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STUDIES ON THE UTILIZATION OF FERMENTED CACTUS FRUIT (*Opuntia ficus-indica*) FOR OLIVE FLOUNDER, *Paralichthys olivaceus*

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CONTENTS

SUMMARY

This study was used on the cactus fruit (*Opuntia ficus-indica*) as a commercial diet feed supplement, a fermented stable by effective microorganism to easily dose into fish, and was conducted to investigate the effects on the growth, antibacterial activity, probiotics, feeding stimulants and its commercial value of aquaculture fish.

The growth of fish fed the diet containing fermented cactus fruit fluid (FCFF) was significantly greater than that of fish fed the diet with no FCFF ($P<0.05$) for aquaculture fish, olive flounder *Paralichthys olivaceus* and the preferred level of supplementation is 1.0 %. The growth of fish fed the diet containing 1 % raw cactus fruit was significantly lower than that of fish fed the diet containing 1 % FCFF. These results indicate that a diet with FCFF is more effective than one with unfermented cactus fruit fluid for increasing the growth of aquaculture fish. Thus, FCFF, a land-plant-based herbal supplement, may be an effective feed additive for the improvement of growth and feed efficiency of aquaculture fish.

Fish fed moist pellet-based diets containing 0.08 % fermented cactus fruit powder (FCFP) were significantly higher ($P<0.05$) growth among 0 %, 0.02 %, 0.04 %, 0.08 %, 0.16 % FCFP for olive flounder. Body composition of fish fed diet containing 0.08 % FCFP was significantly higher ($P<0.05$) in crude lipid content. However, glutamic-oxaloacetic transaminase (GOT)and glutamic-pyruvate transaminase (GPT) values clearly decreased with increasing dietary FCFP levels. Therefore, FCFP might be an effective ingredient for the growth, immune system and the feed efficiency of aquaculture fish.

Extruded pellet (EP) diet contained FCFF formulated with 50 % crude protein and 8 % crude lipid mainly might be an effective ingredient for the growth and the feed efficiency of olive flounder, and preferable EP diets were

assumed to be diet containing 2.0 % FCFF for olive flounder.

Fish were fed an experimental diet supplemented with *L. plantarum* (CNU001) culture (about 10^9 colony-forming units mL^{-1} isolated from cactus fruit) by volume to determine the effect of *Lactobacillus plantarum* (CNU001) on the growth and resistance against pathogenic bacteria of olive flounder. In vitro tests found that this lactic acid bacteria effectively inhibited the growth of *Streptococcus* sp., *E. tarda* and *Vibrio* sp. The mortality of fish fed a diet supplemented with *L. plantarum* (CNU001) was lower than that of the control group. These results suggest that *L. plantarum* (CNU001) may have antibacterial properties that increase the immunity of aquaculture fish and result in increased growth and survival rates.

FCFF, Chitosan, β-Glucan, and Obosan as a feed additive were conducted to determine on the growth of olive flounder. The growth was best value in fish fed diet containing FCFF among groups. Otherwise, daily feed rate (DFR) of fish fed diet containing FCFF was significantly lowest ($P<0.05$) than that of control diet. The results indicate that FCFF might be recommended as a feed additive compared to another additives for the growth and the feed efficiency of aquaculture fish.

Essential amino acids (EAAs), taurine and sucrose as a feeding stimulants were conducted to determine on the growth of olive flounder. Fish fed diets supplemented lysine, valine, tryptophan and methionine were significantly improved ($P<0.05$) growth among EAAs groups. Taurine and sucrose were also better. The results suggest that lysine, methionine, tryptophan, valine as a EAAs, taurine and sucrose requirements of aquaculture fish changes with growth and feed efficiency.

요약문

이 연구는 손바닥선인장 열매를 유용미생물을 이용해 양식어류가 이용하기 쉽게 발효시키고, 손바닥선인장 열매 발효액(FCFF)이 양식넙치에 미치는 성장효과, 병원성 미생물에 대한 발효미생물의 probiotic 효과 그리고 섭이자극효과 및 첨가제로써의 상업적 가치를 평가하고자 수행하였다.

FCFF는 넙치 성장에 유의적인 효과를 나타내었으며 사료에 대한 적정 첨가농도는 1 %였다. 한편, 발효하지 않은 1 % 손바닥선인장 열매를 섭이한 어류에서는 1 % FCFF 보다 유의적인 낮은 성장을 보였다. 이러한 결과는 FCFF가 발효하지 않은 선인장 열매에 비해서 양식어류의 성장에 더욱 효과적인 것으로 보이며 따라서 육상식물인 FCFF는 양식어류의 성장 및 사료효율 개선에 효과적인 사료첨가제로 사료된다.

일반적인 습사료 (MP)에 0.08 %의 손바닥선인장 열매 발효분말 (FCFP)을 첨가하여 섭이한 넙치에서가 0 %, 0.02 %, 0.04 % 및 0.16 % FCFP를 첨가한 그룹들에 비해 유의적인 성장차를 보였다. 또한 체조성면에서도 조지방 함량이 유의적인 증가를 보인 반면, GOT, GPT 값은 FCFP 첨가농도가 증가할수록 낮아지는 값을 보였다. 그러므로 FCFP는 양식어류의 성장, 사료효율 개선 및 면역력 개선에 효과적으로 작용하는 것으로 생각된다.

50 % 조단백질과 8 % 조지방을 기본으로 하여 FCFF를 첨가하여 만든 고형사료 (EP)를 양식넙치에 섭이케 한 결과 EP-FCFF는 이들 어류의 성장과 사료효율을 개선시키는 것으로 나타났으며, EP사료 조성시 FCFF 적정 첨가농도는 2 %로 각각 나타났다.

손바닥선인장 열매에서 분리한 유산균을 약 10^9 colony-forming units mL^{-1}로 배양 후 이 유산균이 넙치 성장과 병원성미생물들에 및는 영향을 조사하였다. *in vitro*에서는 유산균은 연쇄구균 (*Streptococcus* sp.) 에드워드균 (*E. tarda*) 그리고 비브리오균 (*Vibrio* sp.)의 성장을 효과적으로 저해하였다. *in vivo*에서는 유산균을 섭이한 어류에서 대조구에 비해 낮은 사망률을 보였다. 이는 유산균의 길항특성 (probiotics)으로 양식어류에 있어서 면역력을 증가시키고 또한 성장과 생존율을 높

이는 것으로 보인다.

FCFF와 시판되고 있는 양식사료 첨가제인 키토산, 베타글루칸 그리고 어보산에 대하여 넙치성장에 미치는 영향을 조사하였다. 성장과 사료섭이율 (FGR)에 있어서는 FCFF가 다른 첨가제들보다 가장 높은 값을 나타냈다. 반면에 일간섭이율 (DFR)은 FCFF에서가 가장 낮은 값을 나타냈다. 이는 FCFF가 양식어류에 있어서 다른 사료첨가제들에 비해 양식사료 첨가제로써 상대적으로 효과가 좋은 것으로 인정된다.

