Studies on Application of Probiotic *Lactococcus lactis* Strain K-C2 as Fish Feed Additives in Aquaculture

（水産増養殖における養魚飼料添加物としての

プロバイオティクス*Lactococcus lactis* K-C2株の応用に関する研究）

Department of Applied Biological Science

Interdiscriplinary Graduate School of Agriculture and Engineering

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September, 2018

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Abbreviations

AAs	Amino acids
1-MetHis	1-methylhistidine
3-MetHis	3-methylhistidine
GABA	γ-Aminobutyrate
Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
Cit	Citrulline
Cys	Cysteine
Glu	Glutamate
Gln	Glutamine
Gly	Glycine
His	Histidine
HyPro	Hydroxyproline
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Orn	Ornithine
Phe	Phenylalanine
Pro	Proline
Sar	Sarcosine

Ser   Serine

Thr   Threonine

Trp   Tryptophan

Tyr   Tyrosine

Val   Valine

Tau   Taurine

cfu   Colony-forming unit

DGGE   Denaturing gradient gel electrophoresis

FCR   Feed conversion ratio

LAB   Lactic acid bacteria

LC-MS   Liquid chromatography-mass spectrometry

PCR   Polymerase chain reaction

Chapter 1: General Introduction

Introduction

In the last five decades, it should be realized that the global aquaculture production has grown steadily, besides the capture production has a downtrend (FAO, aquaculture 2014). To obtain the high productivity of fisheries which conduced to losses and negative impact to environment such as the mangrove conversion to shrimp ponds result in loss of essential ecosyste. Or the collection of wild seed and brood-stock or catching adult animal to provide eggs for hatcheries of some cultured species which lead to loss substantial number of native stocks. The introductions and transfers of aquatic animals outside natural range to aquaculture purpose effect on the degradation of host environment, genetic degradation of animal and the spread of infectious and parasitic diseases (Primavera 2006).

Obviously, environmental degradation, increase of pathogens and reducing resistant of animal have been cause of outbreak of diseases in aquaculture industry in recent years. Risk of diseases particularly has been described in cultured animals (Lightner 2003; Sakai et al. 2007) and the infected agents in aquatic animal are usually virus, bacteria, fungi and parasites (Lightner and Redman 1998). Several reports showed many economic losses in aquaculture when epidemic disease outbreak, especially infectious diseases (Bondad-Reantaso et al. 2005). Beside that, the intensive aquaculture (shrimp and fish farming) has led to growing problems with bacterial diseases, and the treatment of which requires the intensive use of antimicrobials including disinfectants (e.g., hydrogen peroxide and malachite green), antibiotics (e.g., sulfonamides and tetracyclines) and anthelmintic agents (e.g., pyrethroid insecticides and avermectins). Antibiotis are used to treat bacterial diseases because of their capacity to kill or inhibit the growth of microorganisms. The use of antimicrobial drugs in aquaculture has also differences from their use

in terrestrial animals. In aquaculture, antimicrobials are regularly added to the feed, which is then placed in the water where the fish are kept; in some cases, antimicrobials may be added directly to the water; both of these methods may result the presence of antimicrobial residues in aquaculture products and the environment. On the other hand, several bacterial species may survive unfavorable conditions or environmental changes after selecting mutations to adapt themselves in the new conditions, it is cause of leading to the development and spread of antimicrobial-resistant bacteria and resistance genes. Also, the use of antimicrobials in aquaculture may effect on intestinal microflora of host and environmental bacteria (Holmstrom et al. 2003; Romero et al. 2012).

Recent years, using probiotics or immune-stimulant compounds or some chemicals to disinfectant for enhancement of animal health and prevention of pathogen invasion and friendly with environment have become more common in aquaculture industries as an alternative to antibiotics using in aquaculture. In which, probiotic are gaining increasing scientific and commercial interest and have a quite common place in health-promoting functional foods as well as therapeutic, prophylactic and growth supplements, thus probiotics are opening a new era in the health management strategy from human to aquatic species including fish and shellfish (Bidhan et al. 2014).

What is probiotics?

Following Fuller (1989) the term "probiotic" was defined as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance"; through some applications, probiotic was also indicated as non-viable microbial components that acted with benefit not limited to the intestinal region (Salminen et al. 1999). Probiotics can stimulate the immunity of the host, indicated by increasing phagocytosis, antibody production and superoxide

anion production. It produces several compounds that may inhibit the growth of competing bacteria (*e.g.* bacteriocins, sideropheros, protease …). It can be competitive for nutrients, adhesion sites to prevent and exclusion the pathogenic bacteria in the digestive tract of cultured species (Itami et al. 1998; Sakai 1999; Verschuere et al. 2000; Irianto and Austin 2002; Farzanfar 2006).

Overview on the modes of probiotic action as beneficial microbes in aquaculture

Competition for space

Several bacterial pathogens require attachment to the mucosal layer of the host gastrointestinal tract to initiate the development of a disease. Therefore, probiotic supplementation has been suggested to use in early stage as laviculture for competition adhension sites with pathogenic bacteria. Attachment of probiotics may be non-specific, based on the physicochemical agents, or specific, based on the adhesion of the probiotics on the surface of the adherent bacteria and receptor molecules on the epithelial cells. The ability of bacteria to colonize the gut and adhere to the epithelial surface and consequently interfere with the adhesion of pathogens is a desirable criterion in the selection of probiotics (Zorriehzahra et al. 2016).

Production of inhibitory substances

In several studies for using probiotics in aquaculture, probiotics such as *Leuconostoc mesenteroides*, *Bacillus subtilis*, *Lactococcus lactis*, *Lactobacillus buchneri*, *L. acidophilus*, *L. fermentum*, *L. plantarum*, *Sterptococcus salivarius*, etc. had proven their antibacterial activity towards fish pathogens namely *Aeromonas hydrophila*, *A. salmonicida*, *Yersinia rukeri*, *Vibrio* sp.,

or *Staphylococcus aureus* (Zorriehzahra et al. 2016).

Interestingly, other probiotics including *Pseudomonas*, *Vibrio*, *Aeromonas* spp. and *Coryneforms* had antiviral activity against infectious hematopoietic necrosis virus (IHNV); or feeding with a *B. megaterium* strain increased the resistance to white spot syndrome virus (WSSV) in the shrimp *Litopenaeus vannamei*; or application of *Lactobacillus* probiotics as a single strain or mixed with *Sporolac* improved disease resistance against lymphocystis viral disease in olive flounder (Zorriehzahra et al. 2016).

Beside that, there are few studies regarding the antifungal effect of probiotics even though *A. media* isolated from eel (*Anguilla australis*) have been reported a strong inhibitory activity against *Saprolegnia* sp.; *Pseudomonas* sp. M162, *Pseudomonas* sp. M174 and *Janthinobacterium* sp. M169 enhanced immunity against saprolegniasis in rainbow trout; or *L. plantarum* FNCC 226 exhibited inhibitory activity against *Saprolegnia parasitica* A3 in catfish (*Pangasius hypophthalamus*) (Zorriehzahra et al. 2016).

Competition for available energy and chemicals

Many microorganisms, including the known probiotic group lactic acid bacteria, may have ability to compete for energy and metallic ions that are essential for the growth of a number of pathogens in the same environment. Evidently, siderophore-producing bacteria can sequester ferric iron in an iron-low environment, hence making it unavailable for the growth of pathogenic bacteria; or a culture supernatant of *P. fluorescens*, grown in iron-limited conditions, inhibited growth of *V. anguillarum*; also *P. fluorescens* can competitively inhibit the growth of the fish pathogen *A. salmonicida*, by competing for free iron (Zorriehzahra et al. 2016).

Improving the water quality

Bacillus spp. are well-known as the beneficial bacteria in improving the quality of the water system. Several species of this genus have a more efficient ability in converting organic matter into carbon dioxide in comparison to the Gram-negative bacteria, which converts a greater proportion of organic matter into bacterial biomass or slime. The temperature, pH, dissolved oxygen, NH_3 and H_2S in rearing water were found to be of higher quality when probiotics were added, hence maintaining a positively healthy environment for shrimp and prawn larval in green water system (Zorriehzahra et al. 2016).

Competition for nutrients

There are reports demonstrating that some probiotics have a positive effect in the digestive processes of aquatic animals. These bacteria may contribute in the digestion process by producing extracellular enzymes, such as proteases, lipases, as well as growth- promoting factors. Others are capable of supplying vitamins, fatty acids and essential amino acids to the host. Besides bacterial probiotics, many strains of yeast have been used as dietary supplements in a number of fish species (Zorriehzahra et al. 2016).

Interference of quorum sensing

Many bacteria are using quorum sensing system to communicate, and regulate to physiological activities or cell-population density. Application of this system from bacteria is considered a potential anti-infective strategy in aquaculture. For example, some probiotic bacteria such as *Lactobacillus*, *Bifidobacterium* and *B. cereus* strains degrade the signal molecules of pathogenic bacteria by enzymatic secretion or production of autoinducer antagonists, or *L. acidophilus* was

demonstrated that it was able to secrete a molecule for inhibiting the quorum sensing or interacts with bacterial transcription of *Escherichia coli* O157 gene (Zorriehzahra et al. 2016).

Immunodulation

Probiotics has also been reported as a host immune system stimulants. They may stimulate the pro-inflammatory cytokines on the activity of immune cells, increase the phagocytic activity of leucocytes, increase the levels of antibodies, acid phosphatase, lysozyme, complement cytokines and antimicrobial peptides, or improve the intestinal microbial balance, etc. (Zorriehzahra et al. 2016)

For fish, administration of probiotics (*L. sakei* BK19) with herb (*Scutellaria baicalensis*) in tilapia (*Oreochromis fasciatus*) reduced the mortality, altered haematological parameters and enhanced innate immunity against *Edwardsiella tarda*; also this probiotic improved growth, blood biochemical constituents, and nonspecific immunity of olive flounder (*Paralichthys olivaceus*). In other researches, fish were fed the diet containing *L. plantarum* showed the upregulation of mRNA levels of several immune parameters like cytokine IL-8 in the intestinal fish significantly by *L. plantarum* after *Lc. garvieae* infection. The probiotic *Pediococcus acidilactici* treatment on Nile tilapia (*O. niloticus*) caused upregulation of the gene expression of the pro-inflammatory cytokine TNF-a in the fish group fed probiotic. Or presence of *B. subtilis* C-3102 in the diets of hybrid tilapia juvenile (*O. niloticus* and *O. aureus*) may cause upregulation of cytokines such as IL-1b, TGF-b, and TNF-a in the intestine of fish (Zorriehzahra et al. 2016).

Improverment of the effects of stress

Water parameters play important roles and may have a side effect on the physiological and

behavioral aspect of aquatic animals. The stress factors may have have negative effects on fish including thermal, anoxia, hypoxia, chemicals, toxins, nutritional or high density (Zorriehzahra et al. 2016).

There were several researches which demonstrated the positive effect of probiotic supplementation on the cultured fish against stress factors such as supplementation of *L. delbrueckii* subsp. *delbrueckii* in the diet of European sea bass (*D. labrax*) decreased in cortisol level during temperature stress; administration of *Bacillus* spp. to olive flounder (*P. olivaceus*) during transport reduced handling stress by influencing the cortisol level; or out probiotic administration on gilthead bream (*Sparus aurata*) improved tolerance to stress with under high stocking density (Zorriehzahra et al. 2016).

Overview on the use of probiotics in aquaculture

Using probiotics in aquaculture was reported in several studies. (Bairagi et al. 2004) showed that the diet containing leaf meal and two strains of genus *Bacillus* increased growth, feed conversion ratio and protein efficiency ratio of carp, *Labeo rohita*. (Vine et al. 2004) demonstrated the probiotics were isolated from the intestinal tract of the common clownfish that inhibited attachment of *V. alginolyticus* when probiotics were added to intestinal fish mucus after addition of the pathogenic *V. alginolyticus in vitro.* Also, the results in the study of (Sharifuzzaman and Austin 2009) revealed that a two-week feeding regime with *Kocuria* SM1 at concentrations of 10^8 cells/g feed leads to higher disease protection in rainbow trout (*Oncorhynchus mykiss*, Walbaum), with protection linked to stimulation of immune parameters. And *Enterobacter* sp. and may be useful as a potential alternate strategy for rainbow trout protection against *Flavobacterium psychrophilum* after either intramuscularly or intraperitoneal injection of this occurring bacterium, either alive or dead, was at least in part dependent on the enhanced immune function(s) of the

treated fish (Lapatra et al. 2014). In other case, *B. amyloliquefaciens* FPTB16 displayed as a potential probiotic species and could be used in aquaculture to improve health status of catla (*Catla catla*) and disease resistance from pathogens *E. tarda, A. hydrophila, V. parahaemolyticus and V. harveyi* with an optimal dietary supplementation of 10^9 cfu/g (Das et al. 2013). Beside that, the tilapias (*O. niloticus*) were supplemented with the probiotic *Enterococcus faecium* ZJ4 at a final concentration of 1×10^7 cfu/ml showed significantly better final weight and daily weight gain than those fed the basal diet ($p < 0.05$) after 40 days (Wang et al. 2008).

In the field of cultured shrimp, Castex et al. (2009) showed shrimp *Litopenaeus stylirostris* that was fed the probiotic (Gram-positive lactic acid bacteria *Pediococcus acidilactici*) were induced to response of increase in antioxidant enzyme activities and reduced oxidative stress level when they were exposed to *V. nigripulchritudo*. In addition, Moriarty (1999) reported on successful experiences of using probiotic bacteria instead of antibiotics to control luminous *Vibrio* species in shrimp farms in Negros, Philipine. Vaseeharan and Ramasamy (2003) showed the reducing cumulative mortality of black tiger shrimp by *Vibrio harveyi* infection when they were treated by *B. subtilis*. Alavandi et al. (2004) selected two bacterial strains (*Pseudomonas* sp. PM 11 and *V. fluvialis* PM 17) which were isolated from gut of shrimp as candidate probiotics to study the effect on enhancement of immunity of sub-adult black tiger shrimp. Both of bacterial strains had the ability to produce extracellular enzymes and siderophores, but did not show any expected probiotic effects and improvement of the immune system of shrimp *in vivo*. Because of beneficial application of probiotics for aquaculture, the field of probiotics intended for cultured animals is now attracting considerable attention and a number of commercial products are available, particularly directed at shrimp larval culture (Kesarcodi-Watson et al. 2008).

For other cultured species, probiotic has been already applied on abalone in the study of (Macey and Coyne 2005). The results have shown that *Haliotis midae* fed a probiotic-supplemented diet have an improved survival and growth rate compared to animals not fed

probiotics. The growth rate of small (20 mm) and large (67 mm) abalone was improved by 8 % and 34 %, respectively, in two separate eight-month farm growth trials. Also, after seven days challenging with *V. anguillarum*, the probiotic-fed animals had 62 % survival compared to 25 % survival for non-treated animals. Histological analysis showed that the digestive glands of animals receiving probiotics were bacteria-free, whereas the digestive glands of 70 % of the animals receiving the non-supplemented feed had a high bacterial load. The microorganisms tested in this study therefore have tremendous potential as probiotics for commercially produced *H. midae*.

Briefly, several benefit bacteria have been choosen as potential probiotic to study on different cultured species; and they have demonstrated their positive effects on aquatic animals. Among of them, lactic acid bacteria (LAB) are one of bacterial group famous in fermented food processes and dairy industry, recently they have been considered and studied for applying in aquaculture.

What is lactic acid bacteria?

As the review of Ringø and Gatesoupe (1998), LAB is well-known as Gram-positive bacteria, non motile, nonsporulating bacteria that produce lactic acid as a product of fermentative metabolism. LAB with different species have been able to isolate from gastrointestinal tract of animals and human, seafood products, milk and dairy products or on some plant surfaces. LAB has been considered as probiotics for use in aquaculture due to they may inhabit the digestive tract on larval stages, juveniles and ongrowing fish; also some of them are able to colonise the gastrointestinal tract, adhere to mucus, and inhibit fish pathogens as antagonistic activity.

Overview on the use of lactic acid bacteria in aquaculture

Recently, the application of lactic acid bacteria (LAB) on aquaculture has been reported by

several researches. Gatesoupe (2007) had updated that most LAB are harmless, exception the strains of *Streptococcus* and *Lc. garvieae* are pathogenic to fish; some LAB strains have been recognized for beneficent effects on fish health or could stimulate the immune system in fish; also some bacteriocins of LAB were characterized, which may be of interest not only for aquaculture, but also for food preservation.

Particularly, Natesan Sivakumar and Muthuraman Sundararaman (2012) demonstrated the probiotic potential of *L. acidophilus* was antibacterial activity against *V. parahaemolyticus*, *V. cholerae*, *V. harveyi* and *V. alginolyticus in vitro* test; and it was able to control pathogen by feeding to juvenile shrimp (*P. monodon*) through feed (supplemented with 10^5 cfu/g) for 30 days before and after an immersion challenge with *V. alginolyticus* (10^5 cfu/ml) with result in only 20 % final mortality as compared to 86.7 % in the control group. Besides, LAB commercially available cultures (*L. acidophilus*, *S. cremoris*, *L. bulgaricus*) were administered orally to juvenile *Penaeus indicus* that demonstrated their beneficial role on growth and survival of shrimp following the research of Fernandez et al. (2011). For LAB applying to cultured fish, Panigrahi et al. (2005) showed that the probiont *L. rhamnosus* JCM 1136 with the viable forms (live spray or freeze-dried) adding to the diet induced better phagocytic activity and complement activity compared to that of the non-viable heat- killed form after rearing trial with juvenile rainbow trout (average 126 g); also the plasma immunoglobulin level showed an increasing trend in the fish groups that received the viable probiont, but the trend did not exist towards the end of the study; upon withdrawal of the probiotic diets, the LAB disappeared from the intestine and the elevated immune parameters returned to the prefed level; this study elucidates that probiont viability could probably influence the immune responses they induce. In the other way, Talpur et al. (2012) reported when the addition to rearing water with three LAB (*L. plantarum*, *L. salivarius*, *L. rhamnosus*) isolated from gut of female blue swimming crab *Portunus pelagicus*, the surivival rate of larvae significantly improved around 11 %; also these isolates lowered pH and increased

digestive enzyme activity particularly protease and amylase compared to that of the control group which did not receive any bacteria. Lamari et al. (2014) had isolated LAB from rotifer cultures in marine hatchery to aim looking for potential probiotics for marine animals, in which the *L. casei* X2 finally was selected as candidate probiotic because this strain was ability to antibacterial activity against *Vibrio* sp., highly adhesive, ability for surface colonization and the best growth performances of *Artemi*a with or without the pathogen.

The diversity and abundance of LAB strains may supply to aquaculture as potential probiotic that was reported in several studies. Mohamed et al. (2015) resulted the LAB isolated from intestinal tract of Nile tilapia (*O. niloticus*) and Common carp (*Cyprinus carpio*) including *Lc. lactis, L. animalis, L. plantarum, L. fermentum*, *L. raffinolactis* and *L. acidophilus* that were able to against pathogenic bacteria (*A. cavie* and *P. fluorescence*) *in vitro* test. Also, Maji et al. (2016) studied the diversity of putative LAB in freshwater fish that were identified as different strains of *L. plantarum, L. pentosus, L. fermentum, L. delbrueckii subsp. bulgaricus, L. brevis, L. reuteri, L. salivarius, Pc. pentosaceus*, *Pc. acidilactici*, *Weissella paramesenteroides*, *W. cibaria*, *Enterococcus faecium* and *E. durans*; these isolates showed good survival in acid and bile tolerance tests and antimicrobial activity against fish pathogen *A. hydrophila*. Ouissal et al. (2015) identified the enterococcal isolates were recovered from the skin and intestines of the sea bream (*S. aurata*) as *E. faecium*, *E. faecalis*, *E. sanguinicola*, *E. casseliflavus*, *E. gallinarum*, *Carnobacterium* sp., *Aerococcus viridans* and *Vagococcus carniphilus*; the isolates were sensitive to vancomycin but were resistant to several antibiotics, and were ability against 39 bacterial indicators including food-borne and fish pathogenic bacteria in aquaculture as well as other spoilage bacteria.

Latococcus lactis in aquculture industry

Among of LAB, *Lc. lactis* is one of popular LAB that is widely applied in industrial fermentation

and used for the production of cheese (Sanders et al. 1999a). In aquaculture, probiotic feeding could play a crucial role in developing microbial control strategies, since disease outbreaks are recognized as important constraints to aquaculture production and the fear of antibiotic resistance, and *Lc. lactis* is one of LAB that can be used as probiotic in aquaculture feeding (Haziyamin et al. 2012).

