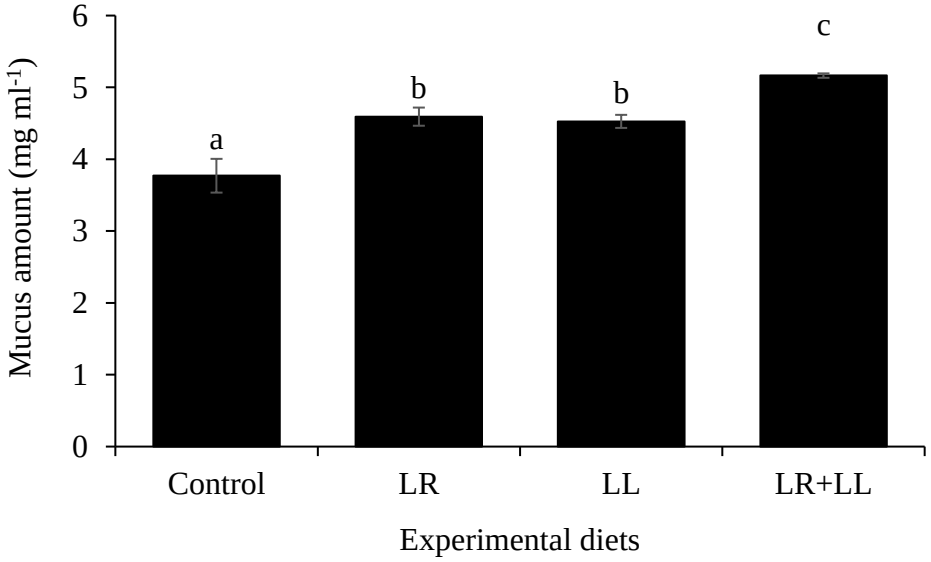


Fig. 6.8: Amounts of mucus secretion on body surface of red sea bream. Values are expressed as mean relative means ± pooled SEM (*n*=9 fish). Values with the same letter are not significantly different ($P > 0.05$). Means with different alphabet are significantly different ($P < 0.05$).

6.5 Discussion

A growing number of studies have demonstrated the ability of probiotics to increase the growth performance, feed utilization, immune response, and modulate gut microbiota of fish, usually after receiving a single species probiotic treatment (Dawood *et al.*, 2015b,c). However, very little effort has been directed towards the possible synergistic effects resulting from the simultaneous administration of different bacteria species to fish (Balca´ zar *et al.*, 2007; Beck *et al.*, 2015; Giri *et al.*, 2014; Sun *et al.*, 2012; Salinas *et al.*, 2008). Therefore, the present study was conducted to evaluate the effects of dietary supplementation of *L. rhamnosus* (LR) or/and *L. lactis* (LL) on the growth, gut microbiota and immunity of red sea bream, *P. major*.

The obtained results clearly demonstrated that feeding of (LR) or (LL) significantly improves growth performance of red sea bream compared with the control group after 56 days. This observation is in agreement with earlier studies that probiotics (*Lactobacillus sp.*) supplementation significantly increased the growth performance of red sea bream (*P. major*) (Dawood *et al.*, 2015b), gilthead sea bream (*Sparus aurata*) (Suzer *et al.*, 2008), and olive flounder (*P. olivaceus*) (Beck *et al.*, 2015). Additionally, *L. lactis* also increased growth performance of grouper (*E. coioides*) (Sun *et al.*, 2012) and olive flounder (*P. olivaceus*) (Heo *et al.*, 2013). Moreover, the obtained results revealed that fish fed the mixture of LR and LL showed a higher Fn wt, WG, and SGR than the groups fed an individual LR or the control diet. In line with our findings, Beck *et al.* (2015) demonstrated that a mixture of two live probiotic organisms, namely *Lactobacillus sp.* and *L. lactis* in diet resulted in better growth performances of olive flounder. In the present study, significant increase FI and feed utilization parameters (FER, PER, and PG) were observed

in fish fed the LR or/and LL-containing diet, this might suggest that the probiotic supplement influenced the feed palatability, and therefore slightly increased the FI and growth rate of fish. To our knowledge, this is the first report of dietary administration of *L. lactis* improved the growth and feed utilization of red sea bream. Suzer *et al.* (2008) suggested that sea bream is primarily affected by probiotic through the enhancement of host nutrition due to the stimulation of digestive enzymes resulting in a higher growth and feed efficiency ratio. Furthermore, the presence of probiotic microorganisms in the intestine improves microbial balance, which in turn improves nutrient absorption and utilization (Gatesoupe, 1999; Lara-Flores, 2011). Interestingly, growth rate and feed utilization significant improvements were observed in fish fed LR and LL-containing diets for 56 days, suggesting the probiotic mixture-fed fish utilized dietary nutrients more efficiently. Although LR and LL were increased weight gain of fish when compared with the control group at the same concentration (10^6 cell g^{-1}), *L. lactis* seemed to be more effective in improving growth performance of *P. major*. Therefore, their probiotic effects may be affected by other factors, like differences in their survival rates in the gut and interaction with host microbiota. This supports the suggestion that each probiotic strain may interact with host in a different way (Bomba *et al.*, 2002; Díaz-Rosales *et al.*, 2006), and further studies would be necessary to optimize the dose and frequency of administration, to maximize their benefits.

Growth performance and feed utilization improvements could be also attributed to the enhanced intestinal digestive functions of fish by probiotic supplementation (LR or/and LL), which might include the increased digestive enzymes activities. Indeed, significant improvements in protease activity (PA), digestibility coefficients of dry matter (ADCD),

protein (ADCP), and lipid (ADCL) were observed in the present study, as further supported this hypothesis. Previous studies have demonstrated that *Lactobacillus sp.* supplementation resulted in the improved digestibility of nutrients (Dawood *et al.*, 2015a,b). It has been known that the digestive enzymes activities are positively correlated with the digestive capacity of fish (Hakima *et al.*, 2006; Perez-Casanovaa *et al.*, 2006) which, to some extent, increased its ability to obtain nutrients from food (Furne *et al.*, 2005). In the present study, intestine protease activities increased significantly with supplementing both dietary LR or/and LL, indicating an improved digestibility coefficients of red sea bream. Similarly, it has been reported that feeding mixture of probiotics could improve the protease activity of rohu (*L. rohita*) (Mohapatra *et al.*, 2012). It is reported that the digestive organs are very sensitive to food composition and cause immediate changes in activities of the digestive enzymes (Bolasina *et al.*, 2006; Shan *et al.*, 2008), which is finally reflected in fish health and growth. Moreover, bacteria also secrete proteases to digest the peptide bonds in proteins and therefore break down the proteins into their constituent monomers and free amino acids, which can benefit the nutritional status of the animal (MacFarlane and Cummings, 1999). Bacterial enzymatic hydrolysis has been shown to enhance the bioavailability of dry matter, protein, and lipid (Ling and Hanninen, 1992), which may result in higher growth and nutrient utilization as observed in the present study. The higher protease activities obtained in the probiotic-supplemented fish are mainly the outcome of stimulation by probiotic itself or exogenous enzyme produced to synthesize endogenous digestive enzyme which might have improved nutrient digestibility leading to better growth performance and feed efficiency in fish. Similar observations have also been reported for other fishes in which the nutrient digestibility increased considerably with the use of

probiotic-supplemented diet (Lara-Flores *et al.*, 2003; Mohapatra *et al.*, 2012; Yanbo and Zirong, 2006). The increased protease activity in probiotic-supplemented diet groups might have resulted better protein digestion and hence better growth and protein gain in fish. Significant improvements in total intestinal bacteria (TBC) and lactic acid bacteria (LAB) counts were observed in the present study, as further supported the enhanced growth and feed utilization. Similar to Mohapatra *et al.* (2012), significant improvement in TBC counts was observed when probiotic-supplemented diets were fed to the fish, which might have resulted better health and immunity of fish and hence more growth. It is reported that the colonization rate of bacteria in the digestive tracts depends on the dietary bacteria level (Bagheri *et al.*, 2008). In our study, the higher degree of adhesion of microorganisms that are supplemented through diets may be the reason for enhanced growth and nutrient utilization of fish. Several reports suggested that most of probiotics exert their effect through colonization in host and excretion of several growth-enhancing nutrients (Ahilan *et al.*, 2004; Bagheri *et al.*, 2008).

In the present study, high protein and lipid contents of whole body was observed in fish fed LR or/and LL-supplemented diets. Again, it seems that supplementing diets with LR or/and LL improved protein and lipid utilization in red sea bream. This is similar to the results of Mohapatra *et al.* (2012). Biometric indices (CF, HSI, and VSI) of red sea bream were not significantly altered by LR or/and LL. However, the results reflect the improvements in body weight together with body length of fish fed LR or/and LL diets when compared with the fish fed the control diet.