FCFF가 넙치 성장에 효과적인 원인을 알아보고자 섭이자극물질들로써의 필수아미노산, 타우린, 그리고 과당 (sucrose)에 대한 효과를 조사하였다. 필수아미노산중에서는 라이신, 발린, 트립토판, 그리고 메티오닌 등이 유의적으로 넙치성장을 개선하였다. 타우린과 과당 역시 보다 효과적인 것으로 나타났다. 이러한 결과들은 필수아미노산중에서는 라이신, 발린, 트립토판, 메티오닌 등이 양식어류의 성장과 사료효율을 변화시킨다고 여겨지며 타우린 및 과당도 긍정적인 역할을 하고 있다고 보아진다.

GENERAL INTRODUCTION

The Indian fig *Opuntia ficus-indica*, which is native to Mexico, Latin America, South Africa, and the Mediterranean, was introduced to Jeju Island, Korea, in the 20th century. The species is now widespread there and is cultivated for its sweet fruit, with 3,000 tons harvested annually. The fig improves digestive function, reduces the symptom of rheumatic arthritis, and is used to treat wounds thus, it has been used as a folk remedy as well as a decorative plant (Song et al., 1989). While recent laboratory studies on mammals have reported the fig's many beneficial effects, including antitumor (Shin et al., 1998), antioxidant (Kuti, 2004), antiviral (Ahmad et al., 1996), antiulcer (Lee et al., 1998), cholesterol-lowering (Palumbo et al., 2003), and antibacterial (Chung, 2000) effects, little information exists on its effects on fish.

The olive or Japanese, flounder (*Paralichthys olivaceus*) is a commercially important marine fish cultured in Korea and Japan. Its farming progressed rapidly with the advent of artificial feeding methods in the late 1980s and the development of aquaculture facilities on land in Korea. The aquaculture production of olive flounder has increased from only 1,000 tons in the 1990s to 34,533 tons in 2003 (Statistical Year Book of Ministry of Maritime Affairs and Fisheries, 2003).

Recently, with the adoption of seed production and feeding methods, the production per area squared has increased and it is being produced en mass. These mass-feeding methods have created new problems, including environmental pollution (Kikuchi et al., 2000), and various stresses and diseases (Murata et al., 1996). In addition, because the feed used for these fish is expensive, it is critical to raise the productivity of aquaculture fish (Kikuchi et al., 1992), while reducing costs with the development of inexpensive feed.

In addition, the treatment of microbial diseases in fish using antibiotics or chemotherapeutics is difficult, not particularly effective ,and costly, and might involve environmental hazards (Immanuel et al., 2004). Hence, many researchers are seeking to develop more efficient feeds using natural additives that maximize the feed efficiency, while reducing microbial diseases and decreasing environmental discharge, thus increasing profits without requiring antibiotics and chemotherapeutics.

Few data are available on the use of *Opuntia* in feeding fish, although we know that it contains a great deal of cellulose, the incorporation of which in the diet of fish results in poor feed utilization. Therefore, we fermented Opuntia fruit using microorganisms to facilitate its use by fish. Our study had the following objectives:

1. To investigate the effects of adding fermented cactus fruit fluid (FCFF) to a commercial diet on the growth of the olive flounder and to determine the optimum dose.
2. To investigate the effects of fermented cactus fruit powder (FCFP) in a moist pellet diet on the growth of the olive flounder and to determine the optimum dose.
3. To determine the utilization of an extruded pellet (EP) diet containing FOF on the growth of the olive flounder.
4. To determine the growth and antibacterial activity of lactic acid bacteria, Lactobacillus plantarum (CNU001), isolated from *O. ficus-indica* in fingerling olive flounder.
5. To compare the effects of FCFF as a feed additive with commercial chitosan, β-glucan, and obosan on the growth of fingerling olive flounder.
6. To investigate the effects of essential amino acids (EAAss), taurine, and sucrose as feeding stimulants on the growth of fingerling olive flounder.

REVIEW OF LITERATURE

1. CACTUS (*Opuntia ficus-indica*)

The cactus *Opuntia* (genus *Opuntia*, subfamily Opuntioideae, family Cactaceae) is a xerophyte producing about 200-300 species (Stintzing and Carle, 2005) and is common in Mexico, much of Latin America, South Africa, the Mediterranean, and Korea. Cactus pear or prickly pears are regionally consumed as fresh fruit, juice, sweets, etc. Mexico, one of main cultivation country related to cactus, is the only country planting cladodes for commercial use on 10,000 ha with a total production of 600,000 tons per annum (Rodriguez-Felix and Villegas-Ochoa, 1997). On the other hand, presently this cactus pear (*Opuntia ficus-indica*) plant is widespread in Jeju Island, Korea, and is cultivated for its sweet fruits. The annual production of fruit is over 3,000 tons.

Cactus pear has been used in traditional medicine in many countries for its curative properties (Shin et al., 2004). Cactus (*O. ficus-indica*) is well-known as a medicinal herb, has been known to have an effective property for improving digestive function, treating wounds, and reducing the symptom of rheumatic arthritis for long time, so it has been partly used as a folk remedy, by which it is well-known as a medicinal herb, as well as a preference for decorative plants (Song et al., 1989). Recently, the use of prickly pear fruit is recommended for their beneficial and therapeutic properties applying to mammals and making it a laboratory level subject following as

1.1. General composition

Cladode composition varies depending on the edaphic factors at the cultivation site, the season and the age of the plant (Batista et al., 2003). The composition of cladodes has been investigated by Malainine et al. (2003), who reported that in 100 g dry matter, 19.6 g ash, 7.2 g lipids and waxes, 3.6 g lignin, 21.6 g cellulose, and 48 g other polysaccharides were found, while crude proteins were not assessed. During growth, the fibrous framework decomposed in the core but developed in the cortex. In total, however, proteins and fibers decreased with age.

1.2. Phamacological profile

1.2.1. Anti-oxidant capacity

Many vegetables are known to act as antioxidants because they may contain vitamin C and various forms of flavonoids. The major components of *O. ficus-indica*, cactus are carbohydrate-containing polymers, which consist of a mixture of mucilage, pectin, and flavonoid (Shin et al., 2004). Recently, supplementation with cactus pear fruit was found to decrease oxidative stress in healthy humans (Tesoriere et al., 2004). Three fravonoid compounds were isolated and identified, namely quercetin, kaempferol, isohametin from *O. ficus-indica*, cactus (Burrer et al., 1982). They are widely distributed in food and hence could play a role in the defence of the organism against oxidative stress (Beck et al., 2003; Jung et al., 2003; Miliauskas et al., 2004) and antidiabetic (Vedavanam et al., 1999). On the other hand, nothing has been reported information available to fish. The total phenols in an ethanolic cladode extract from lyophilized *O. ficus-indica* var. *saboten* were held responsible for the radical scavenging activity towards superoxide and hydroxyl anions (Stintzing and Carle, 2005). When the antioxident activity of cladode extract were monitored, conclusive results were not obtained.