Early, *Lc. lactis* was recognized as a potential probiotic on the the growth performance of a rotifer and its inhibition against *V. anguillarum* (Harzevili et al. 1998), in which the growth rate of the rotifers in suboptimal feeding conditions was significantly higher in the treatment receiving AR21 and *V. anguillarum* than in the treatment where only *V. anguillarum* was added. Zhou *et al.* (2010) investigated the inhibition ability of probiotic, *Lc. lactis* RQ516, against *A. hydrophila in vitro* and its immunostimulatory effect in tilapia, *O. niloticus* as growth promoter; the inhibition ability assay *in vitro* showed the final diameter of the inhibition zone ($p < 0.05$) was 14.77 ± 1.17 mm at 24 h; after 40 days of probiotic treatment as water additives, the result indicated that probiotic *Lc. lactis* RQ516 was beneficial for tilapia in terms of increasing final weight, daily weight gain, the concentrations of serum protein and globulin, and enhancing immune responses by the higher respiratory burst activity, lysozyme content, myeloperoxidase and superoxide dismutase activities were observed in the treated group ($p < 0.05$) than those in the control group. For marine fish, Nguyen et al. (2017) reported the beneficial effects of *Lc. lactis* WFLU12 as a host- derived probiotics in olive flounder, *Lc. lactis* WFLU12 was found to confer to olive flounder protection against streptococcosis caused by *S. parauberis* through competitive exclusion and increased innate immune responses, also this promising probiotic strain significantly promoted fish growth along with better feed conversion. Interestingly, *Lc. lactis* subsp. *lactis* was also effect on the growth, intestinal microbiota, digestive enzyme activity, and disease resistance of white leg shrimp, *L. vannamei*, in the study of Adel et al. (2017); results revealed that growth rate, survival, and body protein level of shrimp were increased with dietary

supplementation of *Lc. lactis*, the activities of digestive enzymes (cellulose, lipase, amylase, and protease) were significantly higher in the groups fed with diets containing 10^7 or 10^8 cfu/g *Lc. lactis* than those in the control, in addition, higher level of *Lc. lactis* supplementation decreased the *Vibrio* counts, moreover *L. vannamei* fed diet supplemented with 10^8 cfu/g of *Lc. lactis* exhibited significantly the highest hematocyte count and post-challenge survival rate (79.2 %) after immersion in Caspian Seawater (10.8 ppt) contaminated with 10^6 cfu/g pathogenic *V. anguillarum* for 2 h. Beside that, the effect of dietary administration of probiotic *Lc. lactis* MM1 for 60 days on the autochthonous microbiota in the foregut, midgut and hindgut of juvenile grouper, *Epinephelus coioides*, was recorded by the growth of many potentially beneficial and unidentified bacteria, whereas the depression of some potential harmful species, like *Staphylococcus saprophyticus*, under the probiotic stimulation (Sun et al. 2012). On the other hand, Itoi et al. (2008) isolated lactic acid bacteria from the intestinal tract of the pufferfish Takifugu, the result showed the tolerance of these *Lc. lactis* subsp. *lactis* isolates to salt was higher than that of *Lc. lactis* subsp. *lactis* from the cheese starter culture by the tolerance in MRS-agar plates containing 200 % seawater or 6 % sodium chloride, this was the first report of the isolation of halotolerant strains of *Lc. lactis* subsp. *lactis* from a marine environment. In 2006, Bernbom et al. (2006) examined the ability of pure nisin, nisin-producing *Lc. lactis* strain CHCC5826, and the non-nisin-producing *Lc. lactis* strain CHCH2862 to affect the composition of the intestinal microbiota of human flora-associated rats; their results indicated that the presence of both the nisin-producing and the non-nisin- producing *Lc. lactis* strains significantly increased the number of *Bifidobacterium* cells in fecal samples during the first 8 days but decreased the number of enterococci/streptococci in duodenum, ileum, cecum, and colon samples as detected by selective cultivation, however, profiles of the microbiota from animals dosed with nisin did not differ from the controls. Also, a strain bacteriocin-producing *Lc. lactis* TW34 was isolated from marine fish by Sequeiros et al. (2015), TW34 bacteriocin inhibited the growth of the fish

pathogen *Lc. garvieae* at 5 AU/ml (minimum inhibitory concentration), whereas the minimum bactericidal concentration was 10 AU/ml; moreover, sequence analysis of nisin structural gene confirmed that *Lc. lactis* TW34 was a nisin Z producer, this nisin Z-producing strain with probiotic properties might be considered as an alternative in the prevention of lactococcosis, a global disease in aquaculture systems.

Outline of this thesis

The aim of this thesis was to isolate lactic acid bacteria in difference materials; to study biochemical properties and antagonistic activity of the isolated strains *in vitro*, to assess the survival rate of a selected strains after exposure to gastrointestinal fluids; and to determine the effect of microorganisms after use of the strain was selected as a live supplement for cultured marine fish. The content of each chapter is as follows: **Chapter 2**, is a study on the screening of lactic acid bacteria isolated from fermented food as potential probiotics for aquacultured carp and amberjack; **Chapter 3**, is a study on the identification and characteristics of selected candidate to be used as a potential probiotic for aquaculture; **Chapter 4**, is a study on the *Lc. lactis* strain K-C2 supplementation in amberjack (*Seriola dumerili*) and to evaluate the effect of this strain on fish growth performance, amino acid content and intestinal microflora after feeding trial for amberjack; **Chapter 5**, is a study on effect of *Lc. lactis* strain K-C2 on the growth performance, the resistance to pathogen of Japanese eel (*Anguilla japonica*); **Chapter 6**, is a general discussion.

Chapter 2: Screening of lactic acid bacteria isolated from fermented food as potential probiotics for aquacultured carp and amberjack

Abstract This study aimed to isolate lactic acid bacteria from fermented foods and evaluate their probiotic properties for application to aquaculture. Sixty-five bacteria strains were isolated using MRS (de Man, Rogosa & Sharpe) media. Using a double layer agar method, three strains, GYP 31, L 15 and K-C2, showed antagonistic activities against all test pathogens belonging to *E. tarda*, *Streptococcus dysgalactiae, S. iniae* and *L. garvieae*. These strains were able to survive in a pH range from 2.0 to 9.0 and at NaCl concentrations of 0 %, 3 %, and 5 %. In the tolerance test, strain K-C2 displayed higher tolerance than strains GYP 31 and L 15 under the stimulation of acidic pH buffers and artificial gastric-intestinal juices. Thus, strain K-C2 was selected as a probiotic candidate for cultured fish. Strain K-C2 adhered to carp and amberjack intestinal mucus at bacterial densities of 10^{10} cfu/ml in *in vitro* tests with adhesion rates of 62 % and 58 %, respectively. Thus, strain K-C2 was considered to be a potential probiotic candidate for use in sustainable aquaculture.

Introduction

Lactic acid bacteria (LAB) are widespread microorganisms that have been isolated from several sources including fermented food, soil, and plants. They are also part of the useful microbial flora in the gastrointestinal tracts of terrestrial and aquatic animals. LAB are a group of Gram-positive rods and cocci; they are non-motile, non-sporing, do not reduce nitrate, and are catalase-negative and oxidase-negative. These bacteria may use carbohydrates as an energy source and produce lactic acid either as the sole product of metabolism or as the major end-product. There are LAB

that belong to the genera *Streptococcus, Enterococcus, Lactobacillus, Aerococcus, Carnobacterium, Leuconostoc, Lactococcus* and *Pediococcus.* (Ringø and Gatesoupe 1998)

As a source of functional ingredients, LAB have become widely used as probiotics in human nutrition, the veterinary field, and aquaculture as biological control pathogens (Verschuere et al. 2000; Herich and Levkut 2002; Wedajo 2015). Also, LAB play an important role in the fermented food industry in the production of fermented foods made from dairy products, meat, vegetables, etc. Fermented foods are known to be natural sources of probiotics and safe components of the human diet. LAB are important in lactose digestion and are useful in preventing several types of diarrhea, controlling irritable bowel syndrome and inflammatory bowel diseases, and enhancing immune system function at the intestinal and systemic levels, and have also shown promise against stomach ulcers (Wedajo 2015). The benefits of LAB from fermented foods for human health have been demonstrated, but there has been little research on the effect of these beneficial bacteria on aquatic animal health. For use in aquaculture, putative probiotics have been obtained from aquatic environments (water, sand or mud), the skin mucus of fish, the gastrointestinal tracts of aquatic animals, and from commercial products (Newaj-Fyzul et al. 2014); however, there is no clear evidence that probiotics isolated from a host or from their ambient environment perform better than isolates originating from different habitats (Sahu et al. 2008). Therefore, the beneficial microorganisms in fermented foods should be considered for use in the aquaculture industry. On the other hand, different bacteria in the same living environment may freely exchange genetic material, and they can adapt quickly to the positive or negative effects of the ambient environment (Aminov 2011); meaning that the antagonistic activity of beneficial bacteria against harmful bacteria may be unstable everlasting, pathogenic bacteria may appear more and it may be more difficult for destroying them. Thus, the important characteristics of fermented microorganisms such as their resistance to intestinal pathogens, their enhancement of the immune response system of the host, and their stimulation of the digestive process should

be examined for their potential application in aquaculture for controlling and limiting the growth of pathogens.

In this study, we aimed to isolate LAB from several different sources such as fermented vegetables, rice bran, and the intestinal tracts of herbivorous animals in order to investigate their probiotic properties. To identify potential candidates for application to sustainable aquaculture, we administered screening tests such as an antagonistic activity test, an acid tolerance test, an artificial gastro-intestinal juices tolerance test, a test for growth at different pH levels and NaCl concentrations, and a mucus adhesion test.

Materials and methods

Isolation of LAB

Fermented cabbage, Chinese cabbage, cucumber, seaweed and rice bran were purchased at supermarkets in Miyazaki, Japan, and feces were collected from the intestinal tracts of cows raised at Sumiyoshi Live Stock Science Station, University of Miyazaki, and transferred to the laboratory for the isolation of LAB. Each 1 g of the samples was suspended in 9 ml of a sterile physiological saline solution (0.85 % NaCl [w/v]), shaken vigorously and kept for 10 min at room temperature for sedimentation. Then, the suspension was serially diluted in sterile physiological saline solution, and each dilution from 10^{-1} to 10^{-6} was plated on MRS agar (2 g glucose, 0.5 g yeast extract, 1 g peptone, 1 g meat extract, 0.5 g sodium acetate, 0.2 g ammonium citrate, 10 mg $MgSO_4.7H_2O$, 5 mg $MnSO_4.5H_2O$, 0.2 g $K_2HPO_4.7H_2O$, 2 ml tween 80 [50 mg/ml distilled water], 1.5 g agar, 100 ml distilled water, pH 6.5). The agar plates were incubated at 28 °C for 24-48 h for the collection of colonies (Shelar et al. 2012).

The selected colonies were then streaked on the GYP agar (1 g glucose, 1 g yeast extract,

0.5 g peptone, 0.2 g meat extract, 0.2 g sodium acetate, 0.5 ml salt solution [MgSO$_4$.7H$_2$O 40 mg, MnSO$_4$.5H$_2$O 20 mg, FeSO$_4$.7H$_2$O 2 mg, NaCl 2 mg per 1 ml distilled water], 1 ml tween 80 [50 mg/ml distilled water], 1.5 g agar, 100 ml distilled water, pH 6.8) containing 0.5 % CaCO$_3$ (w/v) as an indicator. The plates were incubated at 28 °C for 24 h. The acid production was judged by the formation of a clear zone around a lawn of colonies, and these colonies were purified by streaking from single colony on a fresh MRS agar plate several times.

Antagonistic activity test

Twelve strains of fish pathogens representing three genera (*Lactococcus*, *Streptococcus* and *Edwardsiella*) were kindly provided by the Fish Diseases Laboratory at the Department of Marine Biology and Environmental Sciences at the University of Miyazaki. These strains were cultured in BTH (Bacto Todd Hewitt, BD Bacto™, USA) broth for 24 h at 28 °C.

The antagonistic activity was evaluated according to the method described by Geis et al. (1983) with slight modification. The isolates and pathogens were incubated overnight in 5 ml MRS broth and 5 ml BTH broth, respectively, at 28 °C. Five µl of each suspension of isolates at 1 x 10^8 cfu/ml were spotted onto an MRS agar (1.5 %, [w/v]), and the plates were kept on the clean bench for 1 h to completely allow the spots to attach to the surface of the agar plates. The overnight cultured pathogenic strains were centrifuged at 5,000×g for 10 min, and the cell pellets were washed and suspended in 0.1 ml of saline solution. A 0.1 ml volume of the cell suspension of each of the pathogens was mixed with 5 ml of soft BTH agar (0.5 % [w/v]) at a final concentration of 1 x 10^7 cfu/ml and overlaid on the MRS agar plates with isolated bacterial strain spots. The plates were incubated at 28 °C for 72 h, and the diameters of the inhibition zones around the spots of isolates were measured to determine the antagonistic activity of the isolates.

Preparation of washed bacterial cell suspension

The candidate strains were cultured in MRS broth at 28 °C for 24 h. Cells were collected by centrifugation (5,000×g for 10 min, 25 °C), washed, and suspended in 10 mM phosphate-buffered saline (PBS) buffer (pH 7.0) as a washed cell suspension. The density of the cell suspension was based on the total viable counts as determined by the agar plate counting method with MRS agar plates.

Growth of LAB at different pH and NaCl concentrations

MRS broths with different pH values (2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, and 9.0) were prepared by adding HCl or NaOH solutions. MRS broths containing different NaCl concentrations (0, 3, 5, and 10 % [w/v]) were prepared by adding sodium chloride (Nacalai Tesque, Inc., Kyoto, Japan), and the pH was adjusted to 6.8. Aliquots of the washed cell suspensions (0.1 ml) were added to 10 ml of each MRS broth and incubated at 28 °C for 72 h. The turbidity was measured as optical density (OD) at 600 nm to monitor the growth of the test strains.

Acid tolerance test and artificial gastro-intestinal juices tolerance test

The tolerance of the selected candidates under acidic conditions were determined according to the method of Sahadeva et al. (2011) with slight modification. The acidic pH buffer was prepared using 0.1 M glycine-HCl buffer (pH 2.0, 2.5, 3.0, 3.5, 4.0 and 7.0 as a control). The artificial gastric juice was prepared by dissolving pepsin (Nacalai Tesque, Inc., Kyoto, Japan) in 0.1 M glycine-HCl buffer (pH 2.0, 2.5, 3.0, 3.5, 4.0, and 7.0 as a control) to obtain a final concentration of 3 g/l. The artificial intestinal juice was prepared by dissolving pancreatin (Sigma-Aldrich,

USA) in 0.1 M Tris-HCl buffer (pH 8.0 and 7.0 as a control) to obtain a final concentration of 1 g/l with or without 0.45 % (w/v) gall powder (Wako Pure Chemical Industries, Ltd., Osaka, Japan). These solutions were sterilized using a membrane filter (DISMIC-13cp, pore size 0.2 µm, Advantech MFS, Japan).

According to the method reported by Huang and Adams (2004), an aliquot (0.2 ml) of the washed cell suspension was mixed with 0.3 ml of 0.9 % saline solution and 1 ml of the acidic pH buffer, the artificial gastric juice (pH 2.0-4.0), or intestinal juices (pH 8.0). The mixtures were then agitated for 10 s and incubated at 28 °C. After 60, 90, 120 and 180 min periods of incubation, 0.1 ml of the mixture was taken to determine the viable cell counts in all of the MRS agar plates after 48 h. Each assay was performed in triplicate.

In vitro **mucus adhesion test**

This experiment was carried out following the method described by Namba and Hirose (2005) and Tsukatani et al. (2008) with slight modification. Briefly, mucus extract was prepared from the intestinal tracts of five healthy common carps (average body weight 20 g) and three healthy amberjacks (average body weight 67 g). Mucus was scraped from the intestinal walls with a sterilized pincette, homogenized with 5-fold volumes of 10 mM PBS (pH 7.2) and centrifuged at 15,000×g for 10 min at 4 °C. The supernatant was collected and stored at -20 °C. The protein content of the mucus extract was determined by the Lowry method (Lowry et al. 1951) using albumin from bovine serum (Sigma-Aldrich Inc., USA) as a standard and adjusted to 1.0 mg/ml with PBS. This solution was used as a mucus extract.

One hundred µl of the mucus extract was added to each well of a 96-well microtiter plate and incubated overnight at 4 °C. Each well was washed three times by PBS. Next, 100 µl of each serial dilution of the bacterial suspension from 10^{11} to 10^5 cfu/ml was added to each of three wells

and incubated for 1.5 h at 28 °C; then each well was washed three times by PBS. Then 95 µl of standard medium (0.25 g yeast extract, 0.5 g peptone and 0.1 g glucose in 100 ml distilled water, pH 7.0) and 5 µl of a WST-1-electron mediator mixture (including 9 parts 11 mM water soluble tetrazolium salt WST-1 (2-[4-Iodophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium, Dojindo laboratories, Kumamoto, Japan) and 1 part 0.8 mM electron mediator (2, 3, 5, 6-tetramethyl-p-benzoquinone dissolved in dimethyl sulfoxide)) were added to each well and continuously incubated for 4 h at 28 °C. The transformation of WST-1 into a water-soluble formazan by mitochondrial dehydrogenase in viable bacterial cells was measured at OD 450 nm by SkanIt RE of Varioskan Flash 2.4.5 software using a Varioskan flash microplate reader (Thermo Scientific, Japan).

In vivo mucus adhesion test

Healthy common carps (*Cyprinus carpio*) were obtained from a commercial fish farm (Mie Prefecture, Japan). The carps were maintained in dechlorinated tap water with a water temperature, pH, and dissolved oxygen of around 26 °C, 7.0-8.0, and 8-9 mg/l, respectively. The carps were acclimated in 30-l aquaria at a density of six individuals with a closed rearing system and were fed a commercial diet (Kyorin Ltd., Fukuoka, Japan) for one week before the experiments were performed.

Also, healthy amberjack (*Seriola dumerili*) were obtained from a commercial fish farm (Miyazaki Prefecture, Japan). The fish were maintained in artificial seawater (Sea Life, Marine Tech Co., Tokyo, Japan) with a water temperature, salinity, pH, and dissolved oxygen of around 22 °C, 30 ‰, 7.0-8.0, and 8-9 mg/l, respectively. The fish were acclimated in 30-l aquaria at a density of six individuals with a closed rearing system and were fed a commercial diet (Hamachi EP d5, Feedone Co Ltd., Kanagawa, Japan) for one week before the experiments were performed.

Two treatment groups for each fish species with three simultaneous replications were used. These groups were as follows: (i) a control group fed a commercial diet without selected candidate strain K-C2; (ii) a treated group fed a commercial diet containing strain K-C2 (1 x 10^{11} cfu/g). The test fish with an average body weight of 20 g were starved for two days before being fed each diet one time in an amount equal to 1 % of their body weight. After being starved for the next two days, the intestinal tracts of the test fish were sampled. Each 0.1 g of the intestinal mucus was suspended in 0.9 ml of a sterile physiological saline solution (0.85 % [w//v] NaCl) and shaken vigorously. Then, the suspension was serially diluted in sterile physiological saline solution, and each dilution from 10^{-1} to 10^{-6} was plated on MRS agar. The agar plates were incubated at 28 °C for 24 h to recover strain K-C2 from the treated group. Randomly, about 10 colonies per plate were selected for the colony PCR (following the method of Yi et al. (2010)) using species-specific primers to identify evidence of the presence of the candidate strain in the fish intestine.

Results

Isolated candidates and antagonistic activity test

A total of 65 strains of LAB were isolated from different sources of fermented foods that showed different antagonistic activities against 12 species of pathogenic bacteria in aquatic animals. The results of the antagonistic activity assay are shown in Table 1. Three strains named GYP 31, L 15 and K-C2, which were isolated from rice bran, the feces of cows, and fermented Chinese cabbages, respectively, showed antagonistic activity against all of the test pathogens. Whereas others exerted no effect (data not shown) or showed an effect only against some or all of the

tested pathogens with low inhibitory effects. Based on these results, strains GYP 31, L 15 and K-C2 were selected as probiotic candidates for further investigation.

Growth at different pH and NaCl concentration

Fig. 1 shows the results of the relative growth of probiotic candidates in MRS broth at different pH levels. Strain GYP 31 grew well in the range of pH from 5.0 to 8.0 (more than 84 % of relative growth), the growth of this strain was strongly suppressed in the range of pH from 2.0 to 4.0 (less than 13 % of relative growth) and was almost fully inhibited at pH 9.0. Strain L 15 grew well in the range of pH from 5.0 to 9.0 (more than 72 % of relative growth), and the growth of this strain was strongly suppressed in the range of pH from 2.0 to 4.0 (less than 2 % of relative growth). Strain K-C2 grew well in the rage of pH 6.0 to 9.0 (more than 95 % of relative growth), and the growth suppression (57 % of relative growth) was observed at pH 5.0, and the growth of this strain was strongly suppressed in the range of pH from 2.0 to 4.0 (less than 30 % of relative growth). In the range of pH from 2.0 to 4.0, the relative growth of strain K-C2 was higher than those of strains GYP 31 and L 15.

All three of these strains showed high relative growth of over 70 % in the range of NaCl concentration from 0 % to 5 % (Fig. 2).

Test of tolerance of artificial gastric and intestinal juices

Acid tolerance test

Viable counts of the probiotic candidates are presented in Fig. 3. The viability of the strains GYP 31, L 15, and K-C2 decreased at pH levels from 2.0 to 4.0 within the initial 60 min. Subsequently,

31

strain GYP 31 had no viability at pH 2.0 (data not shown) and the level did not change much until 180 min of incubation, except that the viability slightly increased at pH 3.0 and 3.5. The viability of strain L 15 remained stable at pH 3.0, 3.5, and 4.0, declined at pH 2.0 until 180 min, and showed a small increase at pH 2.5 beginning at 120 min of incubation; strain K-C2 showed viability had no fluctuation at pH levels ranging from 2.0 to 4.0 until 180 min, except that at pH 3.0 the viability declined until 120 min and then maintained the same level at 180 min of incubation.