Blood parameters results in the current study are considered to be within the accepted values for juvenile red sea beam, compared to those of the previous findings (Dawood *et*

al., 2015a,b,c). Dietary supplementation of LR or/and LL significantly enhanced hematocrit of red sea bream as a general health response towards nutritional strategies and environmental changes. Similarly, Dawood *et al.* (2015b) reported that hematocrit level was enhanced by the supplementation of *Lactobacillus sp.* in red sea bream diets. In the present study, the plasma total protein of the probiotic fed groups was higher than the control group, and the elevated levels of the former may imply a better health status in fish. This change in the plasma protein is also thought to be an adaptive response that contributes to regaining homeostasis after tissue injury or infection (Gerwick *et al.*, 2002). It has also been reported that the total protein in plasma was significantly higher in rainbow trout fed with LR supplementation (Panigrahi *et al.*, 2010). Results of the present study also revealed that the plasma total cholesterol and triglyceride levels decreased after feeding probiotic microorganisms incorporated diet for 56 days compared to the control group. In agreement with the current findings, plasma total cholesterol and triglycerides have been reported to decrease after probiotic supplementation in red sea bream (Dawood *et al.*, 2015b,c). Results over several years from animal and human studies suggest a moderate cholesterol-lowering action of dairy products fermented with strains of LAB (Chen *et al.*, 2014; Falcinelli *et al.*, 2015; Periera and Gibson, 2002). Among the reasons, probiotic bacteria ferment food-derived indigestible carbohydrate to produce short chain fatty acids in the gut, which can then cause a decrease in the systemic levels of blood lipids by inhibiting hepatic cholesterol synthesis and/or redistributing cholesterol from plasma to the liver (Falcinelli *et al.*, 2015). Possible modulation of the expression of genes involved in lipid metabolism may also have contributed towards lowered plasma lipids by probiotic supplementation (Falcinelli *et al.*, 2015). Further studies are required to enhance our

understanding of the roles that gut microbes play in lipid metabolism. Recently, Oxidative stress was measured using the free radical analytical system assessing the derivatives of oxidative stress by measuring reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) in fish plasma samples (Dawood *et al.*, 2015a,b,c). Ballerini *et al.* (2003) and Pasquini *et al.* (2008) reported that, fish with higher value of d-ROMs indicate a more oxidative condition, therefore, higher BAP value indicate they have stronger tolerance against oxidation. Using these parameters our study showed that fish fed diets supplemented with LR and/or LL were more tolerant of oxidative stress indicating a higher health status, which indicated high BAP and low d-ROMs values. Similarly, Dawood *et al.* (2015a,b) illustrated that dietary supplementation of *Lactobacillus sp.* also stimulated the oxidative status of red sea bream. To date there remains a lack of explanation about how these additives work to effect on these parameters, so more studies are needed. It has been reported that the interaction between probiotics and intestinal epithelial cells plays an important role in fish gut mucosal immunity which controls the physical as well as immunological barrier property of intestine leading to an immune response stimulation (Liu *et al.*, 2013; Shida and Nanno, 2008; Thomas and Versalovic, 2010; Wells, 2011). Results of the current study illustrated that dietary administration of LR or/and LL enhanced immune parameters, including lysozyme and bactericidal activities of serum and mucus, alternative complement pathway (ACP), peroxidase, and superoxide dismutase (SOD). Similarly, the health promoting effects of dietary combined probiotics have been reported to stimulate the innate immune responses in gilthead seabream (*S. aurata*) (Salinas et al., 2005, 2008), olive flounder (*P. olivaceus*) (Beck *et al.*, 2015), grouper (*E. coioides*) (Sun *et al.*, 2012), and *L. rohita* (Giri *et al.*, 2014). Salinas *et al.* (2005) reported that,

combination of bacterial strains that complement each other and occupy different niches within the gut microflora environment could result in an enhancement or prolongation of the desirable effects on the host immune response and health.

Several studies have demonstrated that external factors such as nutrients, vitamins and probiotics influence the alternative complement pathway (ACP) activity in fish (Cerezuela *et al.*, 2012b; Cuesta *et al.*, 2006). In this study, ACP activity was one of the most influenced immune system parameters, showing a significant increase in all of the treatment groups. The present results are in complete agreement with the findings of previous studies (Giri *et al.*, 2014; Sun *et al.*, 2012). Bactericidal activity (BA) is one of the most important factors in host resistance against pathogenic bacteria. Our results revealed that, the highest serum and mucus BA was found in fish fed LR or/and LL supplemented diets. Similarly, Dawood *et al.* (2015a,c) reported that dietary compound probiotics significantly increased the BA of red sea bream. Lysozyme activities in fish have been reported to be modulated by several probiotic species, like in rainbow trout (*Oncorhynchus mykiss*) by *L. rhamnosus* and *L. plantarum* supplementation (Son et al., 2009; Panigrahi *et al.*, 2004), brown trout by *L. lactis ssp. lactis* (Balca´ zar *et al.*, 2007), and grouper (*E. coioides*) by *L. lactis* (Sun *et al.*, 2012). In line with those previous studies, probiotic LR and LL affected the serum lysozyme activity of *P. major* in this study. Serum peroxidase values were enhanced by the LR or/and LL diet, whereas peroxidase content was not affected by the combined mixture of LR and LL bacteria, confirming previous data in sea bream (Dawood *et al.*,2015a,c; Salinas *et al.*, 2008). Superoxide dismutase (SOD) catalyses the dismutation of the highly reactive $^{-}O_2$ to less reactive H_2O_2 and functions in the main antioxidant defense pathways in response to oxidative stress. In the present study,

SOD activities improved significantly in the treatment groups. Similar observations were reported in *L. rohita* fed diets supplemented with *B. subtilis* singularly or in combination with *L. plantarum* or/and *Pseudomonas aeruginosa* (Giri *et al.*, 2014). Fish have a unique physical barrier composed of skin and epidermal mucus that acts as a part of non-specific defense mechanisms (Hernandez *et al.*, 2010; Palaksha *et al.*, 2008), and prevents fish body from being directly exposed to pollutant and stressor through environmental water. Results of this study showed that higher amounts of mucus secretion were observed in all fish fed LR or/and LL-supplemented diets compared to that of fish fed the control diet. Similarly, oral administration of probiotic increased the secretion of skin mucus in red sea bream (*P. major*) (Dawood *et al.*,2015a,b,c) and gilthead seabream (*S. aurata*) (Salinas *et al.*, 2008). The present results demonstrated that, in general, the diet that contained both *L. rhamnosus* and *L. Lactis* species induced greater effects in the red sea bream on the innate immune system. Therefore, multispecies probiotics were shown to be able to elicit different responses in the host immune system compared with monospecies preparations. There is conclusive evidence that, in higher vertebrates, adequately designed multistrain or multispecies probiotic formulations possess health promoting effects that are lacking in monospecies probiotic diets (Timmerman *et al.*, 2004). Some of the proposed mechanisms include greater survival, growth, viability or adhesion to mucosal surfaces of one species in the presence of another species (Hosoi *et al.*, 2000; Ouwehand *et al.*, 2000), the production of different enzymes or other proteins, the creation of a probiotic niche and additive/synergistic effects of strain specific properties (Timmerman *et al.*, 2004).

The effects of probiotics are very diverse, and depend on conditions including the sources, types, dose, and duration of supplementation (Franz *et al.*, 1997; Perdigón *et al.*, 2001);

therefore, candidate strains for a probiotic mixture need to be wisely selected for maximizing their combinatory effects. In this regard, the mixture of *L. rhamnosus* and *L. Lactis* appears to be an adequate combination. This complementary function enabled by the mixture of *L. rhamnosus* and *L. Lactis* may have resulted in the increased several performances of red sea bream. However, more studies are required to confirm this possibility.

Chapter VII:

Combined effects of probiotics and vitamin C as feed additives on the performances of cultured fish species

Experiment I: Immune responses and stress resistance in red sea bream, *Pagrus major*, after oral administration of heat-killed *Lactobacillus plantarum* and vitamin C

Immune responses and stress resistance in red sea bream, *Pagrus major*, after oral administration of heat-killed *Lactobacillus plantarum* and vitamin C

7.1 Abstract

The present study evaluated the interactive benefits of dietary administration of heat-killed *Lactobacillus plantarum* (LP) and vitamin C (VC) on the growth, oxidative status and immune response of red sea bream (*Pagrus major*). A diet without LP and VC supplements was employed as a control diet. Four other test diets with 0 or 1 g LP kg^{-1} combined with 0.5 or 1 g VC kg^{-1} (2×2 factorial design) were fed to red sea bream (2 ± 0.01 g) for 56 days. A significant interaction was found between LP and VC on final body weight (FNW), weight gain (WG), hematocrit (HCT), serum bactericidal (BA) and lysozyme (LZY) activities, mucus LZY and peroxidase (PA) activities, nitro blue tetrazolium (NBT), catalase, mucus secretion and tolerance against low salinity stress test (LT_{50}) ($P < 0.05$). In addition, FNW, WG, specific growth rate, feed and protein efficiency ratio, serum (BA, LZY, PA and NBT), mucus (LZY and PA), superoxide dismutase, malondialdehyde and mucus secretion were significantly affected by either LP or VC ($P < 0.05$). Furthermore, only LP was a significant factor on survival, plasma total cholesterol, mucus BA and alternative complement pathway ($P < 0.05$). However, VC supplementation affected on HCT and LT_{50}. Interestingly, fish fed with both LP at 1 g kg^{-1} diet with VC at 0.5 or 1 g kg^{-1} diet showed higher growth, humoral and mucosal immune responses, anti-oxidative status, mucus secretion and LT_{50} as well as decreased plasma, triglyceride and total cholesterol levels than the fish fed control diet ($P < 0.05$). These results demonstrated that dietary LP and VC had a significant interaction for red sea bream with the capability of improving growth performance and enhancing stress resistance by immunomodulation.

Keywords: *Pagrus major*, Probiotic, Vitamin C, Blood chemistry, Oxidative status, Immune response, Skin mucus

7.2 Introduction

In intensive aquaculture systems the farmed fish often subjected to stresses that are above and beyond their capacity of endurance, such as high temperature, crowding, water quality deterioration and the invasion of bacteria and viruses. All these adverse environmental factors might disturb the balance and harmony between fish and the environment, causing stress response in fish and, consequently, affecting production negatively (Dawood *et al.*, 2016a). As a result, fish may develop depressed immune systems and compromised non-specific barrier (e.g. skin), enhancing their susceptibility to different stressors (Guardiola *et al.*, 2014). To reduce stress, the fish industry has been using antibiotics and hormones trying to offer an adequate diet to maintain the health of cultured fish (Miranda and Zemelman, 2002). However, the use of antibiotics represents environmental hazards, spreads pathogen-resistant genes, immunosuppression and limits the efficiency of this treatment (Bachère, 2003). Diverse types of feed additives using dietary supplements of probiotics, prebiotics, β-glucans, vitamin C and immunostimulants may help to reduce the susceptibility of fish to stress and diseases (Dawood *et al.*, 2016a; Dawood *et al.*, 2015a,b,c,e).