1.2.2. Anti-inflammatory properties

Reduction of acute inflammation by ethanolic *O. ficus-indica* stem extracts was ascribed to a lower leucocyte migration. In contrast to nonsteroidal inflammation inhibitors, no adverse effects were noted (Stintzing and Carle, 2005). Oral administration of the ethanol extracts of *O. ficus-indica* was shown to suppress carrageenan-induced rat paw edema and leukocyte migration in the carboxymethyl cellulose pouch model in rats (Park et al., 1998). In further experiments with a methanolic extract from *O. ficus-indica* cladodes, the fractions obtained after re-extraction with hexane and ethyl acetate were most efficient to accelerate the healing process (Park and Chun, 2001).

1.2.3. Anti-ulcerogenic effect

Lee et al. (2001, 2002) postulated an antiulcerogenic effect of the cladode or fruit powder from *O. ficus-indica*. Stomach lesions triggered by hydrochloric acid/ethanol or hydrochloric acid/acetylsalicylic acid were reduced, but no anti-inflammatory effect could be proven.

1.2.4. Hypoglycemic and antidiabetic effect

The efficiency of cladode preparation was underlined by Pardanani et al. (1978), who investigated the hypoglycemic potential of *O. ficus-indica* cladodes on non-insulin-dependent diabetics. However, the mechanism of these pharmacological effects could not be clarified.

1.2.5. Anti-hyperlipidemic and Cholesterol lowing properties

Fernandez et al. (1992) demonstrated that the reduction of blood lipids triggered by isolated pectin from *Opuntia* was due to the enhanced blinding of bile acid. On the other hand, Wolfram et al. (2002) reported a reduction of total cholesterol, LDL, apolipoprotein levels, triglycerides, fibrinogen, blood glucose, insulin and urate, while body weight, apolipoprotein and lipoprotein levels were found to remain unchanged. The exact mechanisms, however, still need to be elucidated.

1.2.6. Healing wounds effect

Park and Chun (2001) reported that the methanolic extract of *Opuntia ficus-indica* stems and its n-hexane and ethyl acetate fractions showed significant wound healing activity when topically administered in rats. These findings provide pharmacological support for the use of *O. ficus-indica* stems for wound healing in folk medicine, although the mechanism has not been elucidated.

1.2.7. Anti-viral properties

An extract of cactus plant *Opuntia streptacantha* inhibited intra cellular virus replication and inactivated extra cellular DNA viruses, such as herpes, and RNA viruses such as influenza type A and human immunodeficiency virus (HIV)-1 (Ahmad et al., 1996). It was tested *in vitro* whether *O. ficus-indica* affected infection with coronavirus (transmissible gastroenteritis virus) or herpes viruses (bovine herpes virus, equine herpes virus) and found that *O. ficus-indica* had a minor effect on these viruses (Shin et al., 2004). Only high concentrations of crude cactus or fermented cactus (<1 : 40 : 80 of *O. ficus-indica* fruit) were no significant differences between the crude extract of *Opuntia* fruit and fermented *Opuntia*.

1.3. The role as a feed additive

Recently, with the adoption of seed production and feeding skills for aquaculture, the production per square area has increased and is being operated in mass-production. New problems have been arising due to this mass-feeding, as it often brings environmental pollution (Kikuchi et al., 2000), along with many different stresses and diseases (Murata et al., 1996) and lower marketing value (Nakagawa and Kasahara, 1986).

The treatment of microbial diseases in fish by means of currently applied antibiotics or chemotherapeutics is difficult, not particularly effective and costly, and might involve environmental hazards (Immanuel et al., 2004). In addition, the feed used for the flounder requires more than half of the prime cost, and therefore, it renders the greatest burden to the fish farms (Lee et al., 1995). Hence, many researchers are trying to develop the most efficient feed and maximize the feed efficiency, reduce microbial diseases, decrease environmental discharge, and bring forth a guide to profitable growth by using the natural herbal plant as a feed additive.

Various researches have been reported on the use of natural feed additives like terrestrial herbs (Immanuel et al., 2004; Karunasagar et al., 1994; Sahul Hameed and Balasubramanian, 2000), sea weed (Nakagawa and Kasahara, 1986; Yone et al., 1986a, b; Satoh et al., 1987), algae (Nakagawa et al., 1992, 2000; Nakazoe et al., 1986; Watanabe et al., 1990), aloe (Kim et al., 1999; Kim et al., 2000), green tea (Park et al., 1999), orange (Song et al., 2002), paprika (Hancz et al., 2003), manda (natural fermented food in Japan) (Asida and Okimura, 2005) and so on, to increase the feeding efficiency, production and reduce microbial diseases. In recent years, by direct application of various herbal levels used for human treatment and medicinal supplements at traditional Chinese pharmacies into the aquaculture, many different effects such as enhancement of immunity, antibiotic function, growth, and fleshy tissues have been shown (Hwang et al., 1999; Jung et al., 2002).

1.3.1. Immunostimulant effect

Various immunostimulants have been reported to enhance the nonspecific immunity including chitin (Zampini et al., 2005; Wang and Chen, 2005), glucans (Baba et al., 1993; Santarem et al., 1997; Yano et al., 1989), saponin (Ninomiya et al., 1995), nutrition (Blazer, 1992; Pedersen et al., 2004; Skjermo and Bergh, 2004), vitamins (Kiron et al., 2004; Mileva et al., 2002; Sahoo and Mukherjee, 2003) by one of the measures about antibiotics, chemotherapeutics and vaccines.

Generally vegetable extract is known for antimicrobial effect on all kinds of bacteria and fungi (Immanuel et al., 2004; Karunasagar et al., 1994; Lee et al., 1982; Sahul Hameed and Balasubramanian, 2000). Therefore, using vegetable extract as immunostimulants seems to be an attractive alternative way of controlling fish diseases. Herbal ayurvedic products are used as medicines either in the form of extract or powder and they do have growth inhibitory effect against microbial pathogens (Immanuel et al., 2004). It has been reported that the extracts of herbal plant having activity against pathogenic and non-pathogenic Gram-negative bacteria such as, *E. tarda* and *Vibrio* sp. (Cowan, 1999; Jung et al., 2002; Karunasagar et al., 1994; Kim et al., 1999). However, antibacterial effect of cactus has been examined little. Ethanol extracts of cactus showed some activity against Gram-positive bacteria such as *Bacillus subtilis*, *Micrococcus luteus* and *Escherichia coli* (Chung, 2000). In a previous paper, Heo et al. (2003) reported hat fish fed fermented cactus fruit (*O. ficus-indica*) showed strong activity against Gram-positive bacteria, *Streptococcus* sp. in vitro, although the precise antibacterial mechanism remains to be elucidated. There are two kinds of important bacterial diseases, such as Gram-positive bacterial *Streptococcus* sp., Gram-negative bacterial *Edwardsiella tarda* and *Vibrio* sp. among all kinds of bacteria diseases, showing in flounder aquaculture. Seriousness of the infection damage from these pathogenic bacteria are responsible of important economic losses in olive flounder farming (Kwon et al., 1999; Park et al., 1993; Park et al., 1994; Lim et al., 2003; Bang et al., 1992) however, treatment and relief using the antibiotics or chemotherapeutics is under the difficult situation (Immanuel et al., 2004;

Kwon et al., 2002; Kim et al., 2002; Sivaram et al., 2004).