Artificial gastric and intestinal juices tolerance test

The effects of artificial gastric and intestinal juices on the viability of the strains GYP 31, L 15 and K-C2 are shown in Figs. 4 and 5. There was a downtrend in the viability of these three strains after 60 min of incubation, and then the simulated gastric and intestinal juices seemed no affect the viability of the three strains beginning at 180 or 240 min of incubation. Although the strain GYP 31 showed no tolerance at pH 2.0, the viability was slightly increased at a pH of 2.5 after 120 min and continuously decreased at pH 3.0 until 180 min of incubation time had passed. Moreover, under the initial 60 min of the simulation, the viable counts of strain L 15 had dropped by 2.2×10^9, 2.0×10^6, 1.8×10^5, 1.3×10^5, and 1.5×10^4 times at pH levels of the artificial gastric juice of 2.0, 2.5, 3.0, 3.5, and 4.0, respectively, and dropped 1.2×10^5, 1.1×10^5 times in artificial intestinal juices at pH 8.0, pH 8.0 with 0.45 % gall powder (pH 8.0 + G), respectively. The viable counts of strain K-C2 had decreased by 7.5×10^4, 7.5×10^4, 1.4, 2.2, and 1.8 times in artificial gastric juices with pH levels of 2.0, 2.5, 3.0, 3.5, and 4.0, respectively, and by 8.8, 2.2×10^2 times in artificial intestinal juices at pH 8.0, pH 8.0 with 0.45 % gall powder (pH 8.0 + G), respectively. Thus, strain K-C2 displayed an ability to survive better than the other tested strains in the simulation using artificial gastric-intestinal juices.

Mucus adhesion test

In the results of the *in vitro* test shown in Fig. 6, the adhesion rates of strain K-C2 to carp mucus and amberjack mucus increased from 0 % to 62 % and from 0 % to 58 % with the increase of the bacterial densities from 10^5 to 10^{10} cfu/ml, and diminished to 59 % and 54 % at the bacterial density of 10^{11} cfu/ml, respectively.

In the *in vivo* test, no colonies were detected on the MRS agar plates from the intestinal mucus of carps and amberjacks in the control group. The candidate strains remaining in the intestines of carps in the treated group were determined to have a concentration of about 3×10^6 cfu/g intestinal mucus by counting the growth colonies on the MRS agar plates. All of the growth colonies were small, smooth, round, and opalescent; the colonies were selected randomly and checked by a colony PCR assay that could indicate the existence of a candidate strain in the intestinal carps. Unfortunately, no colonies grew on the MRS agar plates that were streaked with amberjack intestinal mucus in the treated group.

Discussion

In this study, we first aimed to isolate the LAB from different sources and evaluate some characteristics of the isolates using selection criteria in order to consider them potential probiotics that could be applied to aquaculture. The antagonism of the candidate probiotics against pathogens is a major criterion for preselecting a candidate, since probiotics that are effective *in vitro* may be effective in protecting the host against pathogens *in vivo* (Verschuere et al. 2000). In previous studies, the inhibitory effects of LAB were demonstrated by their capability to produce organic acids, hydrogen peroxide, bacteriocins, nisin or antimicrobial peptides; however, although most of the reports showed the ability of LAB to combat Gram-negative pathogens, there have been few reports on the antagonism exhibited by LAB against Gram-positive fish pathogens

(Stoffels et al. 1992; Ringø and Gatesoupe 1998; Vázquez et al. 2005; Sugita et al. 2007; Zhou et al. 2010; Touraki et al. 2012; Ustiugova et al. 2012; Volzing et al. 2013; Zendo 2013; Sequeiros et al. 2015). Interestingly, the selected strains in this study did not inhibit the growth of only three strains of Gram-negative bacteria, but they also inhibited the growth of nine strains of Gram-positive bacteria, which caused several epidemic diseases in fish.

Another important criterion for becoming a good source of probiotics is the tolerance for high acid levels (Sahadeva et al. 2011). According to Page et al. (1976), acidic conditions (pH 2.0-4.0) were presented in the catfish stomach, and the pH slightly increased to alkaline values (pH 7.0-9.0) in the intestinal tract; thus, the exposure to gastrointestinal juices may be the cause of the decreased viability of ingested probiotics (Liong and Shah 2005). In one report, Rallu et al. (1996) found that *Lc. lactis* strain MG1363 had high survivability when it was incubated at pH 4.5 or above, and its survivability markedly decreased with increasing incubation time at pH levels below 4.5. However, Kim et al. (1999) reported that *Lc. lactis* subsp. *lactis* strains can survive at pH levels as low as 2.5 (in M17 medium with HCl). Sanders et al. (1999) reviewed the acid resistance mechanism of *Lc. lactis* and found that the primary mechanism for controlling intracellular pH was the FoF1 ATPase that translocates protons to the environment at the expense of ATP; a second mechanism for pH homeostasis was the arginine deiminase (ADI) pathway; and *Lc. lactis* may express a glutamate-dependent acid resistance mechanism in the presence of chloride. The bile resistance ability of *Lc. lactis* depended on the cellular fatty acid composition such as the amount of hexadecanoic acid or octadecenoic acid (positive) and of hexadecanoic acid or C-19 cyclopropane (negative) (Kimoto-Nira et al. 2009). Also, according to the research of Takanashi et al. (2014), *Lc. lactis* strains isolated from different sources would have differences in their bile tolerances; however, these *Lc. lactis* strains could survive in 0.6 % oxgall. This finding explained how the candidate strain K-C2 could maintain a stable viable count from 60 min to 180 min or 240 min of incubation time in artificial gastric (pH from 2.0 to 4.0) or intestinal fluid (pH

8.0), respectively, and this strain showed quick adaptability and better subsistence in comparison to the others after the initial 60 min of incubation, as pointed out in the results of this study.

On the other hand, the ability to adhere to fish mucus is also essential for the selection of probiotics (Ringø and Gatesoupe 1998). The mechanism of adhesion of LAB to the tissues of the gastrointestinal tract of fish has still not been investigated fully. However, Tannock (1999) suggested that the adherence of lactobacilli to epithelial surfaces was related to the contributions of specific carbohydrates (lectins). Several LAB, such as *Carnobacterium* sp., *Lc. lactis* subsp. *lactis*, *Lc. lactis* subsp. *cremoris*, and *L. curvatus,* displayed adhesion to the intestinal mucus of rainbow trout (Joborn et al. 1997; Balcazar et al. 2007). In this study, strain K-C2 also showed an ability to adhere to carp and amberjack intestinal mucus in an *in vitro* test. Besides, the results of an *in vivo* test with a low percentage (about 0.03 %) or no recovery of strain K-C2 from carp or amberjack intestinal mucus after two days without feeding suggested that it was necessary to feed this candidate strain continuously during the feeding trial to maintain the positive impact from beneficial microorganism to aquatic animal.

In conclusion, the use of the LAB as probiotics in humans or animals has been studied and approved. In this study, strain K-C2 was shown to act against Gram-negative and -positive bacterial pathogens in fish, to have the ability to survive in artificial gastro-intestinal juices, and to adhere to fish mucus, suggesting that it has the potential to be used as a probiotic in the sustainable aquaculture industry.

Table 1 Antagonistic test of candidate strains against fish pathogens with double layer agar method

Isolated strains	Gram negative bacteria			Gram positive bacteria								
	Edwardsi-ella tarda HDK1	*E. tarda* HDK 2	*E. tarda* E09-11	*Steptococ-cus dysgalactiae* KGB	*S. dysgala-ctiae* KSB	*S. dysgala-ctiae* IK	*S. iniae* 99	*S. iniae No3*	*S. iniae 1024*	*Lactococcus garvieae* M593002	*L. garvieae* KT0A1	*L. garvieae* SM071-1
GYP 25	ND	1	2	2	1	4	4	4	3	ND	ND	ND
RB 30	ND	ND	ND	2	1	3	3	4	4	ND	ND	ND
GYP 31	**10**	**10**	**5**	**7**	**9**	**12**	**8**	**12**	**12**	**3**	**4**	**3**
GYP 33	ND	ND	ND	ND	ND	ND	ND	2	4	ND	ND	ND
GYP 38	ND	2	2	3	ND	4	4	ND	ND	ND	ND	ND
GYP 44	1	1	1	3	3	4	4	6	3	ND	ND	ND
GYP 45	ND	ND	ND	ND	ND	ND	2	ND	1	ND	ND	ND
GYP 49	ND	ND	ND	2	ND	ND	2	ND	ND	ND	ND	ND
GYP 64	1	2	1	3	3	4	4	4	3	ND	ND	ND
GYP 69	3	4	3	3	3	3	1	3	4	1	1	1
GYP 70	ND	1	ND	ND	1	ND	3	2	2	ND	ND	ND
GYP 72	ND	ND	ND	1	2	ND	2	3	4	ND	ND	ND
GYP 74	ND	ND	ND	ND	1	ND	ND	1	3	ND	ND	ND
GYP 78	ND	ND	ND	2	ND	ND	2	1	3	ND	ND	ND
GYP 4-2	ND	ND	ND	1	1	ND	2	1	1	ND	ND	ND
GYP 4-5	**10**	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
GYP 4-11	ND	ND	ND	2	1	ND	2	1	3	ND	ND	ND
GYP 4-12	ND	ND	ND	2	1	2	2	1	3	ND	ND	ND
GYP 4-19	1	1	ND	ND	ND	3	2	1	3	ND	ND	ND
GYP 4-20	1	1	ND	2	2	3	3	2	3	ND	ND	ND
GYP 4-21	2	2	1	4	3	3	6	3	6	1	1	1
GYP 4-23	2	2	ND	2	2	1	3	2	4	ND	ND	ND
GYP 4-24	ND	ND	ND	1	1	1	ND	ND	1	ND	ND	ND
K 1-5	ND	ND	ND	1	1	1	ND	ND	2	ND	ND	ND
K 1-6	ND	ND	ND	1	1	2	2	1	1	ND	ND	ND
K 4-1	2	2	2	8	4	3	4	3	4	1	1	2
K 4-2	ND	ND	ND	3	2	3	2	1	2	ND	ND	ND
K 5-1	ND	ND	ND	2	1	2	1	1	1	ND	ND	ND
K-C 2	**2**	**3**	**3**	**9**	**4**	**3**	**4**	**4**	**8**	**2**	**2**	**2**
L4	4	3	4	9	6	4	13	8	6	1	1	1
L8	3	3	6	9	16	10	13	7	8	2	0.5	ND
L9	2	5	3	7	9	10	9	5	12	2	0.5	2
L15	**7**	**8**	**9**	**16**	**12**	**14**	**14**	**12**	**18**	**3**	**4**	**2**
L17	ND	ND	ND	3	ND	3	3	ND	4	ND	ND	ND

Values shows diameters of inhibitory zone ($n = 3$).
ND, not detected.
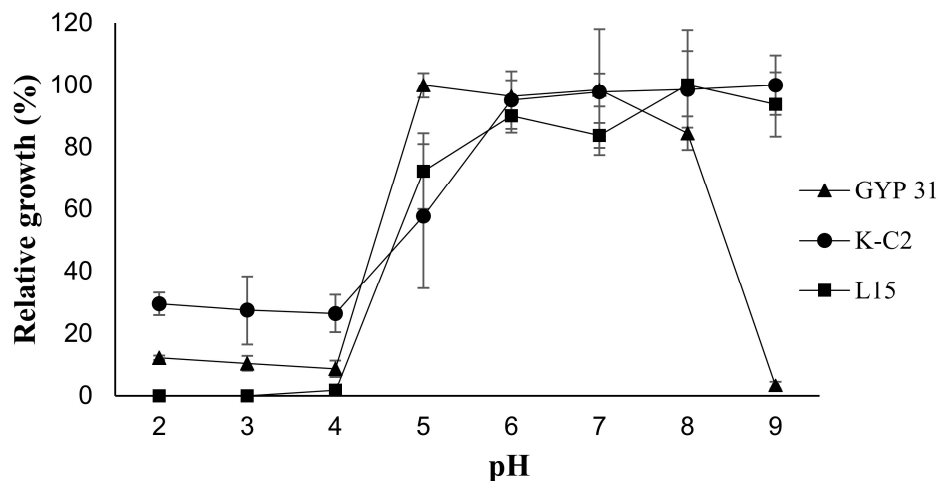
Fig. 1 The relative growth of three strains, GYP31, K-C2 and L 15 at pH levels. Values represent mean ($n = 3$)
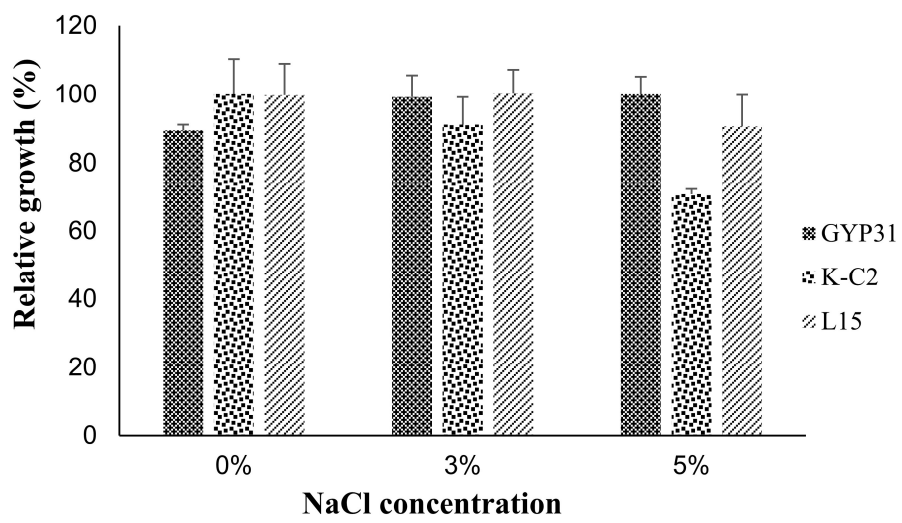
Fig. 2 The relative growth of three strains, GYP31, K-C2 and L 15 at different NaCl concentration. Values represent mean (n = 3)
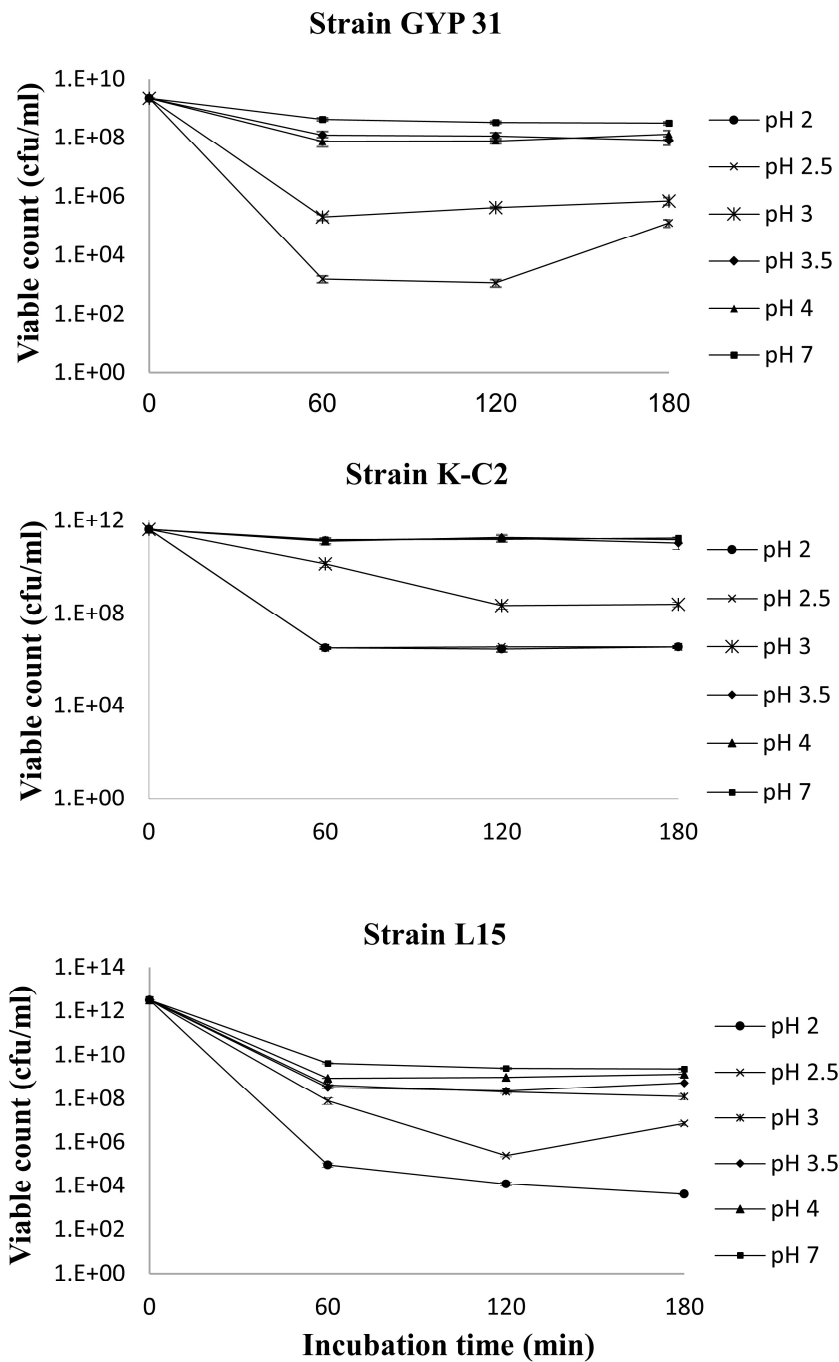
Fig. 3 Effect of acidic pH buffer on the viability of three strains, GYP31, K-C2 and L 15. Values represent mean (*n* = 3)

Fig. 4 Effect of artificial gastric juice on the viability of three strains, GYP31, K-C2 and L 15 at pH levels. Values represent mean (n = 3)