Heat-killed *Lactobacillus plantarum* (LP), namely, Immuno-LP20™, is known as probiotics for their health-promoting effects, including enhancement of the growth performance, immune system, stress resistance and reduction of blood lipid concentrations of red sea bream, *Pagrus major* (Dawood *et al.*, 2015a,b). Although probiotics were

originally defined as live microbial components, non-viable microbes have also been shown to exhibit beneficial effects that are equivalent to, or even greater than, those of live microbes (Dawood *et al.*, 2015c; Isolauri *et al.*, 2002; Naidu *et al.*, 1999; Salminen *et al.*, 1999). The use of probiotics, benefit the host by producing inhibitory compounds, competing for chemicals and adhesion sites, modulating and stimulating the immune function, and improving the microbial balance (Isolauri *et al.*, 2002; Naidu *et al.*, 1999; Salminen *et al.*, 1999). Several studies have demonstrated that killed probiotics can improve growth performance, feed utilization, disease resistance and immunostimulation of cultured fish species (Choi *et al.*, 2008; Dawood *et al.*, 2016a; Dawood *et al.*, 2015a,b,c,e; Diaz-Rosales *et al.*, 2006; Pan *et al.*, 2008; Panigrahi *et al.*, 2010; Rodriguez-Estrada *et al.*, 2013; Salinas *et al.*, 2006; Salinas *et al.*, 2008; Sheikhzadeh *et al.*, 2016).

Vitamin C (VC) is one of the best known feed additives and plays an essential role in normal physiological functions and stimulation of the immune response of fish (Roosta *et al.*, 2014). A number of investigations confirmed the fact that VC plays an important role as water-soluble antioxidant for red sea bream (Gao *et al.*, 2013; Gao *et al.*, 2014; Ren *et al.*, 2008). The major line of antioxidative defense system against radicals by VC is to prevent lipid peroxidation in fish plasma (Gao *et al.*, 2013). Moreover, VC also promotes beneficial effects on the growth performance, serum bactericidal activity, phagocytic activity, serum complement activity, antibody levels, lysozyme activity, and mucosal parameters of several fish species (Lin and Shiau, 2005; Ming *et al.*, 2012; Nayak *et al.*, 2007; Roosta *et al.*, 2014). Additionally, previous research findings indicate that dietary supplementation with immunomodulatory vitamins such as VC can improve immune response and disease resistance of a variety of fish species (Ming *et al.*, 2012; Nayak *et al.*,

2007; Ren *et al.*, 2007; Verhlac *et al.*, 1998). Until now, the interactive effects of probiotics and VC on the health status, immune response and stress resistance of fish are not documented yet.

The red sea bream, *P. major* is a very important cultured species in East Asia countries, particularly Japan due to its high market value, desirable taste and recent supply shortage. Recently, diseases of cultured red sea bream showed an increasing trend, especially in intensive aquaculture system causes disease outbreak. Therefore, the present study was conducted to clarify the possible interactions between dietary LP and VC on growth performance, blood chemistry profile, immune response and tolerances against stress of red sea bream, and the result would suggest new avenues for the alleviation of stress and prevention of fish diseases.

7.3 Materials and methods

7.3.1 *Preparation of the experimental diets*

Beside the control diet, four diets contained 2 levels (0 and 1 g kg^{-1} diet) of heat-killed *L. plantarum* (LP) (House Wellness Foods Corp., Itami, Japan (Dawood *et al.*, 2015a,b)) combined with 2 levels (0.5 and 1 g kg^{-1} diet) of vitamin C (VC) (L-Ascrobil-2-phosphate-Mg) were formulated (2×2 factorial design) to evaluate combined effects of dietary LP and VC. Brown fish meal and casein were used as protein sources, and soybean lecithin and pollack liver oil were the main lipid sources. All ingredients were thoroughly mixed in a food mixer and dry pelleted in a laboratory pellet mill, through a (1.6–2.1 mm) diameter die. Pellets were dried in an oven at 50 °C for 2 h, and then stored in a freezer in airtight bags until use. Chemical analyses of the diets were performed following the Association

of Official Analytical Chemists methods (AOAC, 1998). VC contents of test diets were determined based on the method of Gao *et al.* (2013). Ingredients and proximate composition of the experimental diets are presented in Table 7.1.

Table 7.1: Formulation and proximate composition of the test diets*.

Ingredients (g kg^{-1} dry diet)	Test diets (LP/VC, g kg^{-1})				
	LP0/VC0	LP0/VC0.5	LP1/VC0.5	LP0/VC1	LP1/VC1
Brown fish meal	280	280	280	280	280
Casein	280	280	280	280	280
Dextrin	70	70	70	70	70
α-starch	60	60	60	60	60
Soybean lecithin	50	50	50	50	50
Pollack liver oil	60	60	60	60	60
Vitamin mixture	30	30	30	30	30
Mineral mixture (VC free)	30	30	30	30	30
Activated gluten	50	50	50	50	50
α-Cellulose	90	89.5	88.5	89	88
LP[1]	0	0	1	0	1
VC[2]	0	0.5	0.5	1	1
Proximate composition					
Crude protein (%)	50.8	50.1	50.4	50.5	50.7
Total lipid (%)	11.4	12.1	11.2	11.5	11.5
Moisture (%)	8.9	9.5	9.7	9.8	9.7
Ash (%)	10.8	10.7	10.7	10.8	10.9
Gross energy (KJ g^{-1})	19.6	19.4	19.5	19.6	19.7
VC content (mg kg^{-1})	Trace	581	573.7	1066	1088.7

*According to Dawood *et al.* (2016a).

[1] LP: preparation of heat-killed *Lactobacillus plantarum* made by House Wellness Foods Corp. (Itami, Japan) (Dawood *et al.*, 2015a,b).

[2] VC: vitamin C source (L-Ascrobil-2-phosphate-Mg).

7.3.2 *Growth trial*

The experiment was performed at the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan, with red sea bream (*P. major*) obtained from Tawaki farm, Kumamoto, Japan. The fish were acclimatized for 2 weeks in the laboratory condition and reared in a 500-L tank. During this period, a commercial diet (50% crude protein; Higashimaru, Japan) was supplied to the fish. The trial was run in 15 polycarbonate tanks of 100 L water capacity (26.3±1.2°C water temperature) in a flow through sea water system. Tanks were equipped with an inlet, outlet, and continuous aeration (1.5-2 L min^{-1}) of filtered seawater (33.3±0.5 g L^{-1} salinity) and dissolved oxygen was kept near saturation (6.1±0.5 mg L^{-1}). Thereafter, 20 fish with an initial mean body weight of 2 ± 0.01 g were distributed to each tank and the experimental diets randomly assigned to triplicate groups. The trial lasted 56 days and during that period fish were fed by hand, twice daily, 7 days a week, until apparent visual satiation. Utmost care was taken to avoid feed losses. At the termination of the experiment, the fish were fasted for 24 hours, and then, the total number, individual body weight and length of fish in each tank were measured.

7.3.3 *Calculation of growth, survival and feed utilization*

Body weight gain (WG), specific growth rate (SGR), survival (SUR), feed efficiency ratio (FER), protein efficiency ratio (PER) and condition factor (CF) parameters were calculated using the following equations:

$WG\ (\%) = (W_0 - W_f) \times 100/W_0$; $SGR\ (\%\ day^{-1}) = \{(Ln\ (W_f) - Ln\ (W_0)) / t\ (56\ days)\} \times 100$;

$SUR\ (\%) = 100 \times (N_f / N_0)$

Where W_0 and W_f were initial and final body weight (g) of fish, respectively; t was duration of experiment in days; N_f and N_0 were initial and final number of fish, respectively.

FER =live weight gain/dry feed intake; PER =live weight gain (g)/dry protein intake (g)

CF (%) = weight of fish (g)/ (length of fish)^3 (cm)^3×100

7.3.4 *Plasma, serum and skin mucus collection*

Heparinized (1600 UI ml^{-1}, Nacalai Tesque, Kyoto, Japan) syringes were used to collect blood from the caudal vein of 5 fish in each replicate tank (15 fish per treatment) and pooled. Partial heparinized whole blood was used to analyze the hematocrit and nitro blue tetrazolium (NBT) activity while plasma was obtained by centrifugation at 3000×g for 15min under 4°C. The resulting plasma was frozen at −80 °C for the blood chemistry profile analysis. Serum samples were obtained from the caudal vein of another 5 fish in each replicate tank (15 fish per treatment) with non-heparinized disposable syringes. After clotting at 4 °C during 3-4 h, each sample was centrifuged and the serum removed and frozen at −80 °C until use. Skin mucus samples were collected from red sea bream specimens using the method of Dawood *et al.* (2015a,b). Briefly, skin mucus was collected from dorso-lateral body surface of 5 fish per each replicate tank (15 fish per treatment) by using a small piece of sterilized cotton and immediately suspended in 1 ml of phosphate-buffered saline (PBS, pH=7.4). Sample was then put into a handmade set of two centrifugal tubes, the upper tube had a small filter, with which the mucus in the cotton will be collected in the lower tube while centrifuged. The sets of the double-tube (1.5 ml centrifugal tube) were centrifuged at 3000×g for 5 min under 4 ˚C (MX-160, Tomy Seiko Co., LTD, Tokyo, Japan), and the supernatant in the under tube was transferred into another centrifugal tube (510-GRD, QSP, San Diego, CA, USA) and kept at -80˚C until the analysis.

7.3.5 *Hematocrit, blood chemistry and total serum protein analysis*

Fresh heparinized blood was used for hematocrit determination using the micro hematocrit technique (Dawood *et al.*, 2016a). Plasma total bilirubin (T-BIL), blood urea nitrogen (BUN), glutamyl oxaloacetic transaminase (GOT), glutamic-pyruvate transaminase (GPT), total cholesterol (T-CHO), triglyceride (TG) and glucose (GLU) parameters were measured spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan) as described by Dawood *et al.* (2015a).