These suggested that cactus may have alternative to synthetic antibiotic drugs against pathogenic bacteria, such as Gram-positive pathogen, *Streptococcus* sp. and Gram-negative pathogens *E. tarda* and *Vibrio* sp. and cactus as a natural immunostimulant will be useful against bacterial disease for cultured fish.

1.3.2. Immunomodulatory effect

There is a lack of information on cactus easily used in fish, because its contains with much cellulose constituent. Incorporation of cellulose in the diet caused poor feed utilization by fish. Therefore, this study was used on the cactus fruit, a fermented stable by effective microorganism to easily dose into fish. Most studies concerned with the effects of microorganisms as probiotics on cultured aquatic animals have emphasized a reduction in mortality or conversely, increased survival (Moriarty, 1998), the improved resistance against disease (Gatesoupe, 1991), the ability to adhere to and colonize the gut (Joborn et al., 1997), the ability to reduce the number of bacterial cells in kidneys (Park et al., 2000), the production of polyamines and digestive enzyme activity (Tovar et al., 2002), and the development of the non-specific immune system by means of cellular systems (Irianto and Austin, 2002), the preservation of various products such as milk, meat, poultry, fruits, vegetables and cereals (Lerol et al., 1996), the preservation of fish products (Gelman et al., 2001), anti-microbial (Nuku-Paavolsa et al., 1999), and cinogenic effects (Fernandes and Shahani, 1990).

Aerobic Gram-positive endospore-forming bacteria, *Lactobacillus* spp. have been evaluated as probiotics, the reduction in the use of chemicalsand antibiotics in the aquatic environment (Austin et al., 1995) and in enhanced growth of the farmed species (Cai et al., 1998; Gatesoupe, 1991; Refstie et al., 2005). Similarity in crustaceans, Venkat et al. (2004) reported growth of the fed probiotic fed groups was significantly higher ($P<0.05$) than the control group after feeding experiment with diet containing lactobacillus-based probiotics in the post-larval *Macrobrachium rosenbergii* for 60 days. Chang and Liu (2002), Moriarty (1998) and Queiroz and Boyd (1998) suggested that probiotics improved the survival of larvae, increased food absorption by enhancing protease levels and gave better growth. However, optimum concentration of those probiotics needs to be standardized in the diet of experimental fish. The probiotic bacteria have to be administered at an optimal dose that may depend on the size and species of experimental fish and used probiotic strain as well as environmental condition in rearing tank.

Therefore, although the exact mechanism by which probiotics inhibited bacteria proliferation in fish still remains to be elucidated, fermented cactus fruit may affect immune system of fish and then, have potential to reduce bacterial diseases, and also fermented cactus fruit can induce better growth and survival in culture fish.

1.3.3. Feeding stimulant effect

Many studies show that four types of common, low molecular weight metabolites, acting either alone or as components of mixtures, serve as potent attractions or as stimulants of feeding behavior (Carr et al., 1996). These feeding substances are mostly amino acid, nucleic acid, organic acid, and sucrose as a level with absorptive function less than 1,000 molecular weight (Carr, 1982).

Cactus contains low-molecular weight compound like that

minerals : potassium is main mineral amounting to about 60 % of total ash content (166 mg/100 g fresh weight), followed by calcium (93 mg/100 g fresh weight), sodium (2 mg/100 g fresh weight), and iron (1.6 mg/100 g fresh weight) while magnesium was not detected (Munoz de Chavez et al., 1995).

sugar : the free sugar content was reported to reach 0.32 g/100 g fresh weight (Munoz de Chavez et al., 1995), but also varying with species.

organic acids : *O. ficus-indica* contains mainly malic acid (36 mg/100 g fresh weight) and citric acid (178 mg/100 g) contents , respectively (Teles et al. 1994), but also varying with species and harvest time.

amino acids : Retamal et al. (1987) reported that the amino acid pattern of *O. ficus-indica* cladodes comprising 18 compounds, such as 13.0 % glutamic acid, 10.6 % asparaginic acid, 8.3 % leucine, 7.7 % alanine, 7.0 % valine, 6.5 % proline, 5.9 % lysine, 5.5 % arginine, 5.2 % isoleucine, 5.1 % phenylalanine, 4.8 % glycine, 4.3 % threonine, 4.3 % serine, 4.1 % tyrosine, 2.3 % histidine, 2.1 % methionine, and 0.8 % cysteine.

However, there is no information available above mentioned on the effects of cactus to fish. The fish used for this study are reviewed in the subsequent sections.

2. OLIVE FLOUNDER (*Paralichthys olivaceus*)

Olive flounder also known as Japanese flounder, *Paralichthys olivaceus*, farming has rapidly progressed with development of artificial feeding skills in the late 1980's (Min, 1988) and with the adoption of aquacultural facilities on land in Korea. The olive flounder aquaculture production was only 1,000 tons in the 90's, which has increased to 34,533 tons in 2003 (Statistical Year Book of Ministry of Maritime Affairs and Fisheries, 2003). This fish are carnivorous and its farming requires either minced whole fish like sardine, horse mackerel, mackerel, anchovy as the main ingredient (protein source) in formulated feeds due to its abundance, low price, high nutritive value and palatability at the sea-aquaculture farms (Kikuchi et al., 2000).

It is very important to raise the productivity and to reduce the cost performance with development of low-price formula feed, to led to upgrade for growth in aquaculture fish (Kikuchi et al., 1992). Therefore, finding an alternative protein source for fish meal and reducing protein content in the feeds by improving utilization of dietary protein are required to produce a stable supply of commercial feeds at a reduced price.

It has been carried out to estimate the optimum dietary protein level for maximum growth of olive flounder range from 46.4 % to 51.2 % (Kim et al., 2002). The appropriate dietary protein and lipid levels for this species have also been reported by Lee et al. (2000), who have suggested that a diet high in protein (50 %) and low in lipid (7 %) is suitable for the growth of juvenile flounder. On the other hand, Kikuchi et al. (2000) reported feed efficiency and protein efficiency ratio increased with increasing lipid in the diets containing 55 % protein for flounder. In addition, Saitoh et al. (2003) reported that in extruded soybean meal for olive flounder, final body weight and weight gain tended to be greater with an increase of extruded soybean meal content in the diet, reached a plateau by 44.5 % protein and 14 % lipid, but not statistically significant.