Strain GYP-31

Strain K-C2

Strain L 15

Fig. 5 Effect of artificial intestinal juice on the viability of three strains, GYP31, K-C2 and L 15 at pH levels. Values represent mean ($n = 3$)
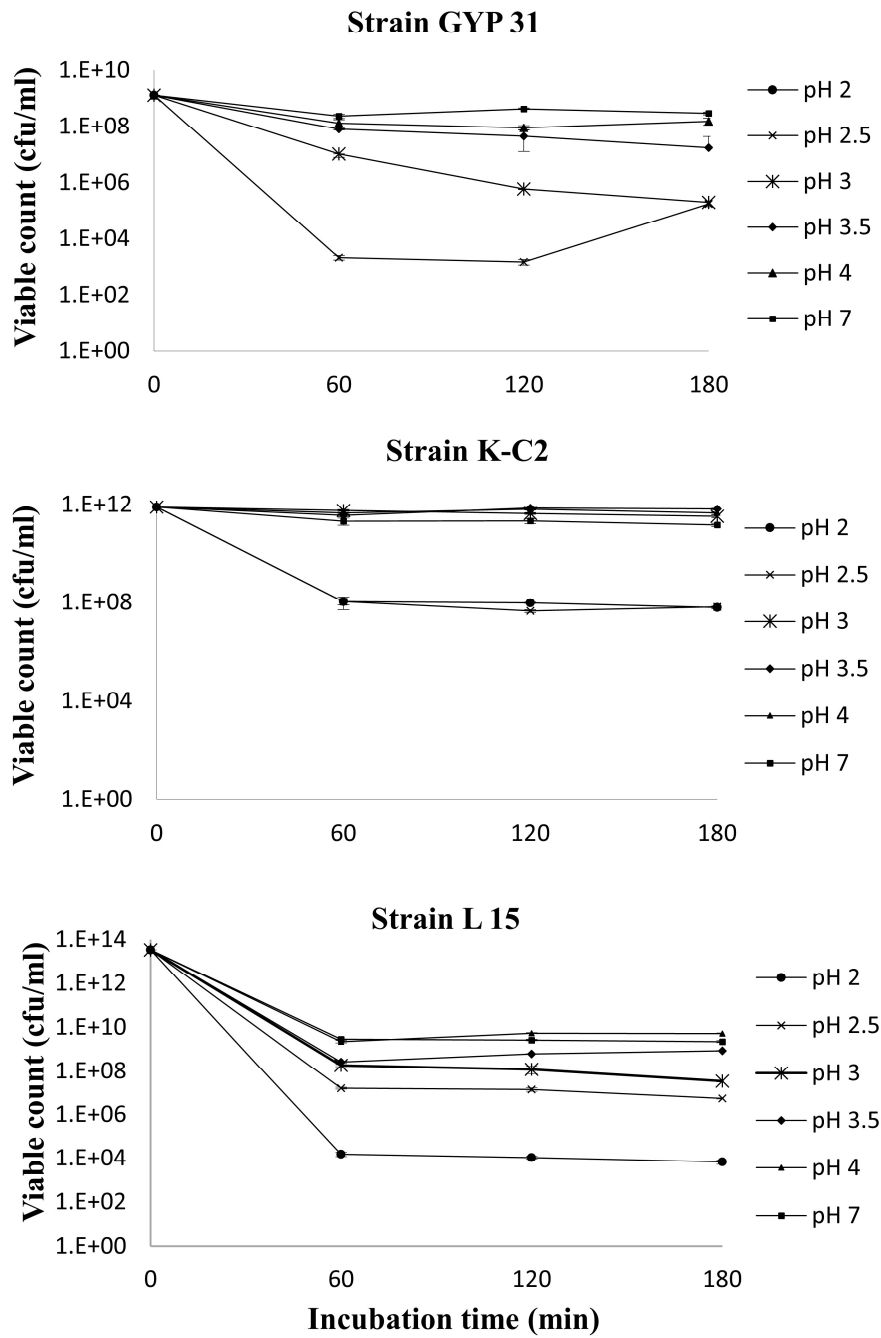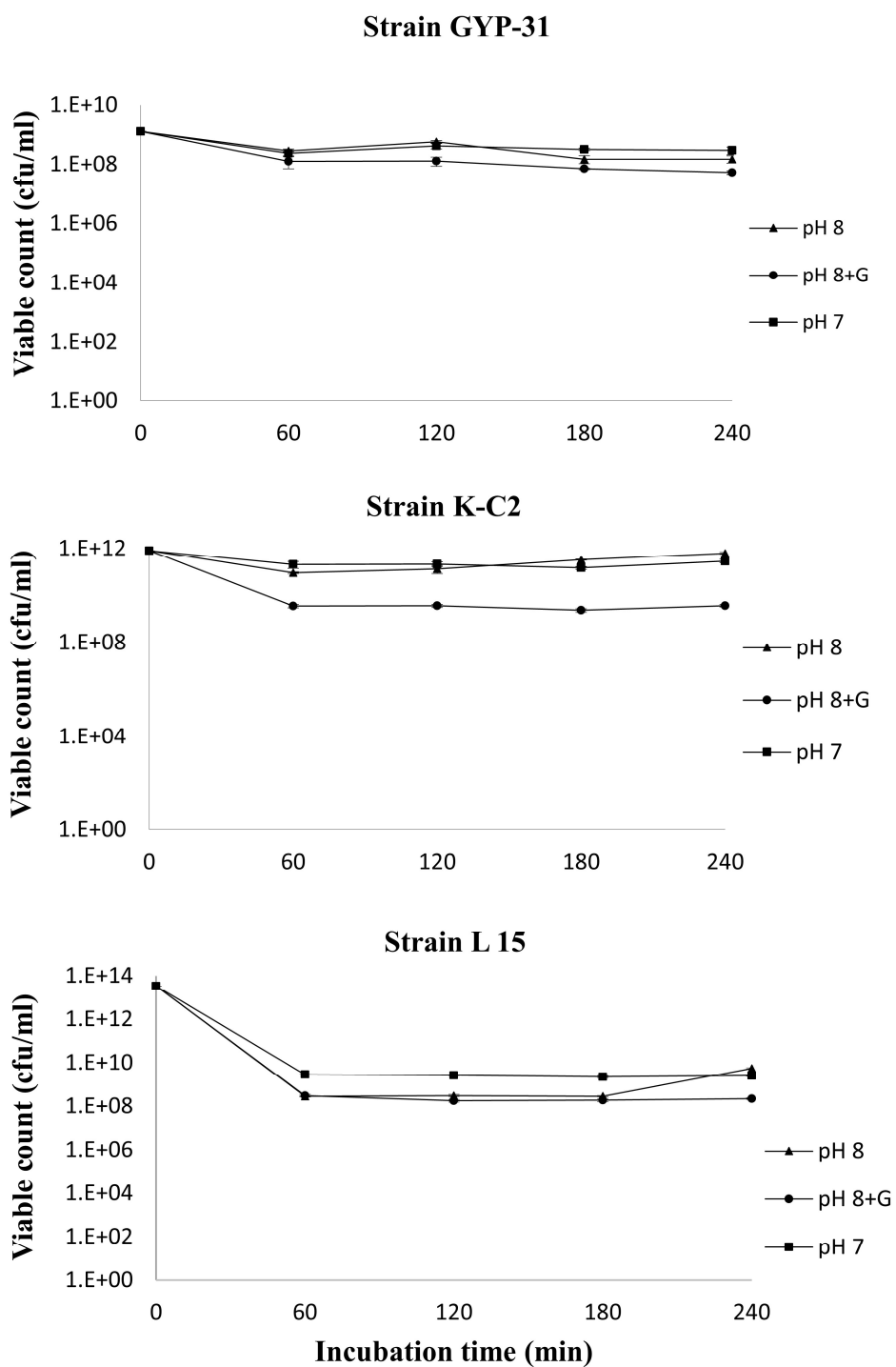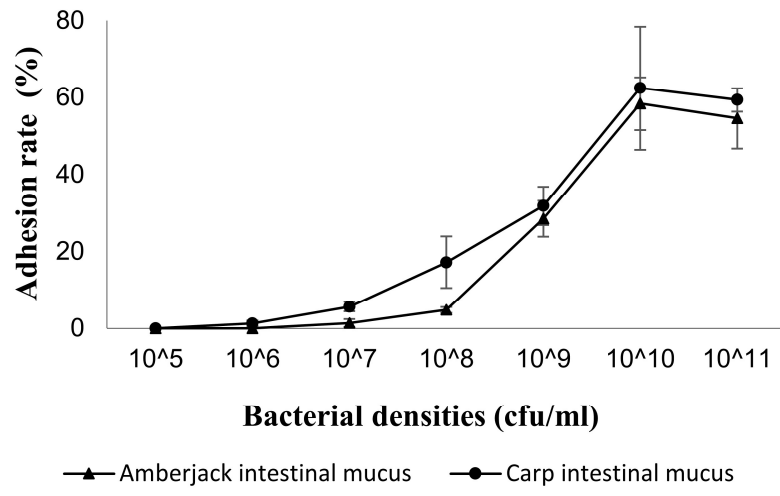
Fig. 6 The adhesion rate of strain K-C2 in fish intestinal mucus. Values represent mean (*n* = 3)

Chapter 3: Identification and the characteristics of selected candidate strain K-C2 to be used as an potential probiotic for aquaculture

Abstract The identification and characterization of selected strain K-C2 were carried out in this study. Based on 16S rRNA gene (1438 bp) analysis, strain K-C2 was identified as *Lactococcus lactis*. This strain was observed to be ovoid in shape by SEM mehod and to be catalase-negative by catalase test in morphological and biochemical investigations. Strain K-C2 was able to ferment almost tested sugars including fructose, galactose, glucose, lactose, maltose, mannose, rhamanose, ribose, sucrose, trehalose, xylose and exception of α-cellulose. By using API ZYM kit to analysis the extracellular enzyme profile, strain K-C2 displayed only strong Naphthol-AS-BI-phosphohydrolase activity; it showed weak esterase (C4), esterase lipase (C8), lipase (C14), acid phosphatase and β- glucosidase activity, and it had no activity with other enzymes. By LC-MS method, there were 14 amino acids were found in bacterial cell of strain K-C2 including Leu, Cys, Met, Phe, Thr, Val, Ala, Asp, Glu, Gly, Ser, Tyr, Pro, Cit. Free amino acid profile of extracted solution from test diet treated with or without strain K-C2 in artificial gastro-intestinal juices showed HyPro, Ser, Gln, His, 1-MetHis, 3-MetHis, theanine, Met, Orn and Lys were significant differences ($p < 0.05$) between the control and treated groups. These results have provided basic and useful informations for further experiments relative to application of strain K-C2 as feed additives for cultured fish.

Introduction

The screening probiotic properties of selected LAB candidaes were examined in previous chapter. Among of the candidates were tested, strain K-C2 showed its ability grew well in broth MRS media with the wide range of pH (2.0-9.0) or NaCl concentration (0-5 %), it had the antagonistic

activities against all tested pathogens and highest tolerance in artificial gastrointestinal juices at pH 2.0. Thus, strain K-C2 was to be selected as the most suitable probiotic candidate for further experiments.

Many diverse bacterial phyla have synthesized and released D-amino acids; these amino acids have been known to be utilized as nutrients to support bacterial growth, to regulate bacterial spore germination, and to be components of the bacterial cell wall (Cava et al. 2011). Besides, the variety of amino acids present in microorganisms have made their detection and identification valuable for differentiating and identifying isolated bacteria. Amino acids have been also useful as chemical markers for detecting, without prior culture, the presence of bacteria in complex matrices such as mammalian body fluids and tissues (Fox et al. 1990). Amino acids from synthesization and releasing of bacterial probiotic may be also one of the sources of nutrients for the host by using as supplementary diet; and several amino acids released by gut bacteria can serve as precursors for the synthesis of short-chain fatty acids (Cava et al. 2011).

On the other hand, LAB are characterized as Gram-positive, usually nonmotile, non-sporulating bacteria that produce lactic acid as a major or sole product of fermentative metabolism (Ringø and Gatesoupe 1998), and they may produce organic acids from sugars fermentation (Hedberg et al. 2008). The use of dietary organic acids in aquaculture has been the focus of much research and commercial interest recently; the use of organic acids in aquafeeds, with emphasis on its impacts on growth, nutrient utilization, mineral availability, gut microbiota and disease resistance of aquatic animal has been reported in several studies (Wing-keong and Chik-boon 2016).

In this chapter, strain K-C2 was identified by the 16s rRNA sequence and characterized by morphological and biochemical observation. Also, analysis of amino acid components from bacterial cell, extracellular enzymes profile and sugar fermentation of strain K-C2 were examined.

Materials and methods

Identification of strain K-C2

DNA extraction and amplification of 16S rRNA gene by polymerase chain reaction (PCR)

Total genomic DNA was extracted from 1-2 ml of strain K-C2 suspension from the overnight culture growth in MRS broth following Vural and Ozgun (2011) with slight modification. Briefly, the cell suspensions in 400 µl of TE buffer (10 mM Tris–HCl [pH 8.0], 1 mM EDTA [Ethylenediaminetetraacetic acid]) were treated with 20 µl protease K (6 mg/ml) and 80 µl sodium dodecyl sulfate (5 %, w/v) at 60 °C for 1-2 h. The lysates were subsequently extracted with phenol/chloroform and precipitated with 100 % ethanol and 3M sodium acetate. The DNA was pelleted by centrifugation at 15000 rpm, 4 °C for 10 min. The DNA pellet was then washed in 70 % ethanol. The dried DNA pellet was re-suspended in 50-100 µl of TE buffer and stored at - 20 °C. Agarose gel (0.8 %, w/v) electrophoresis containing SYBR® Safe DNA gel stain (Invitrogen, Thermo Fisher Scientific Inc., USA) was run at a constant voltage of 100 V. The gel was examined under the LED transilluminator to visually assess the DNA molecular size and concentration.

The 16S rRNA gene region was amplified with the universal primers pair: forward primer-27f (5′ AGAGTTTGATCCTGGCTCAG 3′) and reverse primer-1525r (5′ AAAGGAGGTGAT CCAGCC 3′). A mixture (total volume: 20 µl) containing 2 µl of 10 × Ex *Taq* buffer, 0.4 µl deoxynucleoside triphosphate (dNTP) (10 mM), 0.2 µl of universal forward and reverse primers (1 µM in final concentration), 1 µl of template DNA (1 ng/µl) and 0.1 µl of Ex *Taq* DNA polymerase (5 U/µl) included in the Takara Ex *Taq* kit (Takara Bio Inc., Japan). Conventional PCR amplification from this mixture was performed in a Takara PCR Thermal

Cycler (Takara Bio Inc., Japan) following PCR reaction procedures: initial denaturation of DNA for 2 min at 95 °C, then 30 cycles of denaturation of DNA for 1 min at 95 °C, annealing for 1 min at 55 °C, extension for 1 min at 72 °C and a final incubation for 10 min at 72 °C. The PCR products were analyzed by electrophoresis on a 0.8 % (w/v) agarose gel stained with SYBR® Safe DNA gel stain (Invitrogen, Thermo Fisher Scientific Inc., USA) in 1x TAE buffer (40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA) at 100 V for 25 min. The sizes of the DNA fragments were estimated using standard 1-kb DNA ladder makers (Shinkouseiki, Co., Ltd., Fukuoka, Japan). Then the PCR products were purified by the polyethylene glycol precipitation method (Schmitz and Riesner 2006) and sequenced with an Applied Biosystems 3730xl DNA analyzer (Thermo Fisher Scientific Inc., Yokohama, Japan).

Also, PCR amplification of DNA templates of the selected candidates was carried out using species-specific primers (forward LacF 5' GTACTTGTACCGACTGGAT 3' and reverse LacreR 5' GGGATCATCTTTGAGTGAT 3') (Pu et al. 2002). The prepared mixture used for PCR amplification was as described above, and the cycle conditions were 94 °C, 40 s; 58 °C, 40 s; 72 °C, 1 min (35 cycles).

Accession number of nucleotide sequence, Basic Local Alignment Search Tool (BLAST) analysis and phylogenetic analysis

The nucleotide sequence data of 16S rRNA of strain K-C2 were deposited in the database of the National Center for Biotechnology Information (NCBI). To determine the closest known relatives of the partial 16S rRNA gene sequences obtained, the public data libraries (GenBank) were searched using the BLAST (Saeedi et al. 2015).

The obtained nucleotide sequence data were added to the aligned sequence data set of LAB through an alignment process using CLUSTALX version 1.83 (Thompson et al. 1997). The

corresponding sequences from other bacteria were obtained from the GenBank databases in NCBI. Pairwise evolutionary distances were calculated according to the Kimura 2-parameters model method (Kimura 1980). The phylogenetic trees were constructed by the neighbor-joining statistical method (Saitou and Nei 1987) with Mega version 7.0 (Kumar et al. 2016).

Morphological observation of strain K-C2 with a scanning electron microscope (SEM)

Preparation of bacterial cells for the SEM

Strain K-C2 was cultured in an MRS broth at 28 °C for 48 h. The cultured cells were washed by centrifugation (3,000×g, 5 min) with 0.85 % (w/v) NaCl solution three times. The washed cells were re-suspended in 1 ml of a 2 % glutaraldehyde solution in a 1.5-ml Eppendorf tube. The tube was softly shaken at room temperature for 2 h for fixation. After fixation, the tube was centrifuged at 3,000×g for 10 min, and the supernatant was removed. In the process of dehydration, 1 m of 50 % (v/v) ethanol solution was added to the tube and kept at room temperature for 10 min. The tube was centrifuged at 3,000×g for 5 min, and the supernatant was removed. Subsequently, 1 ml of 99.5 % (v/v) ethanol solution was added to the tube and centrifuged again, and the supernatant was removed. To replace a solvent, 1 ml of t-butyl alcohol was added and kept at room temperature for 10 min. The tube was centrifuged at 1,000×g for 5 min, and the supernatant was removed. This process was repeated three times. A small amount of t-butyl alcohol was added, and the tube was lyophilized with a freeze dryer lyophilizer (ES-2030, Hitachi, Tokyo, Japan).

Observation of cell morphology with an SEM

A small amount of the lyophilized cells was taken and mounted directly on an aluminum SEM stub with carbon conductive tape. The samples were observed and photographed with an SEM (SU3500, Hitachi, Tokyo, Japan).

Biochemical observation of strain K-C2 using a catalase activity test

Strain K-C2 was incubated on MRS agar at 28 °C for 24 h. After the colony grew on the agar plate, it was picked up using a sterilized loop and then dipped in 3 % H_2O_2 solution; no bubble appearance indicated that the tested strain was a catalase-negative bacterium (Taylor and Achanzar 1972).

Sugar fermentation of strain K-C2

Ten dietary sugars were tested including α-cellulose, fructose, galactose, glucose, lactose, maltose, mannose, rhamanose, ribose, sucrose, trehalose and xyclose. These above sugars and the pH indicator (bromocresol purple) were prepared in 2 % aqueous solutions and sterilized by using membrane filter (DISMIC-13cp, pore size 0.2 μm, Advantech MFS, Japan). Also, a modified MRS broth (pH 6.8) without addition of any sugar was employed in the fermentation assay (Hedberg et al. 2008).

The fermentation assay was performed in 96 well-microtiter plate (Home of tissuse culture TPP®, Switzerland); 50 μl of modified MRS broth was mixed with 50 μl of each of the 12 sugars in the wells; 5 μl of pH indicator was mixed with 5 μl each of the washed cell suspension of bacterial strains and then 10 μl of this mixture was added to each sugar in the wells. The plates were incubated at 28 ºC and were examined for color changes after 24, 48 and 72 h of incubation. Each combination of bacteria and sugar was processed in triplicate; the bacteria were replaced by

PBS as negative control. The indicator turns purple at pH > 6.8 was considered negative, the pH indicator between 5.2 and 6.8 was considered weak, the indicator turns yellow at pH < 5.2 was considered positive reaction. (Hedberg et al. 2008)

Analysis the extracellular enzymes profile of strain K-C2

The profile of extracellular enzymes of strain K-C2 was carried out by using API ZYM kit following the manufacture's instructions (bio Merieux). Briefly, 65 µl of bacterial cell suspension (10^8 cfu/ml) was prepared before it was added into each cupule of the strip; place the plastic lid on the tray and incubate generally for 4-4.5 h at 37 °C (optimum temperature). After incubation, adding 1 drop of ZYM A reagent and 1 drop of ZYM B reagent to each cupule. Let the color develop for at least 5 min. Reading the reactions and record the results on the result sheet. A value ranging from 0-5 can be assigned, corresponding to the colors developed: 0 corresponds to a negative reaction, 5 to a reaction of maximum intensity and values 1, 2, 3 or 4 are intermediate reactions depending on the level of intensity (3, 4 or 5 being considered as positive reactions).

Analysis of amino acid profile from cell of strain K-C2

Preparation of sample from strain K-C2 dry cell

Strain K-C2 was cultured in 200 ml MRS (de Man, Rogosa & Sharpe) broth medium at 28 °C for 24 h. Cells were then collected by centrifugation (5,000$\times$$g$ for 10 min, 25 °C), washed by 2 times and suspended in 40 ml of a sterile physiological saline solution (0.85% NaCl [w/v]) as a washed cell suspension. The bacterial densities were estimated by optical density (OD) at 600 nm and total viable counts were estimated by the agar plate counting method with MRS agar plates.

The washed cell suspension above was centrifugated (5,000×*g* for 10 min, 25 °C) once again for collecting the sediment. The sediment was freezed at –80 °C for 1 h before it was lyophilized at –45 °C, 20 Pa by Freeze Dryer (FDU-1200 EYELA, Tokyo Rikakikai Co., LTD) for 12 hrs at least.

The dry cell (approximate 3 mg) was mixed with 1 ml of HCl 6N and 0.02 % ß-Mercapto-ethanol and heated at 110 °C for 22 h. Then the mixture was transferred to the volumetric flask, and HCl 0.02 N was added up to 10 ml. This solution was centrifuged at 3,000×*g* for 10 min, then the supernatant was collected and stored at –20 °C as an extract solution for further amino acid analysis.

Amino acid analysis by liquid chromatography-mass spectrometry

Calibration standards were prepared by mixing Wako Amino Acids Mixture Standard Solutions Type AN-II (1.25 µmol/ml concentrations of *o*-phosphoserine, taurine, *o*-phosphoethanolamine, L-2-aminoadipic acid, DL-2-aminobutyric acid, L-cystathionine; 2.5 µmol/ml concentrations of L-aspartic acid, L-hydroxyproline, L-threonine, L-serine, L-glutamic acid, L (-) -proline, glycine, L-alanine, L-citrulline, , L-valine, L (-) -cysteine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, β-alanine, and DL-3-aminoisobutyric acid; 50 µmol/ml concentration of urea, and 6.25 µmol/ml concentration of sarcosine) and Type B (4-aminobutyric acid, 2-aminoethanol, ammonium chloride, DL-plus- allo-∂-hydroxylysine, L-ornithine, L-lysine, L-1-methylhistidine, L-histidine, L-3-methylhistidine, L-anserine, L-carnosine, and L-arginine, in which the concentration of each amino acid was 2.5 µmol/ml) and solutions (5 µmol/mL) of tryptophan, glutamine, asparagine, theanine and cysteine that were prepared from weighed standards in 0.1 N HCl. This stock (S1) was diluted with distilled water by 3/5 (S3), 1/5 (S5) and 1/20 (S7) to get a series of standard solutions.

The amino acid analysis of the samples was performed according to the method of Le et al. (2014) with slight modification. Briefly, 50 µl of the extract solution and each standard solution were mixed with 50 µl of distilled water and 100 µl of acetonitrile (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in micro tubes with tight caps, and were subjected to the measurement of amino acids by LC-MS (UF-aminostation, Shimazdu Co., Kyoto, Japan) consisting of a vacuum pump (oil mist filter EMF20, Edwards Co. Ltd., Crawley, UK) and a gas generator (N2 model supplier 24F, System Instruments Co. Ltd., Tokyo, Japan).

Samples were injected into a shim-pack column (UF-Amino Shimazdu Co., Kyoto, Japan) (100 mmL cm × 2.1 mm i.d., pore size 2 µm) by a SIL-20AC$_{PT}$ autosampler set at 0.5 µl for the injection sample. Analyses were performed in the mobile phase at a flow rate of 0.3 ml/min. The mobile phase was a mixture of APDSTAG Wako eluent (Wako Pure Chemical), APDSTAG Wako borate buffer (Wako Pure Chemical), acetonitrile (Wako Pure Chemical) and pure water. The amount of each amino acid was calculated from the differences between the peak areas of the extract samples and standard solutions.

Analysis of free amino acid profile of extracted solution from test diet treated with or without strain K-C2 in artificial gastro-intestinal juices

Preparation of washed bacterial cell suspension and test diet

Strain K-C2 was cultured in 5 ml MRS (de Man, Rogosa & Sharpe) broth medium at 28 °C for 24 h. Cells were then collected by centrifugation (5,000×g for 10 min, 25 °C), washed and suspended in 1.5 ml of a sterile physiological saline solution (0.85% NaCl [w/v]) as a washed cell suspension. The bacterial densities were estimated by optical density (OD) at 600 nm and total viable counts were estimated by the agar plate counting method with MRS agar plates.

Afterwards, 750 µl of the washed cell suspension was mixed with approximately 1 g of commercial diet in a glass tube, and kept for drying on a clean bench for 4 h.