7.3.6 *Immune responses and mucus secretion*

Lysozyme, bactericidal and peroxidase activities in serum or mucus were determined as described by Dawood *et al.* (2016a,2015a,b,c). Serum alternative complement pathway (ACH50) was measured as described by Dawood *et al.* (2016a). The nitro blue tetrazolium (NBT) assay as described by Anderson and Siwicki (1995) with some modifications by Kumari and Sahoo (2005). Briefly, Blood (0.1 ml) was placed in microtiter plate wells, to which an equal amount of 0.2% NBT solution (Sigma, USA) was added and incubated for 30 min at room temperature. A sample of NBT blood cell suspension (0.05 ml) was added to a glass tube containing 1m l N, N-dimethylformamide (Sigma, USA) and centrifuged for 5 min at 3000 rpm. Finally, the optical density of supernatant was measured at 540 nm. Dimethylformamide was used as the blank. Amounts of secreted mucus were quantitated based on the method described by Kakuta *et al.* (1996) and Dawood *et al.* (2015c). Bovine serum albumin (Sigma Aldrich) was used as standard and the soluble protein concentration of mucus samples was determined following the method of Lowry *et al.* (1951).

7.3.7 *Antioxidant enzymes activities and lipid peroxidation*

Serum superoxide dismutase (SOD) activity was measured by the percentage reaction inhibition rate of enzyme with WST (Water Soluble Tetrazolium dye) substrate and

xanthine oxidase as described by Dawood *et al.* (2016a). The catalase (CAT) enzyme activity was performed using spectrophotometric determination of hydrogen peroxide (H_2O_2) which form stable complex with ammonium molybdate (Goth, 1991). The serum (50 µl) was added to the 1.0 ml substrate (65 µmol per ml H_2O_2 in 60 mmol l^{-1} phosphate buffer, pH 7.4) and incubated for 60 s at 37 °C. The enzymatic reaction was stopped with 1.0 ml of 32.4 mmol l^{-1} ammonium molybdate ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$). A yellow complex of ammonium molybdate and hydrogen peroxide was formed. The absorbance of this yellow colour was measured at 405 nm with a spectrophotometer (HACH, DR/4000U) against a blank (serum was replaced with distilled water). One-unit CAT decomposes 1 µmol of hydrogen peroxide per minute under assay conditions. CAT activities are expressed as kilo unit per liter. The malondialdehyde (MDA) concentration was used as a marker of lipid peroxidation in fish serum and measured using Colorimetric TBARs Microplate Assay Kit (Oxford Biomedical Research, Inc., USA) according to the manufacturer's instructions. The absorbance was measured at 532 nm. The MDA level was expressed as nmol per ml serum.

7.3.8 *Low salinity stress test*

Low salinity stress test was conducted to determine the lethal time of 50% mortality (LT_{50}) in fresh water according to Dawood *et al.* (2015b). Total 15 fish per treatment (5 fish per each tank) were randomly selected and transferred into a 20-L rectangular glass aquarium equipped with continuous aeration and kept under ambient temperature during the stress test. The city water was de-chlorinated by strongly aerating for at least 24 h and mixed with seawater, and then used as low salinity water.

7.3.9 *Statistical analysis*

Statistical analysis was performed with analysis of variance (ANOVA) using a program (Super ANOVA, Abacus Concepts, Berkeley, California, USA). Two-way ANOVA was used to test the effects of dietary LP and VC levels, and their interactions excluding the control group. The data from each treatment group were compared to the control group using the Tukey–Kramer test (one-way ANOVA). Differences among treatments were considered significant when $P < 0.05$.

7.4 Results

7.4.1 *Growth and nutrient utilization parameters*

Growth performances and nutrients utilization of red sea bream fed LP and VC supplements for 56 days are given in Table 7.2. In two-way ANOVA, dietary LP, VC supplementations and the interaction were all significant factors on the final body weight (FNW) and body weight gain (WG) ($P < 0.05$). Furthermore, dietary LP and VC supplementations were both significant factors on specific growth rate (SGR), feed efficiency ratio (FER) and protein efficiency ratio (PER). Only dietary LP was a significant factor on survival rate (SUR) of red sea bream fed test diets for 56 days. However, dietary LP, VC supplementations and interaction were not significant factors on the condition factor (CF) of fish fed with various diets. Compared to the control group (LP0/VC0), fish fed diets supplemented with LP and VC (LP0/VC0.5, LP1/VC0.5, LP0/VC1 and LP1/VC1 diets) showed a significant higher FNW, WG and SGR ($P < 0.05$; Table 7.2). Additionally, fish fed diets supplemented with VC at 0.5 and 1 g kg^{-1} diet combined with LP at 1 g kg^{-1} (LP1/VC0.5 and LP0/VC1 diets) had significant higher FER and PER than the control

group ($P < 0.05$). Similarly, fish fed LP1/VC0.5, LP0/VC1 and LP1/VC1diets showed significantly higher survival rate than LP0/VC0 group ($P < 0.05$).

7.4.2 *Hematocrit and blood biochemical parameters*

The hematocrit and blood chemical parameters in red sea bream are presented in Table 8.3. No significant effect of both LP and VC supplements was found on glucose (GLU), total bilirubin, blood urea nitrogen, glutamyl oaloacetic transaminase (GOT), glutamic pyruvate transaminase (GPT), triglycerides and total cholesterol (T-CHO) of test fish ($P > 0.05$; Table 7.3). Dietary VC and interaction were significant factors on blood hematocrit levels (HCT). However, only dietary LP was a significant factor on plasma T-CHO of red sea bream. HCT was significantly enhanced in fish fed LP1/VC1 diet when compared with fish fed the control diet ($P < 0.05$). Similarly, GLU and T-CHO levels of fish fed with LP1/VC0.5 diet were significantly higher than that of the control group ($P < 0.05$). Fish fed with LP1/VC1 diet got higher plasma TG than those of fish fed LP0/VC0 diet.

7.4.3 *Serum and mucosal immune responses*

In two-way ANOVA, dietary LP, VC supplementations and the interaction were all significant factors on the serum bactericidal activity (BA), serum and mucus lysozyme activities (LZY), mucus peroxidase activity (PA) and nitro blue tetrazolium activity (NBT) of red sea bream fed with various diets ($P < 0.05$; Table 7.4; Fig. 7.2). Additionally, dietary LP and VC supplementations were both significant factors on serum PA. Only dietary LP was a significant factor on mucus BA and alternative complement pathway (ACP) of red sea bream fed test diets for 56 days (Table 7.4; Fig. 7.1). Compared to the control group (LP0/VC0), fish fed LP1/VC0.5 and LP1/VC1 diets exhibited a significant serum (BA, LZY, PA, ACP and NBT) and mucus (BA, LZY and PA) activities ($P < 0.05$; Table 7.4;

Fig. 7.1; Fig. 7.2). Moreover, fish fed diets supplemented with VC at 0.5 g kg^{-1} diet (LP0/VC0.5) showed significantly higher serum LZY activity than the control group ($P < 0.05$; Table 7.4). Similarly, fish fed LP1/VC0.5, LP0/VC1 and LP1/VC1 diets showed significantly higher NBT activity than the control group ($P < 0.05$; Fig. 7.2).

7.4.4 *Amounts of secreted mucus*

In two-way ANOVA, dietary LP, VC supplementations and the interaction were all significant factors on the amounts of mucus secretion on body surface of red sea bream fed test diets (Fig. 7.3). Interestingly, the amount of mucus secretion in fish fed LP1/VC0.5 and LP1/VC1 diets were significantly higher than that in the fish fed the control diet ($P < 0.05$).

7.4.5 *Oxidative status and lipid peroxidation*

A two-way ANOVA test showed that the specific activities of antioxidant enzymes and lipid peroxidation levels [superoxide dismutase (SOD), catalase (CAT) and malondialdehyde (MDA)] were significantly affected by both LP and VC (Table 7.5), and a significant interaction was found between LP and VC on CAT activity. SOD, CAT and MDA activities were significantly enhanced in fish fed LP1/VC0.5 and LP1/VC1 diets when compared with the fish fed the control diet ($P < 0.05$). Moreover, fish fed LP0/VC0.5 diet showed higher SOD activity significantly than fish fed LP0/VC0 diet ($P < 0.05$).

7.4.6 *Low salinity stress test*

The results of lethal stress test against low salinity water test on LT_{50} obtained by regression analysis are shown in Fig. 7.4. LT_{50} was significantly affected by both VC and interaction factors ($P < 0.05$). Significantly higher ($P < 0.05$) LT_{50} value was obtained in LP1/VC0.5 group when compared to the control group.

Table 7.2: Growth performance and feed utilization parameters of red sea bream fed diets containing HK-LP and VC for 56 days*.

Test diets (LP/VC, g kg^{-1})	FNW (g)[1]	WG (%)[2]	SGR (% day^{-1})[3]	FER[4]	PER[5]	SUR (%)[6]	CF (%)[7]
LP0/VC0	15.77±0.14	692.72±6.2	3.69±0.02	1.07±0.05	2.11±0.2	93.33±1.67	1.56±0.13
LP0/VC0.5	17.42±0.4*	793.61±29.76*	3.91±0.06*	1.21±0.03	2.42±0.08	98.33±1.67	1.69±0.11
LP1/VC0.5	22.88±0.35*	1072.22±15.9*	4.4±0.02*	1.47±0.02*	2.92±0.07*	100±0.00*	1.66±0.08
LP0/VC1	19.12±0.11*	865.98±9.73*	4.05±0.02*	1.22±0.02	2.41±0.06	100±0.00*	1.68±0.06
LP1/VC1	25.54±0.52*	1202.49±24.48*	4.58±0.03*	1.57±0.02*	3.12±0.04*	100±0.00*	1.69±0.06
Two-way ANOVA (*P-value*)							
LP	0.0001	0.0001	0.0001	0.0003	0.0012	0.0077	NS
VC	0.0001	0.0001	0.0001	0.0004	0.001	NS	NS
LP×VC	0.013	0.0183	NS[8]	NS	NS	NS	NS

*Values are expressed as mean ± SE from triplicate groups (*n*=60). Data with an asterisk in a column are significantly different from those of control group (D1) ($P < 0.05$). D1 is not included in two-way ANOVA.