Several authors have reported relationships between immunostimulation and growth promoting activity for olive flounder. Kim et al. (1998) found that the dietary 0.3 % obosan, Chinese medical herbal mixture, for 48 weeks showed significantly higher survival rate than control ($P<0.05$), and also improved yields in weight gain, specific growth rate, feed conversion ratio and condition factor, significantly ($P<0.05$) in olive flounder. Song et al. (2002) demonstrated that adding fermented orange fluid to a commercial feed showed improvement in the growth rate of juvenile olive flounder. Yeo and Rho (2004) reported that dietary fermented herbal mixture to a moist pellet was increased tolerance against pathological microorganisms on plates. On the other hand, it is reported that adding aloe powder to the feed for juvenile olive flounder increased their growth, and the proper concentrate level was about 0.5 % (Kim et al., 2000). Recently, Asida and Okimura (2005) reported manda (natural fermented food in Japan) as a dietary immunostimulant had enhance the non-specific immune response of *P. olivaceus* in vitro although not affected by growth. According to Jung et al. (2002), fish fed medical herbal plant improved physiological activities and then, triggered a cascade for anti-bacterial reaction of olive flounder.

Therefore, fermented cactus fruit, land herbal resource as a feed additive may be an effective influence on the growth, feed efficiency by improving utilization of dietary protein, and non-specific immune response for olive flounder, so it is preferred to use that for a aquaculture fish farms.

Chapter Ⅰ.

Effects of fermented cactus fruit (*Opuntia ficus-indica*) on the growth of olive flounder, *Paralicthys olivaceus*

1-1. Effects of supplementation of fermented cactus fruit (*Opuntia ficus-indica*) fluid to the diets on the growth of olive flounder, *Paralichthys olivaceus*

Abstract

Two feeding experiments were conducted to investigate the effects and to determine the effective dose of fermented cactus fruit fluid (FCFF) from *Opuntia ficus-indica* as a commercial diet feed supplement for the growth of olive flounder *Paralichthys olivaceus*. FCFF was prepared by mixing cactus fruit and commercially available effective microorganisms and letting the mixture stand for 2 weeks at room temperature. Three replicate groups of olive flounder with an initial average weight of 6.6 g (experiment 1) or 5.1 g (experiment 2) were fed experimental diets containing 0, 1, 5 and 10 % FCFF for 8 months (experiment 1) or 0, 0.2, 0.5 and 1 % FCFF and C-1 % (non-fermented cactus fruit) for 2 months (experiment 2) by volume of diet. In experiment 1, the growth, feed gain ratio, and condition factor were greater in fish fed the diet containing 1 % FCFF that in those fed other levels of FCFF. However, the daily feed rate of fish fed the diet containing 1 % FCFF was significantly lower than that of the other groups ($P<0.05$). In experiment 2, the growth of fish fed the diet containing 1 % FCFF was significantly greater than that of fish fed the diet with no FCFF ($P<0.05$), and the growth of fish fed the diet containing C-1 % was significantly lower than that of fish fed the diet containing 1 % FCFF. These results indicate that a diet with FCFF is more effective than one with unfermented cactus fruit fluid for increasing the growth of Japanese flounder. Thus, FCFF, aland-plant-based herbal supplement, may be an effective feed additive for the improvement of growth and feed efficiency of olive flounder, and the preferred level of supplementation is 1.0 %

Introduction

The farming of olive flounder (*Paralichthys olivaceus*) has progressed rapidly with the development of artificial feed in the late 1980s (Min, 1988) and the adoption of land-based aquacultural facilities in Korea. The production of cultured olive flounder was only 1,000 tons in the 1990s but increased to 34,533 tons in 2003 (Statistical Year Book of Ministry of Maritime Affairs and Fisheries, 2003). Recently, with the adoption of seed production and improved feeding, the flounder production per unit square area has increased, resulting in mass production. New problems associated with mass-feeding have arisen because it often causes environmental pollution (Kikuchi et al., 2000), along with stresses and diseases in fish (Murata et al., 1996).

The treatment of microbial diseases in fish using antibiotics or chemotherapeutics is costly, difficult, not particularly effective and may create environmental hazards (Immanuel et al., 2004). In addition, the feed used for flounder production accounts for more than half of the prime cost of production and is therefore the greatest burden to fish farms (Lee et al., 1995). Hence, many researchers have attempted to develop new feed to maximize feed efficiency, reduce microbial diseases, decrease environmental discharge, and produce more profitable fish growth by using natural herbal plant products as feed additives.

Various studies have examined the use of natural feed additives, such as terrestrial herbs (Karunasagar et al., 1994; Sahul Hameed and Balasubramanian, 2000; Immanuel et al., 2004), sea weed (Nakagawa et al., 1986; Yone et al., 1986a, b; Satoh et al., 1987), algae (Nakazoe et al., 1986; Watanabe et al., 1990; Nakagawa et al., 1992, 2000), aloe (Kim et al., 1999, 2000), green tea (Park et al., 1999), orange (Song et al., 2002), and paprika

(Hancz et al., 2003), to increase feed efficiency, enhance fish production and reduce microbial diseases. In recent years, many different effects, such as the enhancement of immunity, antibiotic function, growth and fleshy tissues in fish aquaculture, have been shown (Hwang et al., 1999; Jung et al., 2002) by direct application of various herbs and medicinal supplements traditionally used for the treatment of humans.

Cactus (*Opuntia ficus-indica*) is a species of native plant from Mexico, Latin America, South Africa, the Mediterranean, and Korea. It is known to be effective in improving digestive function, treating wounds and long-term reduction of the symptoms of rheumatic arthritis, so it has often been used as a folk remedy and is well known as a medicinal herb (Song et al., 1989). Recently, laboratory tests have found many beneficial effects of this plant on mammals, for example, anti-tumor (Shin et al., 1998), anti-oxidant (Kuti, 2004), anti-viral (Ahmad et al., 1996), anti-ulcer (Lee et al., 1998) and anti-bacterial (Chung, 2000; Heo et al., 2003) effects, as well as cholesterol-lowering properties (Palumbo et al., 1999). However, there is no information available on the effects of this plant on the growth of fish. Therefore, this study examined the effects of fermented cactus fluid, made stable by the presence of effective microorganisms, on the growth of olive flounder.