Sample preparation from test diet

Approximately 1 g of diet treated with or without probiotic candidate strain K-C2 (6.4×10^{10} cfu/g diet) was immersed in 2 ml of the artificial gastric juice (0.3 % [w/v] of pepsin [Nacalai Tesque, Inc., Kyoto, Japan] dissolved in 0.1 M glycine-HCl buffer at pH 3.0), and the supernatant was pipetted after 3 h. The sediment was continuously immersed in 2 ml of the artificial intestinal juice (0.1 % [w/v] of pancreatin [Sigma-Aldrich, USA] and 0.45 % [w/v] gall powder [Wako Pure Chemical Industries, Ltd., Osaka, Japan] dissolved in 0.1 M Tris-HCl buffer at pH 8.0) for 3 h, and the supernatant was collected. These supernatants were centrifuged at $5,000 \times g$ for 5 min to remove the debris and treated with an equal volume of 10 % (v/v) trichloroacetic acid solution (Nacalai Tesque, Inc., Kyoto, Japan). The above mixtures of these supernatants and trichloroacetic acid solution were centrifuged at $10,000 \times g$ for 15 min after being kept at room temperature for 10 min and then overnight at 4 °C. Finally, the supernatants were collected and stored at –20 °C as extract solutions for further amino acid analysis by the method described in previous section.

Results

Identification of strain K-C2

Partial length of the 16S rRNA gene nucleotide sequence (1438 bp) of strain K-C2 was deposited in the data bank with accession number LC212968. Strain K-C2 was identified based on

morphological observation by SEM (ovoid shape) (Fig. 9), biochemical observation (catalase negative), and phylogenetic analysis. In the phylogenetic tree based on the 16S rRNA gene sequences, strain K-C2 was located in the well-supported monophyletic cluster of *Lc. lactis* (Fig. 8). The 16S rRNA gene sequence of strain K-C2 showed an identity of 99 % with those of *Lc. lactis* FMA558 (accession number HQ721275.1) through the BLAST analysis. Also, the PCR results were the same as above when we applied the species-specific primer set, which consisted of the forward LacF and reverse LacreR, to confirm the species name of this candidate strain (Fig. 7).

Sugar fermentation assay of strain K-C2

As the results showed in the Table 2, strain K-C2 had ability to ferment fructose, galactose, glucose, lactose, maltose, mannose, ribose, sucrose, trehalose and xyclose resulting in a final pH < 5.2; it could not ferment α-cellulose after 24 h of incubation; and it was slightly active to ferment rhamanose in pH value ranging between 5.2 and 6.8 after 48 h of incubation.

The extracellular enzymes profile of strain K-C2

As the results showed in the Table 3, strain K-C2 had only strong Naphthol-AS-BI-phosphohydrolase activity; it showed weak esterase (C4), esterase lipase (C8), lipase (C14), acid phosphatase and β- glucosidase activity, and it had no activity with other enzymes.

Amino acid profile from cell of strain K-C2

The amino acid composition of strain K-C2 was presented in the Fig. 11. Under the condition of hydrolysis, about 14 amino acids were determined from dry bacterial cells including Leu, Lys, Met, Phe, Thr, Val, Ala, Asp, Glu, Gly, Ser, Tyr, Pro, Cit.

Free amino acid profile of extracted solution from test diet treated with or without strain K-C2 in artificial gastro-intestinal juices

After the diets were treated with or without *Lc. lactis* strain K-C2 LC212968 in artificial gastro-intestinal juices, the amino acid profile was determined and is shown in Fig. 12. As a result, HyPro, Ser, Gln, His, 1-MetHis, 3-MetHis, theanine, Met, Orn and Lys showed significant differences ($p < 0.05$) between the control and treated groups.

Discussions

Following the research of Pu et al. (2002), the species-specific primer set including the forward LacF and reverse LacreR primer which was designed to specifically detect the species *Lc. lactis*, that was agreed for strain K-C2 in the result of our study; also 16S rRNA of this strain was closely related to *Lc. lactis* with 99 % similarity in homology research. The morphological observation and catalase activity also indicated strain K-C2 as *Lc. lactis*.

In other hand, understanding the composition of AAs in bacterial cell is one of the way to understand the incorporation of AA into bacterial cell through AA transports. From the review of Dai et al. (2011): the gut bacteria use different strategies in the utilization of different AA and nitrogen source whereas the extracellular environment might affect the patterns of bacterial AA utilization. These AAs are either nutritionally essential or functionally important to the animals. And the transport and incorporation of certain AA into bacterial cells may vary with AA structure.

In ruminants, bacterial proteins synthesized in the rumen are digested and the resultant AA or small peptides are absorbed by the small intestine. This process has nutritional advantages especially in the re-assimilation of ammonia derived from the breakdown of proteins and AA. However, in humans and mono-gastric animals, the proteins in gut bacteria mainly come from the assimilation of dietary and host-derived AA. Because the small intestine is the major site for nutrient digestion and absorption and because the absorption of AA is limited in the large intestine, AA utilization by bacteria in the gastro-intestinal tract represents an event of nutritional waste in humans and mono-gastric animals. After probiotic is provided to the host, it will become a part of gut microflora of host, therefore, the studying on the AAs content in bacterial cell of strain K-C2 to find which AA might be "essential" for the optimal growth of this bacteria, also improving the efficiency of utilization of dietary protein and AA in aquatic fed. Furthermore, the active of potential probiotic adding to the diet in vitro also provided a part of information about the utilization and metabolism AA that have already carried out in this study.

Genus *Lactococcus* is one of famous lactic acid bacteria in fermented food industry. They have been also used as probiotics for human and animals. After probiotics are ingested by host, they become part of the intestinal microflora; and the carbonhydrate nutrients are the main source of their nutrients (Floch 2010). So the ability fermentation of probiotic for a variety of sugars will be useful information for evaluating carbohydrate sources in feed ingredient of aquatic animals are suitable for using these probiotics or not. Strain K-C2 is able to ferment 10 per 12 kind of sugars in this study, therefore it has been recognized as suitable probiotic to apply in aquaculture.

The initial screening and selection of probiotics includes testing of the following important criteria: phenotype and genotype stability, including plasmid stability; carbohydrate and protein utilization patterns; acid and bile tolerance and survival and growth; intestinal epithelial adhesion properties; production of antimicrobial substances; antibiotic resistance patterns; ability to inhibit known pathogens, spoilage organisms, or both; and immunogenicity (Harzallah and

Belhadj 2013). To be a potential probiotic, *Lc. lactis* strain K-C2 had shown the tolerance into gastrointestinal juices or ability adhension to mucosal surface as functional criteria in the previous chapter. *Lc. lactis* strain K-C2 may use several different carbohydrate sources to produce acid by fermentation, and it has extracellular enzyme activities with different levels. By comparing the result of 14 amino acids were found from amino acid profile releasing from strain K-C2 dry cell and 29 amino acids were found from free amino acid profile of extracted solution from test diet treated with or without strain K-C2 in artificial gastro-intestinal juices, we recognized that the strain K-C2 may digest the diet and produce several necessary amino acids, especially the content of taurine in diet treated by strain K-C2 displayed higher than that in case the diet without adding this strain. These results indicated strain K-C2 was able to be applied into fish diet for marine cultured fish as live probiotic.
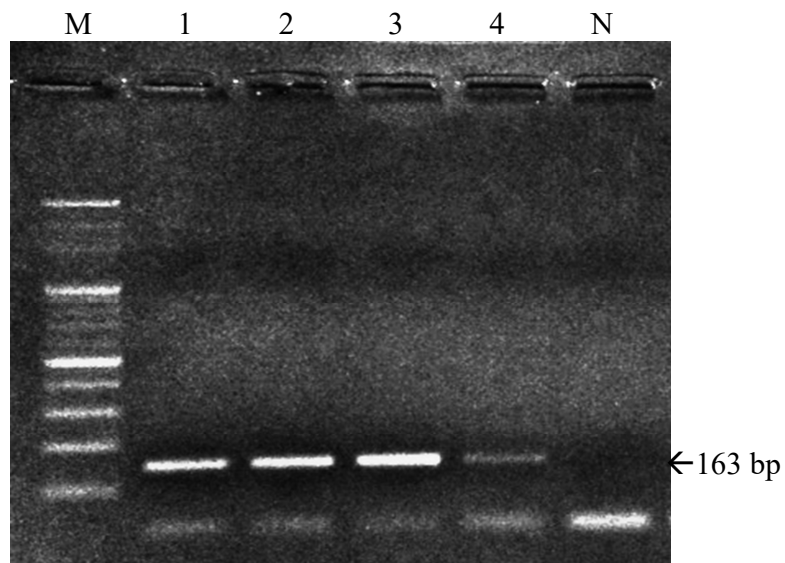
Fig. 7 Polymerase chain reaction (PCR) identification of strain K-C2: PCR amplification products using the *L. lactis* species-specific primer LacF and LacreR. Lanes: M, size marker (100 bp DNA ladder); 1, 2, 3, 4, PCR products from strain K-C2 templates; N, negative control (no template in PCR). Sizes (bp) of PCR products are indicated.
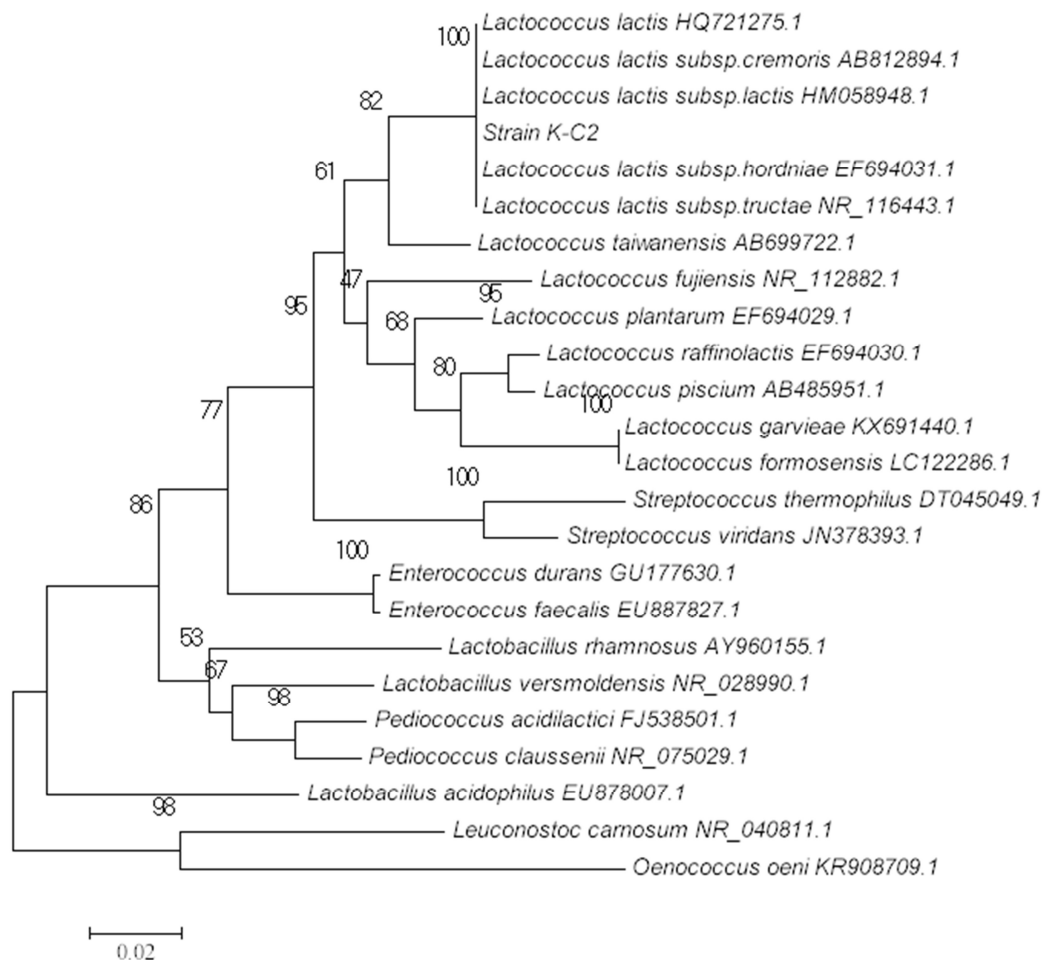
Fig. 8 Phylogenetic tree was constructed by the neighbor-joining method based on the partial sequence of the 16S rRNA gene of strain K-C2 and corresponding region in those for the authentic bacterial genes. Numbers at branches denote the bootstrap percentages of 1000 replicates. The scale at the bottom indicates the evolutionary distance of nucleotide substitutions per site
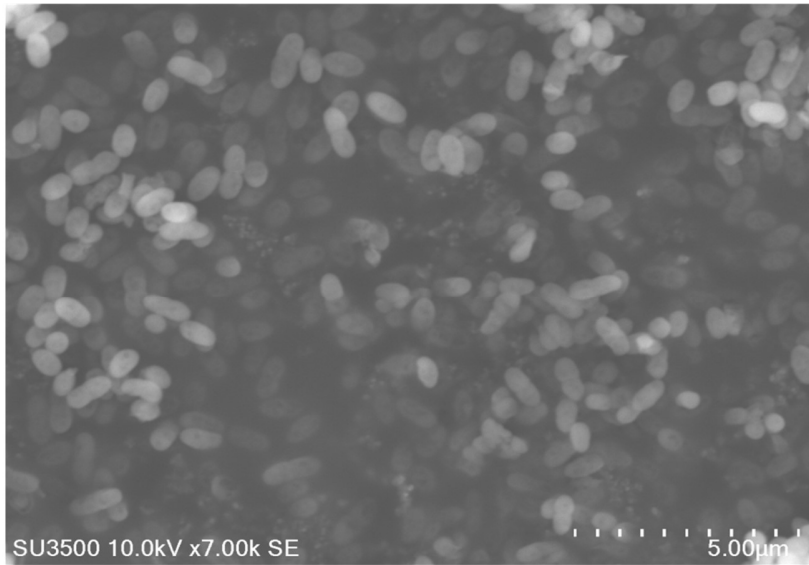
Fig. 9 Morphological observation of strain K-C2 with a scanning electron microscope (scale bar, 5 μm)

Table 2 Fermentation of sugars in an anaerobic atmosphere by strain K-C2

Sugars	Strain K-C2
α-cellulose	–––
Fructose	+++
Galactose	+++
Glucose	+++
Lactose	+++
Maltose	+++
Mannose	+++
Rhamanose	–±±
Ribose	+++
Sucrose	+++
Trehalose	+++
Xyclose	+++

Symbols indicate: "–" negative reaction (pH >6.8), "±" weak reaction (pH 5.2 to 6.8), "+" positive reaction (pH < 5.2) after 24, 48 and 72 h, respectively.

Table 3 The profile of extracellular enzymes of strain K-C2. The number indicated the grade of coloration in APIZYM reading scale (Fig. 10)

No.	Enzymatic activity	Strain K-C2
1	Blank	0
2	Alkaline phosphatase	0
3	Esterase (C4)	1
4	Esterase lipase (C8)	1
5	Lipase (C14)	1
6	Leucine arylamidase	0
7	Valine arylamidase	0
8	Cystine arylamidase	0
9	Trypsin	0
10	Chymotrypsin	0
11	Acid phosphatase	1
12	Naphthol-AS-BI-phosphohydrolase	5
13	α-Galactosidase	0
14	β-Galactosidase	0
15	β-Glucuronidase	0
16	α- Gtucosidase	0
17	β- Glucosidase	1
18	N-acetyl-β-glucosaminidase	0
19	α- Mannosidase	0
20	α- -Fucosidase	0

Fig 10. API ZYM reading scale

Fig. 11 Amino acid components of *Lactococcus lactis* K-C2 cell
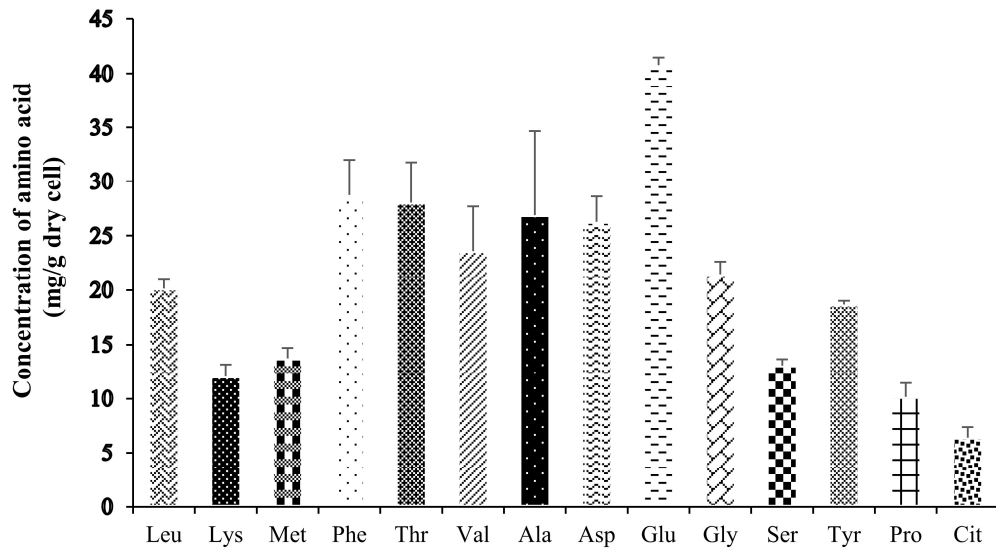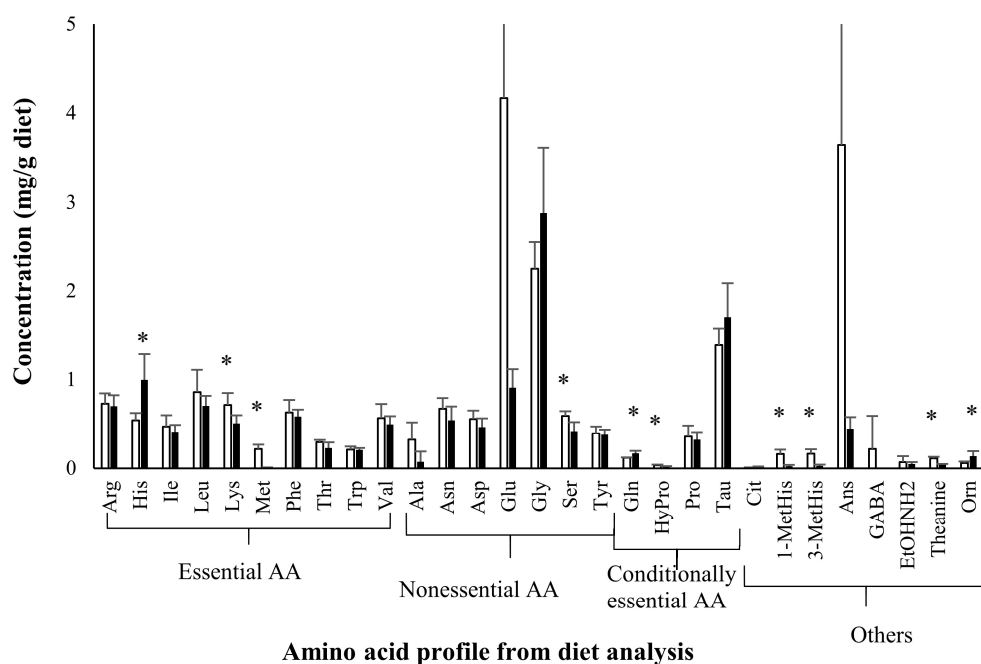
Fig. 12 Free amino acids profile analysis of extracted solution from diet treated with or without *L. lactis* strain K-C2 LC212968 in artificial gatro-intestinal juices. Data (mean ± SD) in the same amino acid with "*" significantly different ($p <$ 0.05) among the control and treated group.

Chapter 4: Effect of *Lactococcus lactis* K-C2 on the growth performance, amino acid content and gut microflora of amberjack, *Seriola dumerili*

Abstract This study aimed to evaluate the effect of *Lactococcus lactis* K-C2 on the growth performance, microbial diversity and release of free amino acid in the intestinal tract, and the edible part of young amberjack, *Seriola dumerili*. Fish were fed a diet with or without strain K-C2 (2 x 10^{10} cfu/g feed) for 25 days. The results indicated that the growth performance of fish in the treated group was significantly higher than those in the control group ($p < 0.05$). The amount of five amino acids (aspartate, sarcosine, taurine, alanine, arginine) in the gut content and thirteen of twenty one amino acids in edible part of fish in the treated group were significantly higher ($p < 0.05$) than those in the control group. *Sphingomonas*, *Propionibacterium* and *Mycobacterium* were observed in gut microflora of fish in both the control and treated groups. *Staphylococcus* and *Kocuria* were detected in one sample from the control and treated groups; *Acinetobacte*r and *Acidobacteria* were found in one sample from the control group. *Lc. lactis* was only found in one sample in the treated group. In conclusion, the dietary administration of probiotic *Lc. lactis* stimulated growth, reduced feed consumption, and improved the nutritional value of cultured amberjack.

Introduction

Amberjack, *Seriola dumerili* is a highly active fish belonging to the genus *Seriola* that is globally distributed in tropical and subtropical area. This species was one of the first intensive marine aquaculture in 1930. It has been an economically important marine cultured fish in Japan (Makino 2011). However, the increase of the number of fish farms around Japan caused frequent disease outbreaks (Nakada 2008). The bacteria have been reported as common pathogens in

Seriola, vaccination and other treatment methods (antibiotics and chemicals) have been studied for protecting fish to against nocardiosis, mycobacteriosis, lactococcicosis and streptococcosis (Itano et al. 2006; Kato et al. 2011; Nakajima et al. 2014); these methods are expensive, and can cause environmental destruction or food safety problems.