[1] FNW: final weight, [2] WG: percent weight gain, [3] SGR: specific growth rate, [4] FER: feed efficiency ratio, [4] PER: protein efficiency ratio, [6] SUR: survival, [7] CF: condition factor, [8] NS: no significant difference.

Table 7.3: Hematocrit and blood chemistry parameters in juvenile red sea bream fed test diets for 56 days*.

Test diets (LP/VC, g kg^{-1})	HCT (%)[1]	GLU (mg dl^{-1})[2]	T-BIL (mg dl^{-1})[3]	BUN (mg dl^{-1})[4]	GOT (IU L^{-1})[5]	GPT (IU L^{-1})[6]	TG (mg dl^{-1})[7]	T-CHO (mg dl^{-1})[8]
LP0/VC0	35±0.58	96.33±5.33	0.53±0.15	5.67±0.33	136.33±24.92	<10	312.67±5.49	263.33±11.89
LP0/VC0.5	36.67±1.77	79.33±9.14	0.43±0.09	6.67±0.88	151.67±40.43	<10	280.33±52.59	246.33±14.45
LP1/VC0.5	37±0.58	71±2.31*	0.56±0.15	5.67±0.33	112±15.28	<10	288.67±18.48	209±7.55*
LP0/VC1	35±1	85±4.51	0.4±0.06	5.67±0.33	159.67±26.34	<10	323±15.95	275.67±6.69
LP1/VC1	40.33±0.88*	79.33±3.76	0.6±0.06	5.67±0.67	143±27.62	<10	252.33±18.22*	217.33±11.98
Two-way ANOVA (*P-value*)								
LP	NS[9]	NS	NS	NS	NS	NS	NS	0.002
VC	0.0007	NS	NS	NS	NS	NS	NS	NS
LP×VC	0.0024	NS	NS	NS	NS	NS	NS	NS

*Values are expressed as mean ± SE from triplicate groups (*n*=15). Data with an asterisk in a column are significantly different from those of control group (D1) ($P < 0.05$). D1 is not included in two-way ANOVA.

[1] HCT: hematocrit, [2] GLU: glucose, [3] T-BIL: total bilirubin, [4] BUN: blood urea nitrogen, [5] GOT: glutamyl oxaloacetic transaminase, [6] GPT: glutamic pyruvate transaminase, [7] TG: triglyceride, [8] T-CHO: total cholesterol, [9] NS: no significant difference.

Table 7.4: Serum and mucus immune responses of red sea bream fed test diets for 56 days*.

	Serum			Mucus		
Test diets (LP/VC, g kg^{-1})	BA (%)[1]	LZY (unit ml^{-1})[2]	PA (OD at 40 nm)[3]	BA (%)	LZY (unit ml^{-1})	PA (OD at 40 nm)
LP0/VC0	40.26±1.62	25.25±0.66	1.24±0.01	30.63±8.45	30.17±0.33	1.54±0.08
LP0/VC0.5	42.99±3.03	30.33±1.02*	1.21±0.18	37.75±10.27	31.17±0.8	1.46±0.09
LP1/VC0.5	67.26±3.43*	40±0.76*	1.57±0.03*	71.87±2.69*	40.46±1.66*	2.06±0.04*
LP0/VC1	43.42±0.9	27.92±1.52	1.29±0.08	53.31±1.46	30.92±0.58	1.554±0.11
LP1/VC1	69.57±3.09*	41.5±2.1*	1.94±0.06*	83.52±1.11*	50.75±1*	2.21±0.07*
Two-way ANOVA (*P-value*)						
LP	0.0005	0.0004	0.0001	0.0032	0.0009	0.0017
VC	0.0007	0.0001	0.0438	NS	0.0007	0.0135
LP×VC	0.0027	0.01	NS	NS	0.0024	0.0015

*Values are expressed as mean ± SE from triplicate groups (*n*=15). Data with an asterisk in a column are significantly different from those of control group (D1) ($P < 0.05$). D1 is not included in two-way ANOVA.

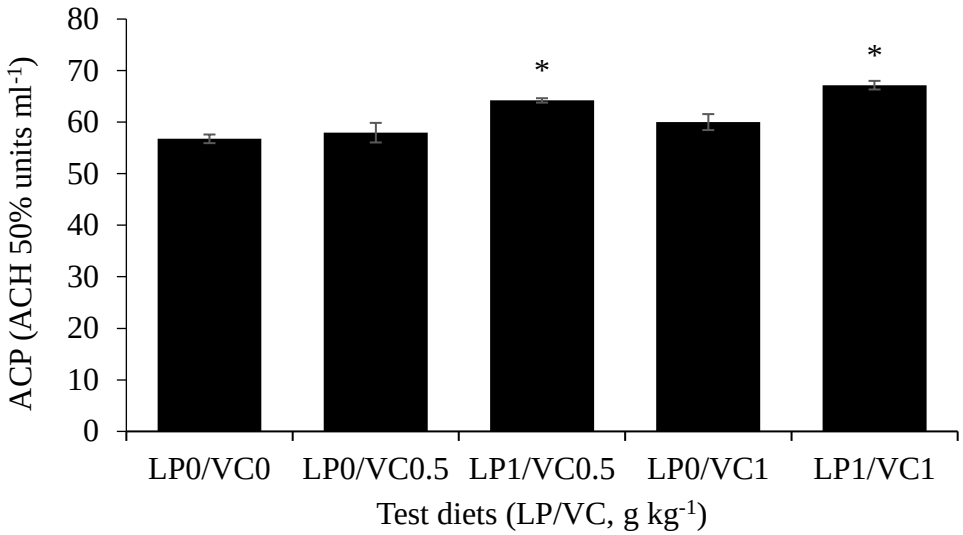
[1] BA: bactericidal activity, [2] LZY: lysozyme activity, [3] PA: peroxidase activity, [4] NS: no significant difference.

Table 7.5: Specific activities of antioxidant enzymes and lipid peroxidation levels of red sea bream fed test diets for 56 days[*].

Test diets (LP/VC, g kg^{-1})	SOD (%)[1]	CAT (KU L^{-1})[2]	MDA (nmol ml^{-1})[3]
LP0/VC0	236.51±5.28	26.22±2.91	11.92±1.44
LP0/VC0.5	264.98±6.25[*]	29.14±2.91	11.52±0.89
LP1/VC0.5	343.56±9.59[*]	55.37±2.91[*]	6.02±0.09[*]
LP0/VC1	269.93±21.54	32.05±5.05	10.31±0.69
LP1/VC1	356±3.52[*]	81.59±7.7[*]	5.73±0.32[*]
Two-way ANOVA (*P-value*)			
LP	0.002	0.002	0.0046
VC	0.0035	0.0063	0.0336
LP×VC	NS	0.0212	NS

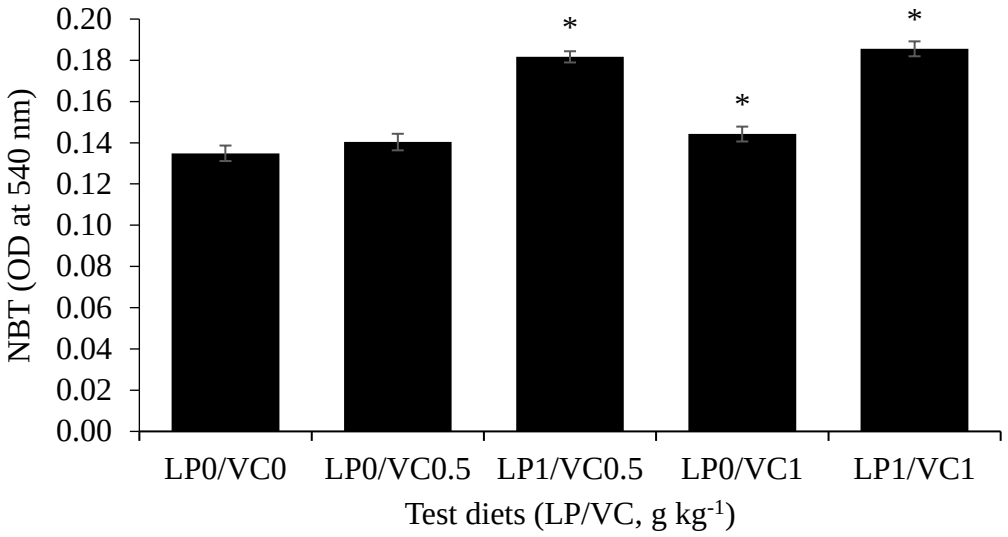
[*]Values are expressed as mean ± SE from triplicate groups (n=15). Data with an asterisk in a column are significantly different from those of control group (D1) ($P < 0.05$). D1 is not included in two-way ANOVA.

[1] SOD: superoxide dismutase, [2] CAT: catalase, [3] MDA: malondialdehyde concentration, [4] NS: no significant difference.