Materials and Methods

Fish and experimental conditions

Juvenile olive flounder (*Paralichthys olivaceus*), 7-8 cm long and 5-6 g in weight, were obtained from a commercial hatchery and were transported to the rearing facilities of the Jeju Province Fisheries Resources Research Institute, Korea. Two experiments were conducted to determine the effective dietary levels of fermented cactus fruit fluid (FCFF) for olive flounder. The first experiment examined the effect of four dietary treatments over 8 months, while the second experiment examined the effect of five dietary treatments over 2 months. All treatments were replicated three times. Cylindrical polypropylene (PP) tanks (1.2 m in diameter, 1.0 m in height)were used as experimental tanks. Each tank was stocked with 200 juvenile flounder, and the water was changed 10-15 times a day. The length and weight of each fish were measured each month. After the measurements, all of the fish were bathed in 100 ppm HCL-oxytetracline (OTC) solution for 1 h.

The water quality parameters in the tank were monitored daily using a YSI system (YSI 556 multi-probe system). The ranges were: temperature, 17.2-20.8 °C; salinity, 31-34 ppt, dissolved oxygen (DO), 6.72-7.32 mgL^{-1}; and pH, 7.30-8.09.

Manufacture of fermented cactus fruit fluid (FCFF)

The cactus fruits (*O. ficus-indica*) used for this research were obtained from the local market on Jeju Island. We used the effective microorganism (EM) product marketed by the Korea EMRO (EM Research Organization) for fermentation. The EM product was composed mainly of lactic acid bacteria and photosynthetic bacteria. The fermentation process involved pulverizing the fruits and mixing them into starch syrup (at 10 % of the cactus fruit) and EM (at 5 % of the cactus fruit). After 2 weeks at room temperature, the EM product reached 10^9 colony-forming units (CFU) mL^{-1}, at which point the FCFF was used in the experiments.

Diet preparation and feeding

The basic experimental diet was in the form of a commercial extruded pellet (Marubeni-Nissin formula feed, Japan). FCFF was added to the diet at 0, 1, 5 and 10 % (experiment 1), and 0, 0.2, 0.5, 1 and C-1 % (non-fermented cactus fruit; experiment 2) by volume of diet. The commercial diet was soaked in the desired concentrations of FCFF to absorb the fluid. The diets were then fed twice daily.

The proximate composition of the commercial diet, the non-fermented cactus fruit, and the FCCF, including amino acids and fatty acids, were analyzed by the Science Laboratory Center Co., Korea, according to standard procedures. These results are shown in Tables 1, 2 and 3, respectively.

The feed gain ratio (FGR), specific growth rate (SGR), daily feeding rate (DFR), and condition factor (CF) were calculated using the following formulae:

$$FGR = TF/TWG$$

$$SGR = [(\ln FW - \ln IW)/t] \times 100$$

$$DFR = (TF \times 100) / [(IW + FW) \times days\ fed/2]$$

$$CF = (BW/TL^3) \times 10^3$$

where BW is the body weight, IW is the initial weight, FW is the final weight, TF is the total amount of feed, TL is the total length, t is the rearing time (days), and TWG is the total weight gain.

Statistical analysis

The data were analyzed using SPSS version 7.5 for Windows (SPSS Science, Chicago, IL, USA); significant differences (at $P < 0.05$) were verified using one-way analysis of variance (ANOVA), followed by Duncan's multiple range tests.

Table 1. A proximate composition of the commercial diet, non-fermented cactus fruit fluid and fermented cactus fruit fluid (FCFF) (%, dry matter basis)

Component	Commercial Diet (%)	Non-fermented Cactus Fluid (%)	FCFF (%)
Moisture	3.36	83.5	85.6
Crude protein	52.0	1.08	1.08
Crude lipid	12.6	0.54	0.60
Crude fiber	1.86	2.51	0.67
Crude ash	12.0	1.28	1.34
Ca	2.56	0.24	**
P	1.90	0.15	0.11
Vitamin C	0.01	0.06	0.01
Vitamin E (a-Tocopherol)	0.06	0.01	**
Carotenoid	* NA	0.02	0.01
β-Glucan	* NA	0.42	0.45
Flavonoid	* NA	0.10	0.07

*, not analyzed; **, less than 0.01 %

Table 2. Amino acid levels in the commercial diet, non-fermented cactus fruit fluid and fermented cactus fruit fluid (FCFF) (%, dry matter basis)

Amino acid	Commercial Diet (%)	Non-fermented Cactus Fluid(%)	FCFF (%)
Aspartic acid	4.84	0.46	0.84
Theronine	2.27	0.19	0.19
Serine	2.18	0.25	0.26
Glutamic acid	8.16	1.06	0.92
Proline	2.53	0.43	0.38
Glycine	3.46	0.39	0.35
Alanine	3.48	0.27	0.31
Valine	2.59	0.21	0.24
Isoleucine	2.19	0.16	0.19
Leucine	4.07	0.35	0.37
Tyrosine	1.74	0.26	0.21
Phenylalanine	2.27	0.21	0.24
Histidine	1.89	0.21	0.20
Lysine	4.22	0.22	0.30
Arginine	2.97	0.54	0.44
Cystine	0.48	0.11	0.09
Methionine	1.26	0.08	0.05
Tryptophan	0.48	-	-

-; not detected

Table 3. Fatty acid levels in the commercial diet, non-fermented cactus fruit fluid and fermented cactus fruit fluid (FCFF) (%, dry matter basis)

Fatty acid	Commercial Diet (%)	Non-fermented Cactus Fluid (%)	FCFF (%)
C10:1	-	0.30	-
C12:0	-	0.76	0.05
C13:0	-	0.84	-
C14:0	6.43	1.09	0.11
C15:0	0.47	0.32	-
C16:0	19.6	27.1	11.4
C16:1	6.76	0.59	0.52
C17:0	0.77	0.63	-
C17:1	-	0.34	11.6
C18:0	3.92	3.13	4.66
C18:1n-9	16.5	11.5	-
C18:2n-6	3.51	25.8	68.0
C18:3n-3	0.76	14.5	1.22
C18:4n-3	2.06	-	-
C19:0	-	0.06	-
C20:0	0.35	1.31	0.48
C20:1	3.52	0.58	0.40
C20:3n-3	-	0.33	0.13
C20:4n-6	1.02	1.02	-
C20:5n-3	12.6	-	0.27
C22:1	3.72	2.26	-
C22:1n-9	-	0.79	0.15
C22:4n-6	0.23	2.56	-
C22:5n-3	1.56	-	0.21
C22:6n-3	12.6	3.92	0.08
Unknown	3.59	0.39	0.75

-; not detected.

Results

Experiment 1

Growth and survival rate

The results for the growth performance of juvenile flounder fed various experimental diets for 8 months are shown in Table 4. The growth of fish fed the diet containing 1 % FCFF was significantly higher than that for any of the other groups receiving different levels of FCFF ($P<0.05$). In contrast, the growth of fish fed the diet containing 10 % FCFF was even lower than that of the group receiving the diet without FCFF (i.e., the control).