The use of probiotics as a live microbial feed supplement has been shown to beneficially affect the host animal by improving its intestinal balance, growth rate and disease resistance in several studies (Fuller 1989; Wang et al. 2008; Saini et al. 2014; Nguyen et al. 2017). *Lactococcus lactis* is one of the most important species of lactic acid bacteria in the fermented dairy industry. This species is a nonpathogenic AT-rich gram-positive bacterium closely related to the genus *Streptococcus* (Nomura et al. 1999; Bolotin et al. 2001). Several strains of this species have been isolated and applied as probiotics in aquaculture for preventing bacterial disease, improving the growth rate for both fresh water and marine fish (Bernbom et al. 2006; Zhou et al. 2010; Sequeiros et al. 2015; Nguyen et al. 2017). *Lc. lactis* could improve the autochthonous microbial diversity along the gastrointestinal tract of grouper, *Epinephelus coioides* (Sun et al. 2012). The benefit of *Lc. lactis* on the gut microbiota has been also observed in white shrimp, *L. vannamei* (Adel et al. 2017), and in human flora-associated rats (Bernbom et al. 2006).

Gastrointestinal microbiota have a role in synthesizing vitamins, essential growth factors and digestive enzymes during digestion and the absorption of nutrients in fish (Nayak 2010). The probiotic role of bacteria may be displayed by the amino acid metabolism directly or indirectly via bacteria-bacteria interactions and cross feeding (Dai et al. 2015). *Lc. lactis* has been reported as an beneficial bacteria species for amino acids biosynthesis (valine, leucine, glutamate, aspartate and sulfur amino acid) *in vitro* conditions (Goupil-feuillerat et al. 1997; Lapujade and Loubiere 1998; Dudley and Steele 2001; Sperandio et al. 2005).

Therefore, this study aimed to investigate the effect of *Lc. lactis* K-C2 on the growth performance, the free amino acid profile and microbial diversity in the gut content, and the

nutritional value of cultured amberjack.

Materials and methods

Microorganism

Lc. lactis K-C2 used in this study was originally isolated from fermented vegetables at Laboratory of Marine Environmental Microbiology, Faculty of Agriculture, University of Miyazaki, Japan. This strain was identified based on 16S rRNA gene sequencing (GenBank accession number LC212968) (Linh et al. 2018).

Experimental design for feeding trial of amberjack, *Seriola dumerili* with *Lc. lactis* K-C2

Source of the fish

Healthy amberjack, *S. dumerili* were obtained from a commercial fish farm (Miyazaki Prefecture, Japan), and transported to the Laboratory of Marine Environmental Microbiology, Faculty of Agriculture, University of Miyazaki, Japan. Before the experiments were performed for one week, the fish were maintained in artificial seawater (Sea Life, Marine Tech Co., Tokyo, Japan) with a water temperature, salinity, pH, and dissolved oxygen of around 22 ℃, 30 ‰, 7.0-8.0, and 8–9 mg/l, respectively; and they were fed with a commercial diet (Hamachi EP d5, Feed One Co Ltd., Kanagawa, Japan). The nutrient composition of a commercial diet containing of 10.5% ± 0.6 moisture, 37.7% ± 1.2 crude protein, 16.4% ± 0.8 crude lipid and 11.4% ± 0.1 crude ash were defined at Laboratory of Marine Environmental Microbiology, Faculty of Agriculture, University of Miyazaki, Japan (un-published data).

Preparation of experimental diet

Strain K-C2 was cultured in 500 ml MRS broth at 28 °C for 24 h. Cells were then collected by centrifugation (5,000×g for 10 min, 25 °C). The pellet was washed and re-suspended in 150 ml of a sterile physiological saline solution (0.85 % NaCl [w/v]) as a washed cell suspension. The bacterial densities were estimated by OD at 600 nm, and total viable counts were estimated by the agar plate counting method with MRS agar plates.

One hundred and fifty ml of the washed cell suspension was mixed with 1 kg of commercial diet (Hamachi EP d5, Feed One Co Ltd., Kanagawa, Japan) in a stainless tray by hand wearing gloves to avoid contamination, and the test diet was dried on a clean bench for 4 h. The test diet was kept at –20 °C for further experiments. The proximate composition of the experimental diets was analyzed as described below, and the result is shown in Table 4.

Experimental design

There were two treatment groups (five fishes per group) where the fish received diet with or without strain K-C2. The control group fed a commercial diet without strain K-C2. The another treatment group fed a commercial diet containing strain K-C2 (2 x 10^{10} cfu/g feed). Each treatment group was conducted in triplicate. The experiment was performed in 30-l plastic tank using closed rearing system. Water was exchanged 30 % daily at 9:00 am. Aeration was supplied through an air stone to each tank. The test fish with an average body weight of approximately 65.4 ± 7.4 g were starved for two days, then they were fed with the experimental diets at 1 % of the average fish body weight once per day for 25 days. The water quality parameters were checked daily at 14:00 pm by D-55 Meter (Horiba, Ltd., Japan) and maintaining the dissolved oxygen 8–9 mg/l, water temperature 20–22 °C, pH 7-9, salinity 30–31 ‰ during the feeding trial.

At the end of experiment, the fish were starved for 24 h prior to sampling. All fish per treatment groups were anesthetized with 2-phenoloxyethanol (Nacalai Tesque, Inc., Kyoto, Japan) at 300 ppm (v/v), then the weight and length of each fish from each tank were checked for growth performance analysis.

Three fishes from each experimental group were randomly picked up. The liver, intestinal tract and fish muscles were dissected from the fish body, and weighed individually to calculate the hepatosomatic index. All fish were filleted, and the edible part consists of all the muscle part on the fish body was store at -80 °C for further experiment. The edible parts were completely homogenized with a food processor before analyzing proximate composition.

The intestinal tract of all experimental amberjack was aseptically opened with a sterile scalpel, and the gut content was put into a 1.5-ml Eppendorf tube. Samples were homogenized using a plastic homogenizer and stored at –20 °C for further free amino acid and intestinal microflora analysis.

Growth performance analysis

Growth performance was identified by below parameters:

Weight gain (WG, %) = 100 x (final body weight –initial body weight)/initial body weight; feed intake = (dry diet given–dry remaining diet recovered) x no. of fish; feed conversion ratio (FCR) = dry feed intake (g)/live WG (g); condition factor (K factor) = (body weight/(body length)^3) x 100; hepatosomatic index (%) = 100 x (weight of liver/weight of fish) (Hossain et al. 2017).

Proximate composition analysis of the edible part of fish

The edible parts of three fishes from three tanks of each experimental group were used for the

proximate composition analysis.

Crude protein was analyzed by the Kjeldahl method. Crude lipid was analyzed as described by Folch et al. (1951). Crude ash and moisture were analyzed according to the guidelines of the Cunniff (1997).

Amino acid analysis of the edible part of fish

The edible part of amberjack (approximate 0.02 g) was mixed with 1 ml of HCl 6N and 0.02 % ß-Mercaptoethanol and heated at 110 °C for 22 h after it was crushed by mortar and pestle. Then the mixture was transferred to the volumetric flask, and HCl 0.02 N was added up to 10 ml. This solution was centrifuged at 3,000×g for 10 min, then the supernatant was collected and stored at –20 °C as an extract solution for further total amino acid analysis.

Preparation of standard solution

The standard solution was prepared by mixing Wako Amino Acids Mixture Standard Solutions Type AN-II (1.25 µmol/ml concentrations of *o*-phosphoserine, taurine, *o*-phosphoethanolamine, L-2-aminoadipic acid, DL-2-aminobutyric acid, L-cystathionine; 2.5 µmol/ml concentrations of L-aspartic acid, L-hydroxyproline, L-threonine, L-serine, L-glutamic acid, L (-) -proline, glycine, L-alanine, L-citrulline, L-valine, L (-) -cysteine, L-methionine, L-isoleucine, L-leucine, L-tyrosine, L-phenylalanine, β-alanine, and DL-3-aminoisobutyric acid; 50 µmol/ml concentration of urea, and 6.25 µmol/ml concentration of sarcosine) and Type B (4-aminobutyric acid, 2-aminoethanol, ammonium chloride, DL-plus- allo-∂-hydroxylysine, L-ornithine, L-lysine, L-1-methylhistidine, L-histidine, L-3-methylhistidine, L-anserine, L-carnosine, and L-arginine, in which the concentration of each amino acid was 2.5 µmol/ml) and solutions (5 µmol/ml) of

tryptophan, glutamine, asparagine, theanine and cysteine that were prepared from weighed standards in 0.1 N HCl. The series of concentration of this stock (S1) was diluted with distilled water by 3/5 (S3), 1/5 (S5) and 1/20 (S7) and the standard solution curve was identified.

Amino acid analysis

The amino acid analysis of the samples was performed by liquid chromatography-mass spectrometry (LC-MS) according to the method of Le et al. (2014) with slight modification. Briefly, 50 µl of the extract solution and each standard solution were mixed with 50 µl of distilled water and 100 µl of acetonitrile (Wako Pure Chemical Industries, Ltd., Osaka, Japan) in micro tubes with tight caps, and were subjected to the measurement of amino acids by LC-MS (UF-amino station, Shimazdu Co., Kyoto, Japan) consisting of a vacuum pump (oil mist filter EMF20, Edwards Co. Ltd., Crawley, UK) and a gas generator (N2 model supplier 24F, System Instruments Co. Ltd., Tokyo, Japan).

Samples were injected into a shim-pack column (UF-Amino Shimazdu Co., Kyoto, Japan) (100 mml cm x 2.1 mm i.d., pore size 2 µm) by a SIL-20ACPT auto-sampler set at 0.5 µl for the injection sample. Analyses were performed in the mobile phase at a flow rate of 0.3 ml/min. The mobile phase was a mixture of APDSTAG Wako eluent (Wako Pure Chemical), APDSTAG Wako borate buffer (Wako Pure Chemical), acetonitrile (Wako Pure Chemical) and pure water. The amount of each amino acid was calculated from the differences between the peak areas of the extract samples and standard solutions.

Analysis of free amino acid profile in gut content

The gut content of amberjack (0.1 g) was mixed with 1 ml of distilled water in a 1.5-ml Eppendorf tube. The mixture was homogenized using plastic homogenizer, then centrifuged at

3,000×g for 10 min. The supernatant was pipetted into another tube and 10% (v/v) trichloroacetic acid solution was added at a volume equal to the volume of supernatant. The mixture was mixed well and centrifuged at 3,000×g for 10 min. The supernatant was transferred to the volumetric flask, and HCl 0.02 N was added up to 10 ml. This solution was centrifuged at 3,000×g for 10 min, then the supernatant was collected and stored at –20 °C as an extract solution for further free amino acid analysis by the method described above.

Gut microbiota analysis

Gut microbiota of three fishes from three tanks of each experimental group were analyzed by denaturing gradient gel electrophoresis (DGGE). DNA of bacteria in the gut content of each fish was extracted using a commercial Qiagen DNeasy® blood and tissue kit (Qiagen, Germany) according to the manufacturer's instructions.

The 16S rRNA gene was amplified using the primer pairs: forward primer-F984 with a GC clamp (5′ AACGCGAAGAACCTTAC 3′) and reverse primer-R1378 (5′ CGGTGTGTACAAGGCCCGGGAACG 3′) (Heuer et al. 1997). A mixture (total volume: 50 µl) containing 5 µl of 10× Ex *Taq* buffer, 1 µl of deoxynucleoside triphosphate (dNTP) (10 mM), 0.1 µl of universal forward and reverse primers (100 mM), 0.2 µl of Ex *Taq* DNA polymerase (5 U/µl) included in the Takara Ex *Taq* kit (Takara Bio Inc., Japan), 2 µl of MgSO$_4$ (25mM), 1 µl of bovine serum albumin (20 mg/ml) and 5 µl of template DNA (1 ng/µl). Conventional PCR amplification from this mixture was performed in a Takara PCR Thermal Cycler (Takara Bio Inc., Japan) following PCR reaction procedures: initial denaturation of DNA for 2 min at 94 °C, then 34 cycles of denaturation of DNA for 15 s at 94 °C, annealing for 30 s at 55 °C, extension for 30 s at 68 °C and a final incubation for 10 min at 68 °C. The PCR products were checked by electrophoresis on a 0.8% (w/v) agarose gel stained with SYBR® Safe DNA gel stain (Invitrogen,

Thermo Fisher Scientific Inc., USA) in 1x TAE buffer (40 mM Tris-HCl, 20 mM acetic acid, 1 mM EDTA) at 100 V for 30 min. The sizes of the DNA fragments were 400 bp that were estimated using standard 100 bp DNA ladder markers (Shinkouseiki, Co., Ltd., Fukuoka, Japan).

DGGE analysis for PCR products was performed in 6% polyacrylamide gels containing 40% acrylamide–biacrylamide (37.5:1, v/v) and a double denaturing gradient of 50% and 70% formamide and urea 7M. Aliquots of PCR products (200 ng/10 μl) were mixed with 10 μl of 2x loading dye, and the mixtures were loaded on the gel using a Dcode system apparatus (Bio-Rad, Hercules, CA, USA). The electrophoresis was run with 1x Tris-acetate-EDTA (TAE) buffer at a fixed voltage of 50 V for 16 h at 58 °C. After the completion of electrophoresis, the gel was stained with SYBR® Safe DNA gel stain (Invitrogen, USA) in 100 ml of 1x TAE buffer for 30 min and scanned under UV light.

All bands were excised from the gel and put in 1.5-ml Eppendorf tube. Partial gel fragments containing bands were washed in distilled water, and then the excised 16S rRNA gene fragments were re-amplified. PCR products were purified by the polyethylene glycol precipitation method (Schmitz and Riesner 2006) and sequenced with an Applied Biosystems 3730xl DNA analyzer (Thermo Fisher Scientific Inc., MA, USA).

The sequence alignments of 16S rRNA gene were deposited in the database of the National Center for Biotechnology Information (NCBI). To determine the closest known relatives of the partial 16S rRNA gene sequences obtained, the public data libraries (GenBank) were searched using BLAST (Saeedi et al. 2015).

Statistical analysis

All data were presented as means ± SD. The significance of statistical differences was analyzed using the Independent-Sample T test data analysis tool in Microsoft Excel 2016 (version 15.27) at the 5 % level of significance.

Results

Growth performance and proximate composition of the edible part

The final body weight and weight gain of fish in the treated group were significantly higher ($p <$ 0.05) than those of fish in the control group (Table 5), and the FCR of fish in the treated group was significantly lower ($p < 0.05$) than that of fish in the control group (Table 5).

Proximate composition of the edible part and somatic parameters of young amberjack after 25 days of feeding were not significant different in both the control and treated group ($p >$ 0.05) (Table 6).

Amino acids composition in the edible part of fish

After the experimental amberjack were fed a commercial diet treated with or without strain K-C2 for 25 days, total amino acids from the edible parts of the experimental amberjack were determined and are shown in Table 7. In detail, the concentrations of essential, nonessential and conditionally essential amino acids in the treated group were higher than those in the control group; specifically, Ile, Leu, Met, Phe, Trp, Val, Asn, Gly, Ser, Tyr, Gln, HyPro, and Pro in the treated group were significantly higher ($p < 0.05$) than those in the control group, and Lys in the treated group was significantly lower ($p < 0.05$) than that in the control group.

Free amino acid profile in gut content

The composition of free amino acids in the gut content of amberjack after treatment with or without strain K-C2 is shown in Fig. 13. The amino acid contents of Arg, Ala, Asp, Tau and Sar

in the treated group were significantly higher ($p < 0.05$) than those in the control group after 25 days of the feeding trial.

Gut microbiota

About 47 bands from six samples were visible in the DGGE gel (21 bands from three samples of the control group and 26 bands from three samples of the treated group) as shown in Fig. 14. From the sequencing result, *Sphingomonas, Propionibacterium* and *Mycobacterium* were identified in all bands of both experimental groups; *Staphylococcus* and *Kocuria* were detected in one of three samples from the control or treated group; *Acinetobacter* and *Acidobacteria* were each only found in one of three samples from the control group; only *Lc. lactis* was observed in one of three samples from the treated group. Several uncultured bacteria were found in both the control and treated groups (Table 8).

Discussion

The role of *Lc. lactis* as probiotic supplements in the dietary effect has been studied on aquatic animal health. The *Lc. lactis* RQ516 was considered as a growth promoter for tilapia, *Oreochromis niloticus* when added in feed (Zhou et al. 2010). Persian sturgeon (*Acipenser persicus*) fed with *Lc. lactis* JF831150 for a period of 56 days showed better growth performances and immunophysiological responses than those using the feed without addition of *Lc. lactis* (Soltani et al. 2016). The growth rate and disease resistance of olive flounder, *Paralichthys olivaceus* were improved when adding *Lc. lactis* WFLU12 isolated from wild marine fish in diet (Nguyen et al. 2017). Adel et al. (2017) stated *Lc. lactis* were isolated from the intestine of healthy white shrimps, *L. vannamei* that could improve the growth performance and disease

resistance of shrimp. Besides that, according to the result from study of Portman (2017), the increasing of dietary moisture content had a negative effect on the growth of California yellowtail fish after feeding at two levels of moisture (5 % and 50 %), so we may understand that the difference of moisture between two test diets (indicated in Table 1) in this study did not effect on the growth of experimental fish. Additionally, dietary ratio for feeding to fish was calculated as 1 % fish body weight (dry weight basis of diet), and amberjack ate food completely during our feeding. Therefore, it is considered that *Lc. lactis* K-C2 had the ability to improve the growth performance of amberjack after a 25-day feeding trial in this study. The high content of amino acids including Ala, Asp, Arg and Tau in the gut might improve the growth performance of fish. Ala and Asp are known as important energy substrates, especially Ala can stimulate the feeding response of fishes; also, a diet containing arginine was demonstrated as growth- and health-promoting in some fishes (Li et al. 2008). Besides, Tau increased fish growth, improved the lipid digestion, and enhanced the immune system (Salze and Davis 2015). Tau, ß-Ala and Sar were proven to be of major importance in regulating cell volume in several tissues in skate fish (Forster and Goldstein 1979).

In this study, there was no Met content in the solution extracted from the diet treated with strain K-C2 in artificial gastrointestinal juices, however Tau content was higher than that in the solution extracted from the diet treated without this strain. *In vivo* test as mentioned in previous chapter, Tau in the gut content of fish after being fed for 25 days on the diet containing this strain increased significantly in comparison with that of the control group. *Lc. lactis* has no bile salt hydrolase activity to directly liberate Tau or Gly from conjugated bile salt (Begley et al. 2006), then Tau metabolism in fish of this study may be carried out indirectly through the pyruvate, Cys or Met metabolism in the presence of *Lc. lactis* as described in the study of Kanehisa and Goto (2000) (Fig. 15). In this study, *Lc. lactis* K-C2 might convert Met into Tau by an indirect metabolism pathway and increased Tau in the gut content of amberjack. *Lc. lactis* K-C2

produced Met during its metabolism process in agreement of Sperandio et al. (2005) and fish then used this Met product for the synthesis of Tau and to meet its other nutritional requirements.

At this study, we found the contents of 20 indispensable AAs in the edible part of the experimental amberjack from the treated group were higher than those in fish from the control group, including Glu, Asp, Ser, Gly and Thr, which are related to the umami and sweet tastes of food (Tanaka et al. 2010, 2016). This result indicated that providing strain K-C2 to amberjack did not only increase the growth efficiency but also improved the nutritional value of the edible part of cultured fish by supplying several the essential amino acids (Arg, Cys, His, Leu, Lys, Met, Thr, Trp, Tyr, Val), non-essential amino acids (Asp, Ser, Ala) or conditionally essential amino acids (Gln, Glu, Gly, Pro, and Tau) for human nutrition (Wu 2013; Mohanty et al. 2014).