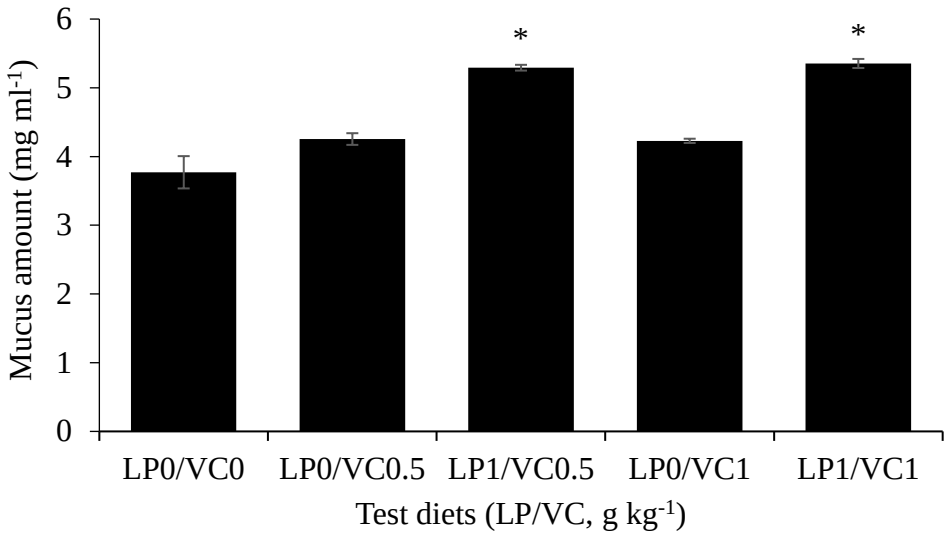
Two-way ANOVA (*P-value*)	
LP	0.0067
VC	NS
LP×VC	NS

Fig. 7.1: Alternative complement pathway (ACP) of red sea bream fed test diets for 56 days. Values are expressed as mean ± SE from triplicate groups (*n*=15). Bars with an asterisk are significantly different from those of control group (D1) ($P < 0.05$). D1 is not included in two-way ANOVA. NS means no significant difference.



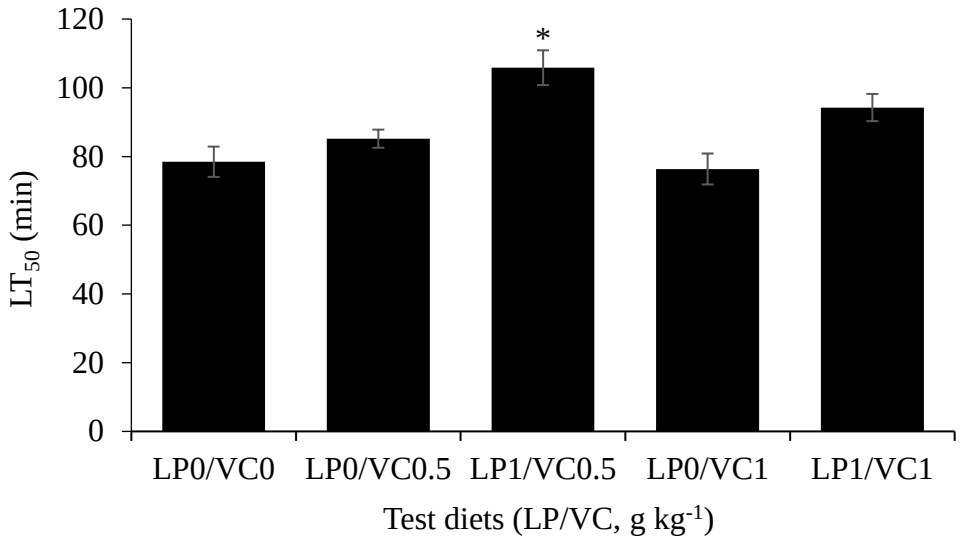
Two-way ANOVA (*P-value*)	
LP	0.0001
VC	0.0001
LP×VC	0.0001

Fig. 7.2: Nitro blue tetrazolium (NBT) activity of red sea bream fed test diets for 56 days. Values are expressed as mean ± SE from triplicate groups (*n*=15). Bars with an asterisk are significantly different from those of control group (D1) ($P < 0.05$). D1 is not included in two-way ANOVA.



Two-way ANOVA (*P-value*)	
LP	0.0004
VC	0.0003
LP×VC	0.0491

Fig. 7.3: Amounts of mucus secretion on body surface of red sea bream fed test diets for 56 days. Values are expressed as mean ± SE from triplicate groups (*n*=15). Bars with an asterisk are significantly different from those of control group (D1) ($P < 0.05$). D1 is not included in two-way ANOVA.



Two-way ANOVA (*P-value*)	
LP	NS
VC	0.0028
LP×VC	0.0281

Fig. 7.4: Time to 50% mortality (LT_{50}) of red sea bream exposed to low salinity water. Values are expressed as mean ± SE from triplicate groups (*n*=15). Bars with an asterisk are significantly different from those of control group (D1) ($P < 0.05$). D1 is not included in two-way ANOVA. NS means no significant difference.

7.4 Discussion

The main challenge facing red sea bream culture is the improvement of immune response and performance through the development of health-promoting diets (Dawood *et al.*, 2016a; Dawood *et al.*, 2015a,b,c,e). Recently, probiotics and vitamin C exhibited various beneficial features particularly highlighted by its capability in modulating the physiological responses of fish that resulted in improved growth and disease resistance (Dawood *et al.*, 2016a; Roosta *et al.*, 2014; Chen *et al.*, 2015; Shahkar *et al.*, 2015). However, there is little information in the literature about their interactive effects on red sea bream. In this regard, the effect of dietary probiotic (LP) and VC in the single and combined forms on some biological indices was discussed below.

In the present study, it is clear that the improvements in growth performance and feed utilization were a function of the LP and VC supplementation in the diets of red sea bream. The growth improving property of *Lactobacillus* sp. has previously been reported in red sea bream (*P. major*) (Dawood *et al.*, 2016a; Dawood *et al.*, 2015a,b), rainbow trout (Rodriguez-Estrada *et al.*, 2013), gilthead sea bream (*Sparus aurata*) (Suzer *et al.*, 2008), and olive flounder (*Paralichthys olivaceus*) (Beck *et al.*, 2015). Evidence is available that indicates beneficial bacterial cells take part in the decomposition of nutrients, improve digestive activity by enhancing the synthesis of vitamins, enzymatic activity (Suzer *et al.*, 2008; Safari *et al.*, 2016; Taoka *et al.*, 2006a), and thus facilitate feed utilization and digestion. It is well documented that, VC is an essential nutrient needed to maintain the normal physiological functions of fish (Chen *et al.*, 2015; Liu *et al.*, 2011). Growth enhancing effects of VC on red sea bream have not been reported previously to the authors' knowledge but enhanced growth performance and feed utilization were reported recently

in Caspian roach (*Rutilus rutilus* caspicus) fry (Roosta *et al.*, 2014), large yellow croaker (*Pseudosciaena crocea*) (Ai *et al.*, 2007), Jian carp (*Cyprinus carpio* var. Jian) (Liu *et al.*, 2011), and cobia (*Rachycentron canadum*) (Zhou *et al.*, 2012). However, dietary administration of VC failed to improve the growth performance of gilthead sea bream (*S. aurata*) (Henrique *et al.*, 1998). Data from this study showed that there was a synergistic effect of LP and VC included in the experimental diets on the growth performance and some biological indices. A likely explanation of this finding may be attributed to the structure–function relationship of LP and VC in order to modulate beneficial microbiota of gastrointestinal tract (GIT) in aquatic species (Das *et al.*, 2014; Khonyoung and Yamauchi, 2012; Liu *et al.*, 2011; Ray *et al.*, 2012). Overall improvement of feed efficiency may be related to enhanced secretion and activity of digestive pancreatic and intestinal enzymes, leading to better feed utilization.

Blood parameters serve as reliable indicators of fish health, and they can vary with season, temperature and nutritional state (Dawood *et al.*, 2016a; Dawood *et al.*, 2015a,b; Ren *et al.*, 2008; Ren *et al.*, 2007). In the present study, dietary supplementation of LP and VC significantly enhanced hematocrit of red sea bream as a general health response. Increased hematocrit levels have also been observed also in red sea bream and rainbow trout fed heat killed probionts (Dawood *et al.*, 2016a; Dawood *et al.*, 2015a,b; Rodriguez-Estrada *et al.*, 2013). Similarly, it has been reported that VC could affect the hematocrit levels of Carp (*Cirrhinus mrigala*) (Zehra and Khan, 2012), Giant catfish (*Pangasianodon gigas*) (Pimpimol *et al.*, 2012) and Indian major carp (*Labeo rohita*) (Tewary and Patra, 2008). Plasma T-BIL, GOT and GPT are often used for the evaluation of liver function as they are released into the blood during injury or damage to the liver cells (Dawood *et al.*, 2016a;

Dawood *et al.*, 2015a,b,c,e). In the current study, neither dietary LP nor VC was a significant factor on blood chemistry profile including (GLU, T-BIL, BUN, GOT, GPT and TG), except for plasma T-CHO content which has been affected significantly by only dietary LP supplementation. Moreover, supplementation of both LP and VC exhibited decreased GLU, TG and T-CHO values when compared with the control group. Plasma GLU concentration is one of the stress indicators in fish, high glucose level often indicates the higher stress status of fish (Eslamloo *et al.*, 2012). In agreement with the current findings, plasma GLU, TG and T-CHO have been reported to decrease after LP or VC supplementation in red sea bream (Dawood *et al.*, 2016a; Dawood *et al.*, 2015b; Pimpimol *et al.*, 2012; Ren *et al.*, 2008). Results obtained from several studies suggested a moderate cholesterol-lowering action of dairy products fermented with probiotic strains (Dawood *et al.*, 2015a,b; Falcinelli *et al.*, 2015). Among the reasons, probiotic bacteria ferment food-derived indigestible carbohydrate to produce short chain fatty acids in the gut, which can then cause a decrease in the systemic levels of blood lipids by inhibiting hepatic cholesterol synthesis and/or redistributing cholesterol from plasma to the liver (Falcinelli *et al.*, 2015). Furthermore, possible modulation of the expression of genes involved in lipid metabolism may also have contributed towards lowered plasma lipids by probiotic supplementation (Falcinelli *et al.*, 2015).