The survival rate of fish in each dietary group was over 88 %, and there was no significant difference among treatments. Fish fed the diet containing 1 % FCFF showed slightly higher survival than those fed the control diet. Above the level of 1 % FCFF, the growth and survival of fish decreased with increasing dietary FCFF levels.

Table 4. Total length (TL), body weight (BW) and survival rate of olive flounder fed experimental diets containing different levels of fermented cactus fruit fluid (FCFF) for 8 months (experiment 1)

Treatment	Initial		Final		Survival rate (%)
	TL (cm)	BW (g)	TL (cm)	BW (g)	
Control	8.47±0.15	6.58±0.31	29.7±0.30^b	302±8.81^b	92.7
1 %	8.44±0.08	6.57±0.23	30.7±0.27^a	336±12.7^a	94.7
5 %	8.51±0.13	6.69±0.29	29.8±0.07^b	302±8.36^b	92.0
10 %	8.47±0.06	6.50±0.19	29.6±0.43^b	300±10.3^b	88.0

Values (mean±SD of three replications) in the same column not sharing a common superscript are significantly different ($P<0.05$).

FGR, SGR, DFR, and CF

Table 5 shows the FGR, SGR, DFR, and CF during experiment 1. The best FGR was obtained when fish were fed the diet containing 1 % FCFF, although the differences among groups were not statistically significant. The SGR of fish fed the diet containing 1 % FCFF was significantly higher than that of other groups ($P<0.05$). In contrast, the DFR was significantly lower in fish fed the diet containing 1 % FCFF, and it increased with increasing dietary FCFF levels.

Fish fed the diet containing 10 % FCFF showed the highest CF, although there were no significant differences among the treatment groups ($P>0.05$).

Table 5. Effect of different levels of dietary fermented cactus fruit fluid (FCFF) on the feed gain ratio (FGR), specific growth rate (SGR), daily feeding rate (DFR) and condition factor (CF) of olive flounder (experiment 1)

Treatment	FGR	SGR	DFR	CF
Control	0.53±0.02	$1.61±0.02^{b}$	$1.65±0.10^{a}$	11.4±0.28
1 %	0.49±0.02	$1.66±0.03^{a}$	$1.46±0.08^{b}$	11.5±0.33
5 %	0.54±0.07	$1.61±0.02^{b}$	$1.66±0.08^{a}$	11.4±0.07
10 %	0.56±0.01	$1.62±0.03^{b}$	$1.73±0.13^{a}$	11.5±0.40

Values (mean±SD of three replications) in the same column not sharing a common superscript are significantly different ($P<0.05$).

Experiment 2.

Growth and Survival rate

The final weight of fish fed the diet containing 1 % FCFF was significantly greater than that of fish fed the diet without FCFF (P<0.05; Table 6). There was no significant difference in the final weight among fish fed 0.2, 0.5, and 1 % FCFF (P>0.05). In contrast, the final weight of fish fed the diet containing 1 % raw cactus fruit (non-fermented) was even lower than that of fish fed the diet with no FCFF (i.e., control).

Survival was lowest in fish the diet containing 1 % FCFF; this was caused by a high mortality rate in one of the rearing tanks. However, there was no significant difference in survival among groups fed diets containing different levels of FCFF.

Table 6. Total length (TL), body weight (BW) and survival rate of olive flounder fed experimental diets containing different levels of fermented cactus fruit fluid (FCFF) (experiment 2)

Treatment	Initial		Final		Survival rate (%)
	TL (cm)	BW (g)	TL (cm)	BW (g)	
Control	8.72±0.19	5.28±0.27	19.7±0.19^{ab}	87.2±0.98^{ab}	90.0
0.2 %	8.58±0.05	5.01±0.17	19.9±0.37^{ab}	92.4±4.44^{bc}	93.3
0.5 %	8.43±0.14	4.72±0.21	20.0±0.14^{ab}	93.3±4.38^{bc}	93.3
1.0 %	8.49±0.25	4.96±0.63	20.1±0.44^{b}	95.0±2.13^{c}	82.3
C-1 %[1)]	8.69±0.10	5.38±0.54	19.4±0.42^{a}	84.8±5.68^{a}	89.5

Values (mean±SD of three replications) in the same column not sharing a common superscript are significantly different (*P*<0.05).

[1)] Feed was supplemented with 1 % raw non-fermented cactus fruit fluid.

FGR, SGR, DFR and CF

The groups of fish that were fed diets containing FCFF showed higher FGR than did the control group (Table 7). Fish fed the control diet had a low SGR; this improved with increases in supplementation with FCFF and reached a plateau by 0.5 % FCFF. Fish fed the diet containing raw cactus fruit had a lower SGR than those fed the control diet. The DFR was highest in fish fed the diet containing 1 % raw cactus fruit (C-1 %). The lowest DFR was observed in fish fed the diets containing 0.2 and 0.5 % FCFF.

The CF was higher in all experimental groups fed diets containing FCFF and raw cactus fruit (C-1 %) than in those fed the control diet, although these differences were not significant. There was also no significant difference among the groups.

Table 7. Effect of different levels of dietary fermented cactus fruit fluid (FCFF) on the feed gain ratio (FGR), specific growth rate (SGR), daily feeding rate (DFR) and condition factor (CF) of olive flounder (experiment 2)

Treatment	FGR	SGR	DFR	CF
Control	0.62±0.04	$2.67±0.05^{ab}$	1.20±0.06	11.4±0.35
0.2 %	0.52±0.07	$2.78±0.05^{ab}$	1.14±0.06	11.7±0.32
0.5 %	0.55±0.07	$2.84±0.09^{b}$	1.14±0.06	11.5±0.31
1.0 %	0.53±0.27	$2.81±0.10^{b}$	1.23±0.27	11.7±0.61
C-1 %[1)]	0.59±0.02	$2.63±0.13^{a}$	1.28±0.08	11.6±0.10

Values (mean±SD of three replications) in the same column not sharing a common superscript are significantly different (*P*<0.05).

[1)] Feed was supplemented with 1 % raw non-fermented cactus fruit fluid.

Discussion

Many studies aimed at enhancing feed quality and antibacterial effects by adding herbal supplements to commercial feed have been performed in recent years (Hwang et al., 1999; Kim et al., 2000; Ashida et al., 2002; Immanuel et al., 2004; Sivaram et al., 2004). However, herbal land plants have mainly been examined for their antibacterial effects (Cowan, 1999). With regard to the effect of herbal supplements on growth, feed supplemented with aloe powder increased the growth of juvenile olive flounder, and the optimal concentration was about 0.5 % (Kim et al., 2000). According to Hwang et al. (1999), a significant growth effect was observed in juvenile Nile tilapia with the addition of 3 % powdered fruits of the Chinese medicinal plant *Lycium chinense* to the feed. Similarly, certain seaweeds, such as *Undaria penatifida* and *Ascophyllum nodosum*, increase growth at supplementation levels of 5 and 10 %, respectively (Yone et al., 1986a). However, the effects of seaweed are related to physiological functions (Amano and Noda, 1985; Yone et al., 1986a; Watanabe et al., 1990; Nakagawa et al., 1997) and disease resistance (Satoh et al., 1987), rather than to the growth of fish. In addition, these results were obtained by adding powdered extracts, whose concentrations would be much higherthan the doses used in the present study. Although the levels of supplementation and the time-frames of the experiments may differ, it is important that the supplement levels be minimized to reduce production costs.