The structure of the gut microflora of amberjack seemed to be very different between the control and treated groups, with the exception that the genera *Acinetobacte*r and *Acidobacteria* disappeared from the treated group, probably because *Lc. lactis* was provided for this group. The finding of *Lc. lactis* only in one of three samples from the treated group may confirm the successful delivery of probiotic candidates to experimental fish through diet supplementation. Microbiota along the digestive tracts of fish play an important role in immunity, nutrition, fish growth, disease outbreaks and fish health management (Nayak 2010). Although, there are several studies on microbial diversity in the intestinal contents of freshwater fish have been conducted (Ringø et al. 2006; Kim et al. 2007; Ray et al. 2012) however, the gastrointestinal microbiota of marine fish has been poorly reported. The genera found in the gut content of amberjack in the present study have either rarely or never been reported previously as a part of the gut microbial community of amberjack. The genus *Sphingomonas* is a group of gram-negative, rod-shaped, strictly aerobic bacteria, containing glycosphingolipids instead of lipopolysaccharides in their cell envelopes, and they typically produce yellow-pigmented colonies (Yabuuchi et al. 1990). Chaudhary and Qazi (2014) showed that *Sphingomonas* sp. was a potential probiotic, as it showed

an ability against *V. anguillarum* that had infected *Labeo rohita* fingerlings. *Staphylococcus* is gram-positive cocci with 1 μm in diameter that forms clumps. Several species belonging to this genus can cause many infections in fish, animals and humans (Foster 1996; Atyah et al. 2010; Peton and Loir 2014); however, other species isolated from fermented fish and meat were considered to be probiotic bacteria (Borah et al. 2016), and others appeared in fish guts as non-pathogen bacteria (Ringø et al. 2016). *Kocuria* is an opportunistic gram-positive cocci from the family Micrococcaceae, which has been reported as a pathogen that causes infections in humans (Chen et al. 2015). This species was also found in the intestines of rainbow trout in a study by Kim et al. (2007). The genus *Propionibacterium* includes gram-positive bacteria that is non-motile, non-spore-forming, anaerobic to aerotolerant, and have high GC content. Some species in this genus are considered potent probiotics but some act as opportunistic pathogens (Rabah and Luiz 2017). Also this genus has been found among the microflora in the digestive tracts of adult fresh water fish (Austin 2006). The genus *Mycobacterium* includes pleomorphic, gram-positive, acid-fast, aerobic, non-motile rods, with several species having long been recognized as significant sources of morbidity and mortality in finfish aquaculture, as well as in wild finfishes (Gauthier and Rhodes 2009). *Acinetobacter* are gram-negative and non-fermenting coccobacilli that may be isolated from the environment, animals and humans. This genus was identified in the gastrointestinal tract of fresh water fish such as Atlantic salmon and rainbow trout (Huber et al. 2004; Atrouni et al. 2016; Ringø et al. 2016). *Acidobacteria* were recently recognized as a phylum with 26 accepted subdivisions; they are all heterotrophic; most species are aerobic or microaerophilic, and some species are facultative anaerobic bacteria. This phylum is particularly abundant in soil habitats (Kielak et al. 2016), and it has also been isolated from carp intestines (Wu et al. 2012). The gut microbiota composition of fishes may result from the host trophic level, habitat salinity, taxonomy or host-specific selection within the gut (Wong and Rawls 2012) and within each individual itself. Also, the research results regarding fish gut microbial diversity may

depend on the different research methods used as suggested by Kim et al. (2007), who found that the diversity generated by DGGE was much smaller than that of the sequences obtained by means of 16S rRNA gene clone libraries in their study. The results regarding the gut microbiota composition of amberjack in our study may be the first such findings and will be useful for reference and further research.

In conclusion, the results of this study presented the significant growth enhancement and high taurine content in the intestinal tracts of experimental amberjack fed a diet supplemented with the probiotic *Lc. lactis* K-C2 for 25 days. This probiotic was isolated from fermented vegetables and could be used as a probiotic in amberjack. It did not only indirectly increase the taurine concentration in the gut, but also stimulated growth, reduced feed consumption and improved the nutritional value of cultured amberjack. However, the effect of *Lc. lactis* on the fish intestinal microbiota in this study is unidentified yet. Therefore, further studies are required to clarify this point.

Table 4 Proximate composition (% wet basis) of the experimental diets for young amberjack

Parameters	Commercial diet (%)	Diet adding strain K-C2 (%)
Moisture	10.5 ± 0.6	13.6 ± 1.0
Crude protein	37.7 ± 1.2	37.0 ± 0.9
Crude lipid	16.4 ± 0.8	16.6 ± 0.8
Ash	11.4 ± 0.1	11.4 ± 0.5

Data represents mean $\pm$ SD ($n = 3$).

Table 5 Growth performance and feed utilization of young amberjack fed test diets for 25 days

Parameter	Diet groups	
	Control group	Treated group
Initial body weight (g)	65.1 ± 8.1	70.1 ± 5.8
Final body weight (g)	75.9 ± 10.3^a	83.9 ± 7.0^b
Weight gain (%)	14.6 ± 4.3^a	19.8 ± 2.7^b
Feed intake (g fish^{-1} 25 days^{-1})	16.4 ± 0.0	19.6 ± 0.0
Feed conversion ratio	2.1 ± 1.3^a	1.4 ± 0.2^b
Condition factor	1.3 ± 0.1	1.4 ± 0.1
Hepatosomatic index	1.3 ± 0.3	1.2 ± 0.2
Viscerasomatic index	0.99 ± 0.2	0.92 ± 0.1

Data (mean $\pm$ SD) in the same row having different superscripts letter are different significantly ($p < 0.05$) among the control and treated group

Table 6 Whole body proximate composition (% wet basis) and somatic parameters in young amberjack fed test diets for 25 days

Parameters	Experimental groups	
	Control group	Treated group
Moisture	77.9 ± 0.00	77.3 ± 0.00
Crude protein	18.4 ± 0.01	19.0 ± 0.04
Crude lipid	5.4 ± 1.0	6.2 ± 1.3
Crude ash	1.7 ± 0.00	1.7 ± 0.00

Table 7 Amino acids profile analysis from the edible part of amberjack from the control or treated group

Amino acid (mg/g edible part)		Edible part of amberjack from the control group	Edible part of amberjack from the treated group
Essential amino acid	Arg	10.6 ± 0.6	11.5 ± 4.6
	His	3.3 ± 0.4	4.7 ± 3.5
	Ile	6.2 ± 0.5^a	7.4 ± 0.4^b
	Leu	29.9 ± 1.9^a	34.7 ± 2.5^b
	Lys	7.1 ± 0.8^a	6.0 ± 0.9^b
	Met	5.1 ± 0.4^a	6.6 ± 0.7^b
	Phe	8.5 ± 0.4^a	12.1 ± 0.9^b
	Thr	4.0 ± 0.07	4.8 ± 2.8
	Trp	1.0 ± 0.1^a	1.4 ± 0.3^b
	Val	7.2 ± 0.5^a	8.8 ± 0.5^b
Nonessential amino acid	Ala	0.8 ± 0.1	0.9 ± 0.3
	Asn	2.4 ± 0.7^a	4.1 ± 1.2^b
	Asp	3.6 ± 0.3	4.2 ± 0.9
	Glu	15.5 ± 2.2	16.2 ± 2.6
	Gly	6.8 ± 0.5^a	9.5 ± 1.7^b
	Ser	5.6 ± 0.4^a	7.1 ± 0.8^b
	Tyr	6.2 ± 0.3^a	8.8 ± 0.8^b
Conditionally essential amino acid	Gln	0.0 ± 0.0^a	0.5 ± 0.2^b
	HyPro	0.6 ± 0.2^a	0.9 ± 0.5^b
	Pro	5.0 ± 0.4^a	6.3 ± 0.8^b
	Tau	1.8 ± 0.4	2.1 ± 1.3
	Total	131.28 ± 0.8	158.7 ± 1.8

Data (mean $\pm$ SD) in the same row having different superscripts letter are different significantly ($p < 0.05$) among the control and treated group

Table 8 Closet relative as determined by BLAST search, similar to the relative for the bands in the denaturing gradient gel electrophoresis (Fig. 3) of gut content samples in amberjack fed the control diet and probiotic diet for 25 days

| Phylogenic group	Band no.	Closest relative	Similarity (%)	Accession number
Proteobacteria	3	*Sphingomonas* sp. hong4-2	94	LC327008
	13	*Acinetobacter* sp. Hop10	100	LC327018
Firmicutes	9	*Staphylococcus sp.* 111	99	LC327014
	10	*Lactococcus lactis* P43-1A (showing 100% coverage of internal nodes with *L. lactis* K-C2 by the test neighbor-joining tree-unpublished data)	99	LC327015
Actinobacteria	5	*Propionibacterium* sp. S4-S3	99	LC327010
	7	*Mycobacterium* sp. CECT 8763	98	LC327012
	17	*Kocuria* sp. RMB42	99	LC327021
	18	*Kocuria* sp. PJC10	99	LC327022
Acidobacteria	12	*Acidobacteria sp.* clone CV-10	94	LC327017
Unclassified bacteria	1, 2	Uncultured bacterium clone 44	99	LC327007
	4	Uncultured organism clone SBYG-4590	78	LC327009
	6	Uncultured bacterium clone SA03D10	92	LC327011
	8	Uncultured bacterium clone 26	98	LC327013
	11	Uncultured bacterium clone EHB-P0710	99	LC327016
	14, 15	Uncultured bacterium clone SIP13C-1E	99	LC327019
	16	Uncultured bacterium clone RH20-3	98	LC327020
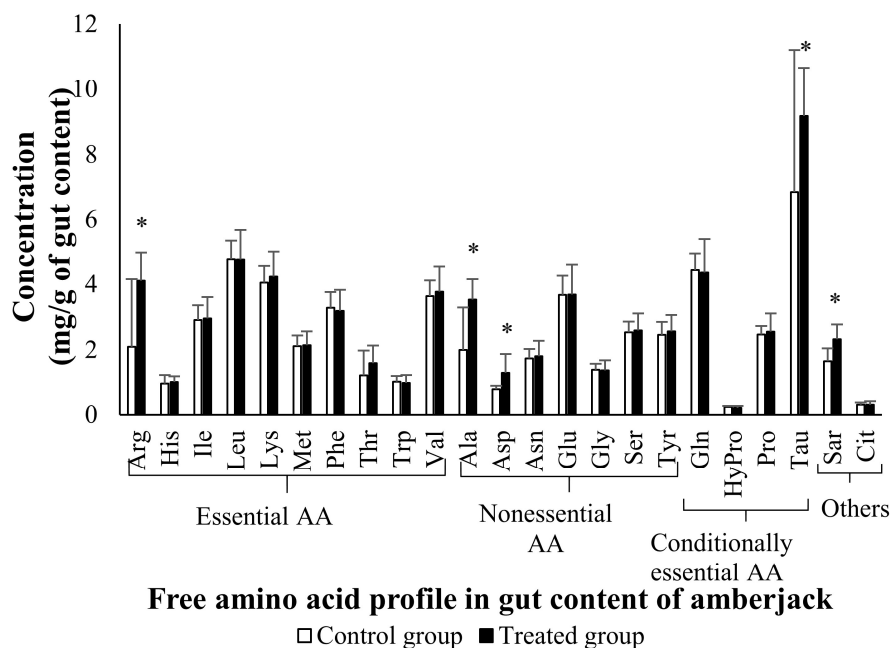
Fig. 13 Composition of free amino acids in the gut content of amberjack in the control and treated group after administration with or without *L. lactis* strain K-C2 LC212968 for 25 days. Data (mean ± SD) in the same amino acid with "*" significantly different ($p < 0.05$) among the control and treated group.
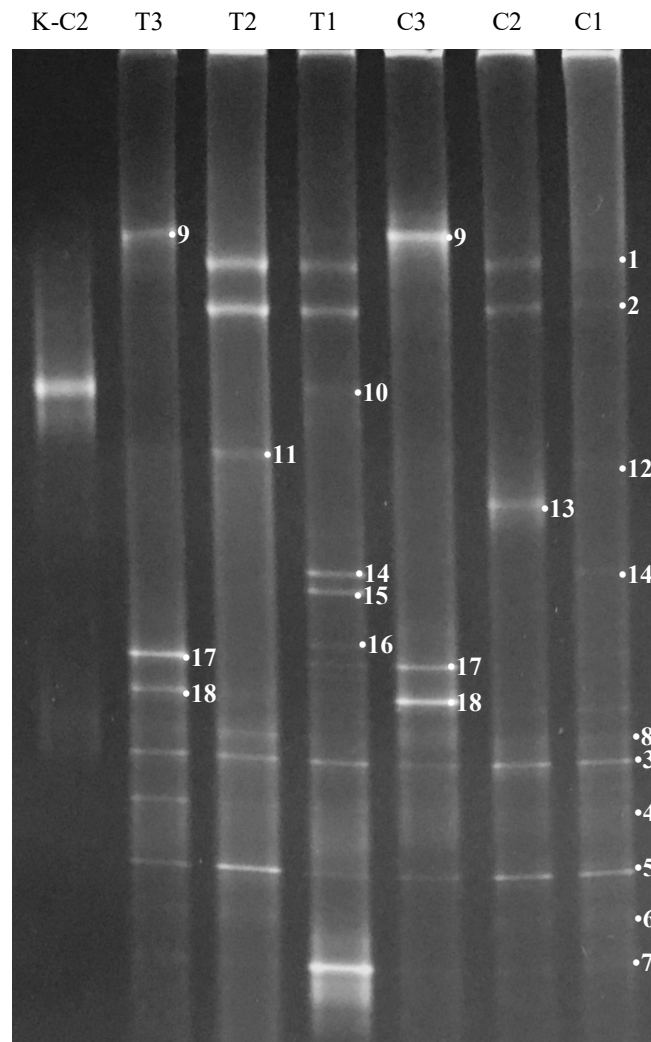
Fig. 14 DGGE profiles of PCR products of the V6-8 region of the 16S rRNA gene from samples of the gut contents in amberjack fed the control diet and probiotic diet for 25 days. C1, C2 and C3 represent the three fish from three groups fed the control diet; T1, T2, and T3 represent the three fish from three groups fed the probiotic diet; K-C2 represent *L. lactis* strain K-C2 LC212968. Bands 1-8 were present in all samples, except bands 1-2 for sample C3. Bands 9, 17, 18 were present in both samples C3, T3; band 14
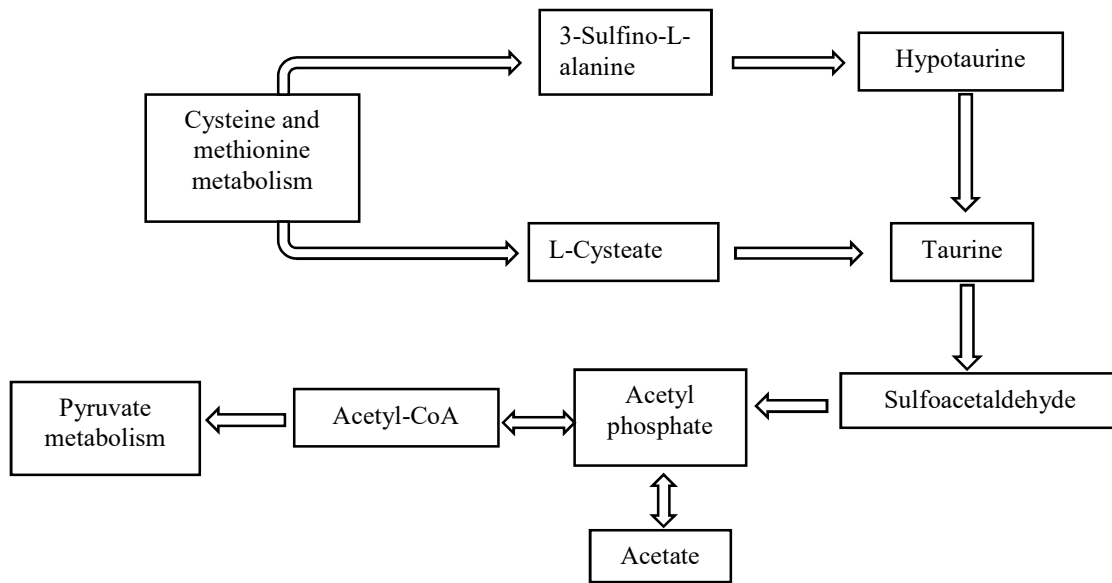
Fig. 15 Taurine and hypotaurine metabolism of *Lactococcus lactis* following to the pathway database of Kyoto Encyclopedia of Genes and Genomes.

Chapter 5: Effect of candidate and commercial probiotic on the growth performace, non-specific immune response and the resistance to pathogen of Japanese eel, *Anguilla japonica*

Abstract This study aimed to evaluate the effect of *Lactococcus lactis* K-C2 and commercial probiotic BA on the growth performance, non-specific immune response and the resistance to pathogen of Japanese eel, *Anguilla japonica*. Fish were fed a diet with or without strain K-C2 (10^8 cfu/g feed) or probiotic BA (10^7, 10^8 cfu/g feed) for 60 days. Unfortunately, the results indicated that the growth performance, somatic parameters, whole body proximate composition and non-specific immune responses among the control and the probiotic-treated groups had no the significant differences in this study. Even eels in experimental groups were challenged by *E. tarda* for 18 days, the result showed the survival rate of eels in T1 and T2 group were higher than that of the control and T3 group. However, the protein content and lysozyme activity from the serum of test fish were no significantly difference between experimental groups ($p > 0.05$). Therefore, the candidate probiotic strain K-C2 and commercial probiotic BA should not be considered as potential probiotic or supplementary feed to Japanese eel.

Introduction

Japanese eel, *Anguilla japonica* was introduced firstly to Asia aquaculture since 1879 in Tokyo, and Japan became the most important eel market in the world. During six decades recently, cultured eel in Japan has declined in quantity after reaching the highest mark in 2000, due to the decrease of Japanese glass eels have been caught from Asia sea. However, the quantity of cultured eel have slightly increased in recent years (FAO, 2015). As a food supply, Japanese eel

has favoured by customer because it may have many nutrients, contain high-quality minerals and proteins and rich taste. Even though it seems scarcity the nutritional studies about this fish species.

On the other hand, the eel aquaculture industry in China, Japan, Korea, Malaysia and Taiwan has developed rapidly in recent years (Lee et al. 2017), it may be reason of fish more susceptible to infection agents. Some disease agents as viruses (*Iridovirus*), bacteria (*Edwardsiella fujianensis*, *A. hydrophila*, *V. anguillarum*), fungi (*Saprolegnia* spp.) and parasites (*Pseudodactylogyrus anguillae*; *P. bini*) have reported as disease outbreaks that decrease growth or increase mortality of Japanese eel (FAO, 2015). To advoid the use of antibiotic and chemicals as treatment methods, the use of probiotic should be one of choices to prevent and control disease that is approved by its effective, environmentally friendly and safe for aquaculture products.

Aslo, the probiotic supplemented to fish diet may increase the nutritional value and umami taste of fish meat. The taste of fish meat usually depends on the concentration of free amino acid in the muscle of fish such as glutamic acid, aspartic acid, serine, glycine and threonine (Tanaka et al. 2016). Besides, the lipid compositions in fish body are an importance source of energy for fish or major components of cell membranes, and are good indices of physiological health in fish (Aziz et al. 2013; Tanaka et al. 2016).

In this study, we supplemented the probiotic *Lactococcus lactis* strain K-C2 LC212968 and BA into fish diet to assess its effects on growth performance, non-specific immune response and the resistance to pathogen of Japanese eel.

Materials and methods

Animals

Healthy Japanese eel, *A. japonica* were obtained from a commercial fish farm (Miyazaki Prefecture, Japan), and transported to the Laboratory of Marine Environmental Microbiology, Faculty of Agriculture, University of Miyazaki, Japan. The eel were maintained in dechloried tap water with a water temperature, pH, and dissolved oxygen of around 22 °C, 7.0-8.0, and 8-9 mg/l, respectively. The eel were acclimated in outdoor cement tanks at a density of nineteen individuals with a closed rearing system and were fed by a commercial mash (Nitto- Fuji Flour Milling Company, Tokyo, Japan) for one week before the experiments were performed.

Preparation of the diet

The strain, *Lc. lactis* strain K-C2 LC212968 was isolated from the fermented vergetable and was identified by cluster analysis on the sequence of 16S rRNA (as the result showed in previous chapter). Probiotic *Lc. lactis* strain K-C2 LC212968 was cultured in MRS broth medium at 28 °C for 24 h. Then, cells were collected by centrifugation ($5,000 \times g$ for 10 min, 25 °C) and suspended in of a sterile physiological saline solution (0.85 % NaCl [w/v]). The bacterial densities were estimated by optical density (OD) at 600 nm and total viable counts by agar plate counting method with MRS agar plates. The bacterial densities were adjusted to 10^{11} cfu/ml. The bacterial suspension was divided in 1.5 ml-Eppendoff tubes (70 tubes in total) with the volume of 1 ml of the bacterial suspension and 250 µl of glycerol 50%, and stored at -20 °C for futher feeding trial.

Four experimental diets were established and were mixed with commercial mash (Nitto- Fuji Flour Milling Company, Tokyo, Japan) everyday for feeding trials, in which a diet without probiotics was used as control diet, two other diets were prepared by supplementing the probiotic BA (provided by TOA pharmaceutical Co., LTD, Japan) at 10^7, 10^8 cfu/g diet, other diet was prepared by supplementing the *Lc. lactis* strain K-C2 LC212968 at 10^8 cfu/g diet.

Experimental groups

Four treatment groups with two simultaneous replications were used. These groups were as follows: (i) a control group fed a commercial diet without probiotics; (ii) a treated group fed a commercial diet containing probiotic BA (10^7 cfu/g) (T1); (iii) a treated group fed a commercial diet containing probiotic BA (10^8 cfu/g) (T2); (iv) a treated group fed a commercial diet containing strain K-C2 (10^8 cfu/g) (T3). The test fish with an average body weight of approximately 114 g were starved for two days, then fish were fed the experimental diets with an amount of diets equal to 2-3 % of fish average body weight once per day for 60 days. The monitored water quality paeameters (mean ± SD) were: water temperature 29 ± 2.2 ᵒC, pH 9.3 ± 0.5, and DO 9.8 ± 2.1 mg/l during the feeding trial.

Sample collection

At the end day of feeding trial, fish were starved for 24 h prior to sampling. All the fish were anesthetized with 2-phenoloxyethanol (Nacalai Tesque, Inc., Kyoto, Japan) at 500 ppm (v/v), then invidual body weight and body length of fish from each tank were measured.

Blood sample was collected from caudal vein of fish by using disposable syringes. Serum samples were obtained by centrifugation at 5,000×g for 10 min, 4 °C using a high-speed refrigerated microcentrifuge (Kitman, Tomy Seiko Co., LTD., Japan). The supernatants were transferred to 1.5 ml-Eppendorf tube and stored at -20 ᵒC for futher analysis.

Liver and intestinal tract were dissected out from fish body, weighed individualy to calculate hepatosomatic index and analyzed microflora.