It has been reported that, probiotics and VC are modulators of immune responses in fish as indicated by the significant changes in the different immunological parameters following probiotic and VC feeding (Guerreiro *et al.*, 2015; Ming *et al.*, 2012; Ren *et al.*, 2007; Safari *et al.*, 2016). All the studied molecules in the present work were conducted in both serum and skin mucus of red sea bream including: bactericidal (BA), peroxidase (PA), lysozyme

(LZY), alternative complement pathway (ACP), nitro blue tetrazolium (NBT) activity and the amount of mucus secretion. Interestingly, some of them are present in the skin mucus in greater levels than in the serum indicating the efficiency of LP or/and VC on the skin mucus. Various peptides such as LZY, antibodies, complement factors and other lytic factors are present in serum acts as a first line defense to prevent adherence and colonization of microorganisms leading to prevention of infection and disease (Guardiola *et al.*, 2014). The alternative complement pathway has an essential role in alerting the host with the presence of potential pathogens and their eventual clearance (Safari *et al.*, 2016). In the current study, alternative complement system of red sea bream was significantly impacted by LP and VC applications, which seems to be a clear tendency that LP and VC are modulators of the complement system in red sea bream. Lysozyme is an important humoural component of the immune system and act as defensive factor against invasive microorganisms in fish (Guardiola *et al.*, 2014). Lysozyme disrupts bacterial cell walls by splitting glycosidic linkages in the peptidoglycan layers (Pan *et al.*, 2008). The PA is an important enzyme that utilizes oxidative radicals to produce hypochlorous acid to kill pathogens. During oxidative respiratory burst, it is mostly released by the azurophilic granules of neutrophils (Diaz-Rosales *et al.*, 2006). Since peroxidases are important microbicidal agents that effectively eliminate H_2O_2 and maintain the redox balance of immune system, it is tempting to consider that peroxidase in serum and mucus will be essential for humoral, mucosal immunity (Guardiola *et al.*, 2014). Many authors have reported the enhancement of LZY, ACP, PA, and BA due to administration of dietary LP and VC in several fish species (Dawood *et al.*, 2016a; Dawood *et al.*, 2015a,b; Nayak *et al.*, 2007; Rodriguez-Estrada *et al.*, 2013; Ren *et al.*, 2008; Shahkar *et al.*, 2015). Results

of the current study illustrated that dietary administration of LP or/and VC enhanced humoral immune parameters, including BA, LZY, PA, ACP and NBT. Similarly, the health promoting effects of dietary combined probiotics and VC have been reported to stimulate the innate immune responses in Indian major carp (*L. rohita*) (Nayak *et al.*, 2007). Dawood *et al.* (2015b) reported that, single supplementation of dietary LP significantly activated the humoral immune response of red sea bream (*P. major*). Similarly, Ren *et al.* (2008) reported that red sea bream (*P. major*) fed diets supplemented with VC obtained an improved humoral immune response by increasing total serum protein, LZY and BA. More recently, Shahkar *et al.* (2015) reported that Japanese eel (*Anguilla japonica*) fed diets supplemented with VC also enhanced serum LZY, PA and NBT. The ability of probiotic cells as well as their components to enhance immunity in fish may be due to the interaction between probiotic cells and intestinal epithelial cells which controls the physical as well as immunological barrier property of intestine through several mechanisms such as tightening the epithelial junction, production of antimicrobial peptides, production of mucosal immunoglobulin and modulating inflammatory reaction, which then triggers a cascade of reactions leading to an immune response stimulation (Safari *et al.*, 2016; Shida and Nanno, 2008; Thomas and Versalovic, 2010; Wells, 2011). VC has been also identified as a good stimulator of immune response (Shahkar *et al.*, 2015). The role of VC as an immunomodulator was emphasized by the presence of intracellular VC in leukocytes at concentrations related to dietary levels (Verlhac and Gabaudan, 1994; Levy *et al.*, 1996). From the immunological point of view, very few efforts have been made in order to evaluate the mucosal responses since they are in direct contact with different environmental stressors and the first line of defense (Guardiola *et al.*, 2014). As a part of innate immunity,

the skin layer is the first line of defense against stressors, plays a frontier role in protecting fish against infections and the skin mucus contains many kinds of biologically active (including defensive) molecules (Ellis, 1999; Fast *et al.*, 2002; Huang *et al.*, 2011; Murty *et al.*, 1984). In this sense, several studies have demonstrated that the presence and activity of immune factors in the skin mucus depends on the environmental conditions and the fish species (Hikima et al., 1997; Subramanian et al., 2008). We carried out this study taking in consideration the efficiency of LP or/and VC supplementations on the skin mucus as the first line of defense and the lack of knowledge in the case of the red sea bream. Results of the current study revealed that red sea bream fed diets supplemented with LP or/and VC showed higher skin mucus BA, LZY and PA activities than the control group, and a significant interaction was found between LP and VC. In agreement with the obtained results, Dawood *et al.* (2016a,2015a,b) found increased mucus BA, LZY and PA activities in red sea bream fed diets supplemented with live and heat killed probionts. Similar to our results, the administration of VC in red sea bream diet significantly increased the LZY and BA activities of skin mucus (Ren et al. 2008). Also, Roosta *et al.* (2014) reported an increase in the mucus Caspian roach (*R. rutilus* caspicus) fry in response to VC supplementation. Results of the present study revealed that, the amount of mucus secretion in fish fed LP and VC was higher than that in the control group, which together with other parameters (mucus BA, LZY and PA activities), implied an improved immune response of the fish fed with supplemented diets. Moreover, the increase of mucus secretion in fish fed with LP and VC can be possibly explained on the basis of increased immune response. Previous research efforts have indicated the importance of the use of immunostimulants for enhancing mucus production in fish. For example, the capacity of heat inactivated

probionts, mannan-oligosaccharide (MOS), β-glucan (BG) and VC to modify the epidermal mucus production in fish (Dawood *et al.*, 2015a,b,c,e; Rodriguez-Estrada *et al.*, 2013; Roosta *et al.*, 2014). Although the mechanisms underlying the effects of dietary supplements such as probiotics and VC on skin mucus protein levels still need to be clarified in future studies, Taoka *et al.* (2006b) stated that elevation of mucus protein contents is an indicator of the amount of skin secretion and increased protein contents represent the possibility of the best responses to different stressors. The elevation of skin mucus protein levels in the present study possibly indicates LP and VC supplementation in the diet contributes to enhancing such defense mechanisms in red sea bream.

During phagocytosis macrophages and neutrophils produce reactive oxygen species (ROS) such as superoxide anions, hydrogen peroxide and hydroxyl free radical, in a mechanism called respiratory burst activity (Murty *et al.*, 1984; Storey, 1966). Besides respiratory burst, free radicals and ROS are continuously being generated as products of cell oxidative metabolism in fish (Martinez-Alvarez *et al.*, 2005; Storey, 1966). ROS are highly bactericidal due to their oxidizing activity (Li *et al.*, 2008; Martinez-Alvarez *et al.*, 2005). However, when ROS production is higher than ROS removal oxidative stress occurs. Fish as other aerobic organisms are also susceptible to ROS attack and developed antioxidant defenses based on substances such as vitamins C and E, glutathione, and carotenoids, besides a complex antioxidant enzymes system (Martinez-Alvarez *et al.*, 2005; Storey, 1966). Serum SOD and CAT are common antioxidant enzymes that can protect organism against damnification by ROS which may lead to many disorders through attack macromolecules (Li *et al.*, 2008; Seifried *et al.*, 2007). MDA is formed as a normal product of lipid peroxidation but overdose MDA can damage cell structure and function (Sies,

1997). Therefore, the antioxidant status in fish can be accurately reflected by the activity of SOD, CAT and MDA. In the present study, the serum CAT and SOD enzymes were significantly influenced by a certain dietary LP and/or VC supplementations. The MDA values of fish fed both LP and VC (LP1/VC0.5 and LP1/VC1 groups) were significantly decreased. These results demonstrated that dietary LP and VC have a positive effect on the antioxidant capacity of red sea bream. The compound increased the antioxidant capacity of red sea bream might by increasing the antioxidant enzymes to decrease the ROS and reducing the MDA contents. Similar studies have demonstrated that heat killed probionts and VC could enhance the antioxidant activity of red sea bream (Dawood *et al.*, 2015a,b; Gao *et al.*, 2013; Gao *et al.*, 2014).

Salinity stress tests have often been used as a final indicator to express the changes in the immune defenses of fish following immunostimulants feeding (Dawood *et al.*, 2015a,b); our results revealed that dietary LP and VC significantly increased red sea bream resistance to low salinity stress. Fish fed LP1/VC0.5 diet showed remarkable tolerance against low salinity stress (LT_{50}) compared to the control group. Increased LT_{50} of red sea bream fed LP and VC supplemented diets indicated improved immune response of red sea bream. Similarly, red sea bream fed probiotics and prebiotics showed greater survival after salinity stress challenge (Dawood *et al.*, 2015a,b). It is interesting to highlight that LP1/VC0.5 was the group that demonstrated significant modulation in their immune responses, especially the BA, LZY, PA, ACP and NBT. These collective observations posit that LP and VC stimulated the different defense factors of the host thus making the fish more resistant to the low salinity stress. However, future studies are required to test this speculative hypothesis.

Interestingly, in the LP and VC combined groups the level of various immune and antioxidant parameters was significantly enhanced compared to the individual LP and VC treated groups. This might be due to the enhanced establishment of the LP in the gastrointestinal tract of red sea bream because the administration of VC along with probiotic bacterium or its products might have promoted the growth of other gut bacteria, which utilized VC efficiently.