Evaluation of growth performance parameters

The following variables were calculated: Weight gain (WG, %) = 100 × (final body weight – initial body weight)/initial body weight; feed intake = (dry diet given–dry remaining diet recovered) × no. of fish; feed conversion ratio (FCR) = dry feed intake (g)/live WG (g); condition factor (K factor) = (body weight/(body length)^3) ×100; hepatosomatic index (%) = 100 × (weight of liver/weight of fish) (Hossain et al. 2017).

Whole body proximate composition analyses

Whole body proximate composition analyses of amberjack were carried out. Crude protein was analyzed by the Kjeldahl method. Crude lipid was analyzed as described by Folch et al. (1951). Crude ash and moisture were analyzed according to the guidelines of the Cunniff (1997).

Non-specific immune response analysis

Lysozyme activity assay

Lysozyme activity was measured from serum with turbidimetric assays (Lygren et al. 1999). Briefly, 10 μl of serum samples was put into a 1.5-ml Eppendorf tube, then added 190 μl of substrate (0.2 mg *Micrococcus lysodeikticus*, lyophilized cell, Sigma, USA)/ml 10 mM phosphate-buffered saline (PBS) buffer (pH 7.2), their absorbance were incubated at room temperature for 1 and 5 min, and then were measured optical density (OD) at 450 nm wavelength using UV/VIS spectrophotometer (V-530, Jasco Corporation, Japan).

Total serum protein

Total serum protein was determined by Lowry method (Lowry et al. 1951) using albumin from bovine serum (Sigma-Aldrich Inc., USA) as a standard and adjusted to 1.0 mg/ml with PBS. Briefly, serum protein samples were diluted to different concentrations by distilled water. Each diluted concentration of serum protein was added 2 ml of the mixture containing 48 ml of 2 % Na_2CO_3 in 0.1 N NaOH, 1 ml of 1 % NaK Tartrate in H_2O, 1 ml of 0.5 % $CuSO_4.5H_2O$ and waited for 10 min at room temperature. Then, 0.2 ml of 1N Folin-Phenol was added to each samle and votexed immediately, kept the samples at room temperature for 30 min. Finally, total serum protein was measured by determining the absorbance at 600 nm wavelength using a V-530 spectrophotometer (Jasco Corporation, Japan).

Challenge test

At the end day of feeding trial experiment, six eels per tank were redistributed in two 30-L aquaria and maintained for futher challenge test. The pathogenic bacterium, *E. tarda* were kindly provided by the Fish Diseases Laboratory, Department of Marine Biology and Environmental Sciences, University of Miyazaki, Japan. The bacterial strain were cultured in BTH (Bacto Todd Hewitt, BD Bacto™, USA) broth for 24 h at 28 °C. The overnight cultured pathogenic strain was centrifuged at 5,000×g for 10 min, and the cell pellets were washed and suspended in saline solution (0.85 % NaCl [w/v]). The bacterial densities were estimated by optical density (OD) at 600 nm and total viable counts by agar plate counting method with BTH agar plates. Three eels per aquarium were injected intraperitoneally (i.p) with 0.1 ml per eel at adjusted concentration of 10^7 cfu/0.1 ml. The mortality of eel in each aquarium was monitored daily up to 14 days. The dead eel was recorded the symptom, dissected and collected the liver, kidney and intestine which were stored at -20 °C until DNA extraction for detecting *E. tarda* infection to dead eel by PCR.

The serum of the survival eels after challenge by *E. tarda* were collected for non-specific immune response analysis as described above.

Results

Fish performance parameters and whole body proximate composition

The result of the experimental fish growth performance is shown in Table 9. At the end of the feeding trial, the final body weight, weigh gain and somatic parameters of fish were no significantly difference between experimental groups ($p > 0.05$).

The whole body proximate composition of eel after 60 days of feeding was not affected by probiotic treatment ($p > 0.05$). The results are shown in Table 10.

Non-specific immune response assay after feeding trial

The result of non-specific immune response assay of test fish after 60 days of trial feeding was presented in Table 11. No significant differences were observed in protein content, glucose content and lysozyme activity from the serum of test fish among the control and treated groups ($p > 0.05$).

Challenge test and non-specific immune response assay

Eels in experimental groups were challenged by *E. tarda* for 18 days, the result was showed in the Fig. 16. The survival rate of eels in T1 and T2 group were higher than that of the control and T3 group.

However, the protein content (Fig. 17), lysozyme activity (Fig. 18) from the serum of test fish were no significantly difference between experimental groups ($p > 0.05$).

Discussion

The introduction of probiotic *Bacillus subtilis* WB60 and *Lactobacillus plantarum* KCTC3928 in diet of Japanese eel, *Anguilla japonica*, was displayed in the research of Lee et al. (2017); from their results, average weight gain, feed efficiency, and protein efficiency ratio of fish fed *B. subtilis* at 10^7 and 10^8 cfu/g diet were significantly higher than those of fish fed other experimental diets ($p < 0.05$); nonspecific enzymatic activities including lysozyme, superoxide dismutase, myeloperoxidase from fish fed *B. subtilis* at 10^7 and 10^8 cfu/g diet were detected to be significantly higher than that from fish fed control diet ($p < 0.05$). Also, the result from the disease challenge test with bacteria *V. angulillarum* showed significantly lower survival rate for fish fed control diet than those of fish fed other experimental diets. These results indicated that oral supplement of *B. subtilis* at 10^8 cfu/g diet could be a more effective source of probiotic compared to *L. plantarum* in Japanese eel. In the contrast, the candidate probiotic strain K-C2 and commercial probiotic BA introduced to Japanese eel have not shown the significant differences in growth rate, weight gain, somatic parameters, whole body proximate composition and non-specific immune responses among the control and the probiotic-treated groups during 60 days of feeding trial in this study. The ineffectness of probiotic to fish may depend on the interation between probiotic and host. Also the diet formulation could be one of factors leading to the infective of applied probiotic on cultured fish (Welker and Lim 2011). Therefore, the candidate probiotic strain K-C2 and commercial probiotic BA should not be considered as potential probiotic or supplementary feed to Japanese eel.

Table 9 Growth performance and feed utilization of young eel fed test diets for 60 days

Parameter	Diet groups			
	Control group	T1 group	T2 group	T3 group
Initial body weight (g)	116.3 ± 17.1	112.0 ± 16.4	116.5 ± 19.6	108.2 ± 22.8
Final body weight (g)	206.0 ± 47.6	194.5 ± 36.9	205.3 ± 32.7	175.7 ± 35.1
Weight gain (g)	89.8 ± 30.9	81.5 ± 22.0	91.2 ± 10.7	64.9 ± 15.7
Feed intake (g fish^{-1} 60 days^{-1})	408.9 ± 0.7	393.7 ± 0.7	408.7 ± 0.7	381.7 ± 0.7
Feed conversion ratio	5.5 ± 3.8	5.4 ± 2.6	4.5 ± 0.5	6.1 ±1.4
Condition factor	1.7 ± 0.5	1.6 ± 0.4	1.6 ± 0.4	1.6 ± 0.4
Hepatosomatic index	2.2 ± 0.7	2.0 ± 0.6	1.8 ± 0.4	1.6 ± 0.3

Values are means of triplicate groups ± SD.

Table 10 Whole body proximate composition (% wet basis) and somatic parameters in young

eel fed test diets for 60 days

Parameter	Diet groups			
	Control group	T1 group	T2 group	T3 group
Moisture	62.8 ± 0.02	60.8 ± 0.03	61.3 ± 0.03	61.9 ± 0.02
Crude ash	1.2 ± 0.0	1.2 ± 0.0	1.3 ± 0.0	1.3 ± 0.0
Crude protein	43.1 ± 7.2	43.4 ± 7.7	45.4 ± 6.6	40.4 ± 5.5
Crude lipid	38.9 ± 3.7	35.6 ± 7.8	37.8 ± 3.6	36.8 ± 4.3

Table 11 Non-specific immune response of young eel fed test diets for 60 days

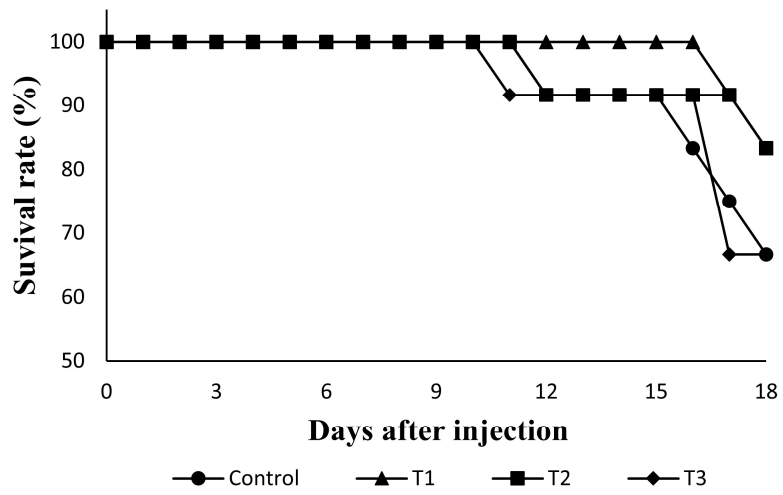| Parameter	Diet groups			
	Control group	T1 group	T2 group	T3 group
Protein content (g/dl)	187.7 ± 45.8	178.7 ± 36.8	176.7 ± 35.7	206.3 ± 39.0
Lysozyme acitivity (unit/ml)	51.9 ± 18.7	45.6 ± 17.2	56.5 ± 26.0	32.6 ± 14.4

Fig. 16 The survival rate of eel in experimental groups treated with or without probiotic after challenge by *E.tarda*
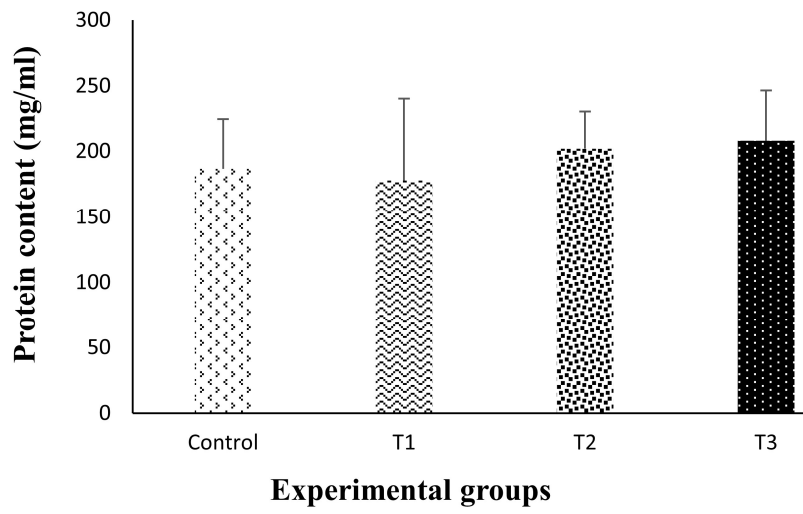
Fig. 17 Protein content in the serum of eel in different experimental groups treated with or without probiotic after challenge by *E.tarda*
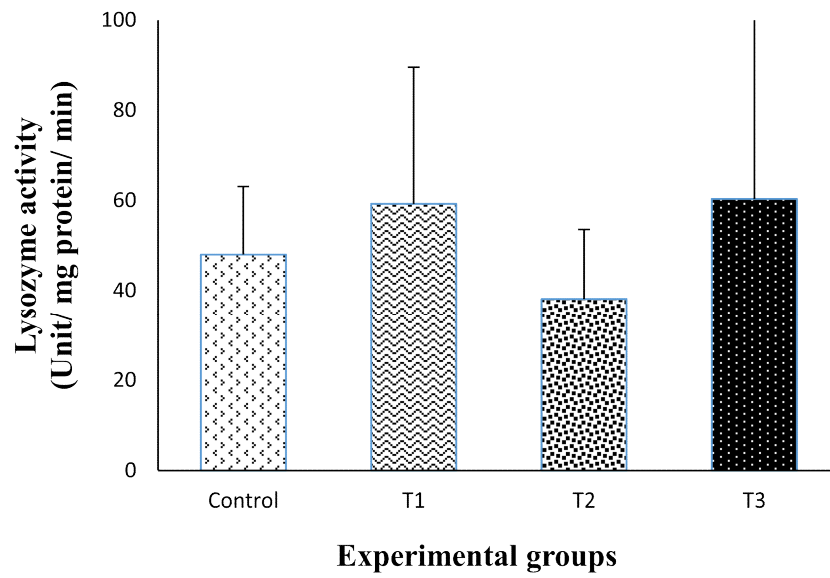
Fig. 18 Lysozyme activity of eel in different experimental groups treated with or without probiotic after challenge by *E.tarda*

Chapter 6: General discussion

Recent years,to obtain high productivity of fisheries conduced to losses and negative impact to environment, especially the mangrove conversion to shrimp ponds result in loss of essential ecosystem. Beside that, the production cost become high and commercial price is low, demand of consumers have become higher that made trend of aquaculture industry in the world downward. The collection of wild seed and brood-stock or catching adult animal to provide eggs for hatcheries of some cultured species (*e.g.* black tiger shrimp *Penaeus monodon*) which lead to loss substantial number of native stocks. The introductions and transfers of aquatic animals outside natural range to aquaculture purpose effect on the degradation of host environment, genetic degradation of animal and the spread of diseases and parasites (Primavera 2006). Obviously, environmental degradation, increase of pathogens and reducing resistant of animal cause outbreak of diseases in aquaculture industry in recent years. Risk of diseases particularly has been described in cultured animals (Lightner 2003; Sakai et al. 2007) and the infected agents in aquatic animal are usually virus, bacteria, fungi and parasites (Lightner and Redman 1998). The use of chemicals and antibiotics can result in toxic to aquatic organisms, human consumers and wild biota, and development of antibiotic resistance and accumulation of residues (Holmstrom et al. 2003). Currently, the use of antibiotics as prevention and therapeutic outbreak of disease in aquaculture is restricted or forbidden in many countries (Shakibazadeh et al. 2011). A growing concern about the high consumption of antibiotics in aquaculture has initiated a search for alternative methods of disease control. Thus probiotic has been one of a chosen strategy and desirable material to utilize in aquaculture for sustainable development.

Probiotics were originally proposed as supplements for the human diet, and the tradition of using probiotic microorganisms to promote human and animal health is now backed by strong scientific evidence for some clearly defined and well characterized strains (Ibrahem 2015). In aquaculture, a number of probiotics are now commercially available and in use, others have been

proven their benefits to aquatic animal by several studies. Commonly, probiotics have been used in aquaculture could be isolated from rearing water, pond sediment or gut microflora of aquatic animal. However, the use of candidate probiotics which isolated from other habitats may create new opportunities for food and nutrition scientists to improve food quality and develop new products with specific health benefits for different hosts. Therefore, in this study we conducted to isolate lactic acid bacteria from fermented food as candidate strains which may be used to reduce the using of antibiotics in aquaculture.

Firsly, the novel lactic acid bacteria have been isolated from rice bran, fermented vegetables and the intestinal tract of herbivores. The candidate probiotics were evaluated as suitable probiotic to apply in aquaculture by antagonistic against fish pathogens, the growth levels in different NaCl concentrations and pH, tolerance in artificial gastrointestinal juices and the adhesion to the fish mucus. *In vitro* tests, the result demonstrated strain K-C2 was able to become as a potetial probiotic and it was identified as *Lactococcus lactis* strain K-C2 LC212968. *Lc. lactis* species is one of the most important groups of lactic acid bacteria that are used in the dairy industry with the production of lactic acid from lactose, hydrolysis of casein and citric acid fermentation during dairy fermentation; their metabolic end products and enzymes directly or indirectly have significant influence in determining the texture and flavour of the final products (Samaržija et al. 2001). In aquaculture, *Lc. lactis* was used as a potential probiotic for the enhancement of growth in the rotifer *Brachionus plicatilis* (Harzevili et al. 1998); it had inhibition ability against *A. hydrophila* and study of itsimmunostimulatory effect in tilapia (*Oreochromis niloticus*) (Zhou et al. 2010); it could modulate the growth performance, digestive enzyme activities, and beneficial intestinal microbiota of *L. vannamei* (Adel et al. 2017); or it improved growth rate and disease resistance in olive flounder, *P. olivaceus* (Nguyen et al. 2017). In our study, *Lc. lactis* strain K-C2 had proved its effect on the growth performance of young amberjack after 25 days of feeding trial.

After feeding *Lc. lactis* strain K-C2 as feed supplement to amberjack for 25 days, several amino acids content in intestinal fish were increased, the amino acids in the fish edible part were also improved. These results in our study may be the first report about the effect of *Lc. lactis* on the improvement of amino acid compositions of the fish edible part. Especially, by the finding about the high concentration of taurine in fish intestine, we hypothesized that probiotic *Lc. lactis* strain K-C2 might play an indirect role in increasing Tau in the gut content of amberjack in two ways: (i) *Lc. lactis* itself converted Met to Tau by an indirect metabolism pathway; (ii) *Lc. lactis* produced Met during its metabolism process, and fish then used this Met product for the synthesis of Tau and to meet its other nutritional requirements. And, with high concentration of taurine was established, the increase growth of amberjack was also performanced.

Unfortunately, in the study of the effect of probiotic candidate *Lc. lactis* strain K-C2 and commercial probiotic (BA) on the growth performance and the resistance to pathogen of Japanese eel (*Anguilla japonica*), these probiotic were orally administered with the diet, the significant differences in growth performances, somatic parameters and disease resistance were not observed among the control and the probiotic-treated groups during 60 days of feeding trial in this experiment.

According to results from *in vitro* and *in vivo* experiments of our study, we may conclude that:

(i) *Lc. lactics* strain K-C2 had ability to against Gram-negative and -positive bacterial pathogens in fish *in vitro* test.

(ii) *Lc. lactics* strain K-C2 might survive in the range of pH from 2.0 to 4.0 and grew well in the rage of pH 6.0 to 9.0 *in vitro* test.

(iii) *Lc. lactics* strain K-C2 showed high relative growth of over 70 % in the range of NaCl concentration from 0 % to 5 % *in vitro* test.

(iv) *Lc. lactics* strain K-C2 had the ability to survive in artificial gastro-intestinal juices, and to adhere to fish mucus *in vitro* test.

(v)   *Lc. lactics* strain K-C2 had the ability to adhere to fish mucus *in vitro* test.

(vi)   A diet supplemented with the probiotic *Lc. lactis* strain K-C2 for 25 days emphasized the significant growth enhancement and high taurine content in the intestinal tracts of experimental amberjack *in vivo* test.

(vii) This strain stimulated growth, reduced feed conversion ratio and also improved the nutritional value of cultured amberjack *in vivo* test.

(viii)   The significant differences in growth rate, weight gain, somatic parameters, whole body proximate composition and non-specific immune responses were not observed among the control and the probiotic-treated groups during 60 days of feeding trial of candidate probiotic strain K-C2 and commercial probiotic BA to Japanese eel (*Anguillajaponica*) *in vivo* test.

Briefly, this study indicated that novel probiotic, *Lc. lactics* strain K-C2 could enhance the growth of amberjack by modification of amino acid flux in the fish intestine, and the growth promoting effect but strain K-C2 was not common among all aquacultured fish species (Fig. 18).
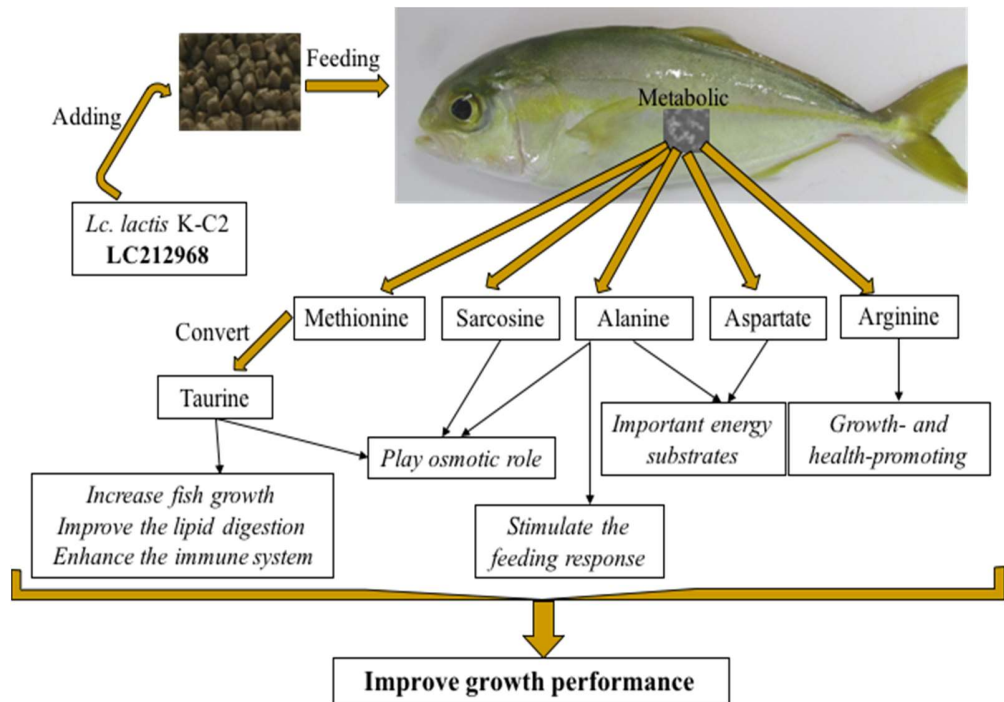
Fig. 18 Activating of *Lc. lactis* **K-C2 as probiotic on amberjack,** *Seriola dumerili*

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