Chapter VIII: General Discussion

8. General discussion

Both red sea bream, *Pagrus major* and amberjack, *Seriola dumerili* are important cultured species in the world because of their high market value and demand. Especially in recent years, red sea bream and amberjack aquaculture have developed rapidly and widely in China, Japan and Korea (Dawood *et al.*, 2015a, b, c and d). However, fish grown in intensive aquaculture systems are often exposed to stressful conditions which have a negative impact on their growth and immunity (Wang *et al.*, 2008a, b). Moreover, the application of antibiotics and chemotherapeutics to control these diseases caused many other problems such as the spread of drug resistant pathogens, suppression of aquatic animal's immune system, environmental hazards and food safety problems (Bachère, 2003). Increasing with the demand for environment friendly aquaculture, works on finding out the alternatives for the antibiotics are urgently needed (Miranda and Zemelman, 2002). Recently, several studies have demonstrated that functional feed additives can improve growth performance, feed utilization, digestibility of dietary ingredients, disease resistance and immunostimulation of aquatic animals (Balca´zar *et al.*, 2006; Diaz-Rosales *et al.*, 2009; Sáenz de Rodrigáñez *et al.*, 2009; Ferguson *et al.*, 2010; Merrifield *et al.*, 2011; Xing *et al.*, 2013; Wang, 2011). Probiotics, prebiotic and other additives, are sustainable ways to manage gut microflora, fish performance and modulate the health of farmed animals.

Probiotics have been defined as live microorganisms that impart positive effects on a host animal by improving the microflora of its gastrointestinal tract (GIT) (Fuller, 1989). Currently, probiotics are defined as the metabolites of live or dead bacterial cells, which function as immunostimulants for modification of enzyme activity or microflora in gastrointestinal tracts (Naidu *et al.*, 1999; Salminen *et al.*, 1999). Some concerns may arise

in fish aquaculture due to the oral delivery of probiotics, which may introduce live bacteria into the environment. Consequently, the use of inactivated bacteria, which would no longer interact with the aquatic environment, was considered (Salinas *et al.*, 2008). Dietary supplementation of several inactivated beneficial bacteria improved the growth performance, immunity and disease resistance of fish (Irianto and Austin, 2003; D´ıaz-Rosales *et al.*, 2006; Rodriguez-Estrada *et al.*, 2013; Salinas *et al.*, 2006; Choi *et al.*, 2008; Pan *et al.*, 2008; Panigrahi *et al.*, 2010).

Prebiotics, on the other hand, are indigestible substances that allow specific changes in the composition and/or activity of gastrointestinal microbiota, which has a positive effect on the nutrition and health status of the host (Song *et al.*, 2014). Previous research efforts have demonstrated the benefits of prebiotics on growth, physiological status and immune responses (Merrifield *et al.*, 2010; Song *et al.*, 2014).

The main beneficial effects of probiotics in fish farming are improvements of growth performances, immunity and tolerance against different stressors (Balca´zar *et al.*, 2006; Irianto and Austin, 2003; Gatesoupe *et al.*, 2007; Lara-Flores, 2011; Tung *et al.*, 2010; Rodriguez-Estrada *et al.*, 2013; Wang *et al.*, 2008a, 2011). In the present research, a higher growth performance and a noticeable increase in nutrient utilization of protein were observed in the experimental groups. These results are in accord with previous studies that demonstrated the application (with different bacteria species and levels) of bacterial cells improves feed conversion and growth rates of Rainbow Trout *Oncorhynchus mykiss* (Bagheri *et al.*, 2008; Rodriguez-Estrada *et al.*, 2013), Cobia *Rachycentron canadum* (Xing *et al.*, 2013), Tilapia *Oreochromis niloticus* (Standen *et al.*, 2013) and grouper *Epinephelus coioides* (Huang *et al.*, 2014). The fish intestine is the direct organ for digestion, absorption

and immunity, as the gut microflora is continuously exposed to bacterial preparations, the latter was thought to affect the production of extracellular enzymes by the microflora within the gastrointestinal (GI) tract of fish, which in turn improves nutrient absorption and utilization (Kesarocodi-Watson *et al.*, 2008; Lara-Flores, 2003; Gatesoupe, 2007; Standen *et al.*, 2013; Tovar-Ramirez *et al.*, 2010, Wang *et al.*, 2008b). It is well known that growth response was strongly related to improved feed utilization. Our results suggested that amongst the tested fish that utilized experimental diets effectively, there were higher feed intake (FI), feed efficiency ratio (FER) and protein efficiency ratio (PER) in fish fed supplemented diets. The increased FI, FER and PER would explain the significant increased growth performance in this group (Dawood *et al.*, 2015a, b, c and d).

Measurements of plasma constituent levels not only are easier compared to immunological parameters or pathogen-challenge trials for evaluating disease resistance and health condition but also can be performed without killing the fish (Panigrahi *et al.*, 2010). These parameters were altered in the current study by various supplementations; these can be explained by their convinced functions in aqua-feed.

In the present study, both dietary probiotic and prebiotic levels enhanced immune parameters, including serum lysozyme activity, serum bactericidal activity, peroxidase content and total serum protein. Moreover, significant interactions between probiotic and prebiotic on the measured immune parameters were observed in the present study. The exact mechanism by which beneficial microorganism cells as well as their components enhance immunity in fish still remains unclear. It is believed that interaction between beneficial bacterial cells and intestinal epithelial cells plays an important role in host gut mucosal immunity which controls the physical as well as immunological barrier property

of intestine through several mechanisms such as tightening the epithelial junction, production of antimicrobial peptides, production of mucosal immunoglobulin and modulating inflammatory reaction, which then triggers a cascade of reactions leading to an immune response stimulation (Thomas and Versalovic, 2010; Wells, 2011; Liu, 2013; Shida and Nanno, 2008).

Synergistic actions between probiotics and prebiotics have been revealed in studies on rainbow trout (Rodriguez-Estrada *et al.*, 2013), koi (*Cyprinus carpio* koi) (Lin *et al.*, 2012) and ovate pompano (*Trachinotus ovatus*) (Zhang *et al.*, 2014). The similar significant interactions on non-specific immunity responses were observed in the present study.

Vitamin C (VC) is an essential micronutrient for normal growth and physiological function of most aquatic animals (Dawood and Koshio, 2016b). In terrestrial animal nutrition, VC has recently spurred scientific interest because of the increasing knowledge on VC involvement in gene expression, cell cycle and reproduction in mammals, in addition recent advances in molecular biology techniques allow the more effective estimation of the effect of VC on metabolism and physiology. In contrast, this information is scarce in aquatic animal nutrition, as studies have essentially focused on the estimation of minimum VC requirement for maximum growth and immune response as well as for the formulation of least-cost diet. This scarcity of information is also due to the lack of well-established indicators of VC status in aquatic organisms. The present study revealed synergitic effects between probiotics and VC on the performance of cultured red sea bream as a result of the imnunostimulation.

In connection with the previous results, future studies are recommended to assess the gastrointestinal microbiota which could be affected directly by the supplementations.

Future research is also needed in order to further investigate the relation and mechanism of the examined feed additives with the significant increase of non-specific immunity and disease resistance in cultured fish species. Therefore, under the experimental conditions, dietary feed additives can significantly improve growth performance and feed utilization of fish. Further, the present work demonstrated a significant interaction on enhancing blood chemistry and immunological parameters of red sea bream, *Pagrus major*. This might be helpful to those involved in aquaculture research and the fish farming industry.

Summary and Conclusion

9. Summary and Conclusion

There is an increasing evidence that natural feed additives can have beneficial effects on aquaculture animals by supporting well-balanced gut microflora and improving fish health. The results obtained from the present study were as:

1. Heat killed *L. plantarum* (HK-LP) significantly enhanced growth performance and feed utilization of red sea bream.
2. Enhancement of *P. major* antioxidant status and immune responses by HK-LP providing them with higher resistance to the stress.
3. The optimum supplement of dietary HK-LP for *P. major* is 0.1%.
4. Dietary supplements of inactivated *P. pentosaceus* (PP) revealed a significant increase in growth performance and feed utilization of red sea bream.
5. The supplementation of PP Prep improves blood parameters, immune response and general health condition of *P. major*.
6. The highest growth parameters and soundest blood and immune parameters were found in fish fed with PP Prep at1.6×10^{12} and 3.2×10^{12} cells g^{-1}.
7. Red sea bream growth performance, blood chemistry and immune responses were improved by dietary β-glucan (BG).
8. BG supplemented diets enhanced antioxidant status and stress resistance of *P. major.*
9. The optimum supplement of dietary BG for *P. major* is 0.1%.
10. Up to 30% soy bean meal (SBM) substitution level with essential amino acid supplementation did not significantly reduce growth, feed utilization and immune response of amberjack, *S. dumerili*.

11. The addition of HK-LP at 0.1% to diets appeared to improve SBM utilization by amberjack.
12. Red sea bream growth performance, blood chemistry and immune responses were improved by dietary BG.
13. BG supplemented diets enhanced antioxidant status and stress resistance of *P. major*.
14. Interactive function of dietary HK-LP and BG has been detected for red sea bream.
15. Increasing dietary HK-LP and BG can improve growth performance, blood parameters and oxidative status of red sea bream.
16. Feeding 0.025% HK-LP combined with 0.1% BG significantly increased most of the detected immune parameters for red sea bream.
17. Oral administration of *L. rhamnosus* or/and *L. lactis* probiotics have positive effects on the growth, feed utilization, health condition, and immune system of the red sea bream.
18. The multispecies formulation was more effective than any of the single-bacteria experimental diets.
19. The appropriate design of multispecies probiotics can have synergistic positive effects on fish health.
20. The potential of *L. plantarum* (LP) and vitamin C (VC) as an immunostimulants for red sea bream.
21. The prospects of LP and VC as immunostimulants are indicated by their capability in improving growth performance and modulating the innate defenses of fish. In particular, one of the significant benefits identified was the improved resistance against the

oxidative and low salinity stressors which were likely a favorable consequence of the LP and VC influences on the different components of red sea bream immune system.

22. Both LP and VC feeding could be recommended to elevate the growth, immunity and stress resistance of red sea bream (*P. major*). The mechanism of their effects needs to be further studied.

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