In this study, FCFF was shown to have a significant effect on the growth and feed efficiency in olive flounder, similar to the findings of Song et al. (2002), who demonstrated that adding fermented orange fluid to feed at 0.1-0.2 % resulted in a greater growth rate of juvenile olive flounder compared with that of the control group.

In the present study, the best FGR and SGR were obtained with a 1 % supplementation of FCFF, although the DFR was slightly lower than that of the control group. This may be related to the taste of the FCFF, which may have decreased feed intake. Fish differ in growth and feed efficiency depending upon the species, age, supplement level and feeding time-span (Nagakawa et al., 1984); in the case of herbal supplements, long-term application would be more effective (Hwang et al., 1999).

The FCFF used here was made by fermentation processing using effective microorganisms (EM) composed mainly of lactic acid bacteria and photosynthetic bacteria. The fermented cactus fruit resulted in significantly faster fish growth (at a 1 % supplementation level) than did the non-fermented cactus fruit.

Fleming (1982) explained that fermentation of vegetable extracts results in the activation of enzymes and a change in vegetable nutrients to an easily digestible and absorbable form. Therefore, fermentation of extracts by microorganisms may be an effective method to minimize the required level of supplementation. Further experiments are needed to elucidate the effects and roles of the microorganisms used in this study on the growth and survival of fish.

We found that supplementation with 10 % FCFF induced lower growth and feed efficiency than in the control. The over-application of a dietary supplement may have negative effects. Yone et al. (1986b) concluded that replacing fish meal with a large amount of algae may eventually depress the amount of digestible protein in the diet.

The fermentation of the cactus fruit resulted in a reduction of crude fiber from 2.51 to 0.67 % (Table 1). The addition of cellulose to the diet resulted in reduced growth and poor feed use in flounder. Generally, dietary fiber is not hydrolyzed by fish (NRC, 1993), and high fiber levels may reduce nutrient absorption (Anderson et al., 1984). Thus, the reduction in dietary fiber by fermentation may overcome problems caused by the raw form of the cactus fruit fluid. However, some minerals, such as calcium and phosphorous, may be reduced by fermentation. FCFF contains lower levels of beta glucan, carotenoids, flavonoids, and vitamin C and E, which supplement radical-scavenger activity (Regnault et al., 1993), than does the cactus fruit. Thus, FCFF may have effective immune activity in fish, and may be useful in rearing cultured fish.

Fermentation resulted in higher levels of the amino acids aspartic acid, valine, isoleucine and lysine (Table 2). Park et al. (2000) reported that free amino acids affect the growth of juvenile olive flounder. Most amino acids, nucleic acids, organic acids, and sugars are well known as feeding stimulants, and those of less than 1,000 molecular weight are easily absorbed (Carr, 1982). Many smaller amino acids differ in their effects, depending upon the fish species (Fukuda et. al, 1989; Kohbara et. al., 1989; Elias and Joseph, 1999; Iwao et. al., 2000). In the present study, it is possible that some amino acids with high molecular weights may have been changed during fermentation to amino acids of lower molecular weight, which subsequently acted as stimulants.

Of the 25 fatty acids analyzed, e.g., myristic acid, magaoleic acid, stearic acid, oleic acid, linoleic acid, eicosapentaenoic acid (EPA), and docosapentaenoic acid (DPA) (Table 3), all were found in greater quantities after fermentation, except for EPA and DPA, which decreased sharply after fermentation.

The results of this study suggest that the supplement FCFF may be effective for increasing the growth and feed efficiency of olive flounder. Thus, this substance is desirable for use in aquaculture, and the optimal level of supplementation in commercial fish feed is 1.0 %.

1-2. Effects of dietary fermented cactus fruit (*Opuntia ficus-indica*) powder with moist pellets on growth, body composition and blood parameters of olive flounder, *Paralichthys olivaceus*

Abstract

Triplicate groups of olive flounder, with initial length and weight of 32.4 cm and 394.1 g were fed moist pellet-based diets containing 0 %, 0.02 %, 0.04 %, 0.08 %, 0.16 % fermented cactus fruit (*Opuntia ficus-indica*) powder (FCFP) for 8 months to determine the effective dose. FCFP was prepared by fermenting the cactus fruit mixture with effective microorganisms followed by freeze drying. The final growth performance and condition factor (CF) of fish fed diet containing 0.08 % FCFP were significantly higher ($P<0.05$) than that of the other groups receiving different levels of FCFP. The best feed gain ratio (FGR) was obtained in fish fed diet containing 0.08 % FCFP, although not statistically different. On the other hand, daily feed rate (DFR) of fish fed diets containing FCFP were lower than that of diet without FCFP. Body composition of fish fed diet containing 0.08 % FCFP was significantly higher ($P<0.05$) in crude lipid content. However, glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvate transaminase (GPT) values clearly decreased with increasing dietary FCFP levels.

The result of the present study demonstrated that FCFP, land herbal resource as a feed additive might be an effective ingredient for the growth and the feed efficiency of olive flounder, and preferable supplemental level to moist pellet type feed is assumed to be 0.08 %.

Introduction

Olive flounder also known as Japanese flounder in Japan, *Paralichthys olivaceus* is one of the commercial important marine finfish species cultured in Korea and Japan and its culture production has increased rapidly since the late 1980's in Korea, reached to 34,533 tons in 2003 (Statistical Year Book of Ministry of Maritime Affairs and Fisheries, 2003). This fish is carnivorous and its farming requires either minced whole fish like sardine, horse mackerel, mackerel, anchovy, and so on asa moist feed (Jeong, 1992), or prepared feeds containing high levels of fish meal (Forster and Ogata, 1998). This means that the feed used for the flounder requires high prime cost. In addition, due to this aquaculture system, many new problems have been arising : environmental pollution (Kikuchi et al, 2000), along with many different stresses and diseases (Murata et al., 1996), lower marketing value (Nakagawa and Kasahara, 1986). Hence, many researchers are trying to develop the most efficient feed and maximize the feed efficiency, reduce microbial diseases, decrease environmental discharge, and bring forth a guide to profitable growth by using the chemotherapeutics, immunostimulants and natural feed additives. The use of chemotherapeutics has several short-comings including the risk of generating resistant pathogens, the problem of drug residues in the treated fish, cost burden and the impacts of environmental pollution (Kim et al., 1999).

Immunostimulants stimulate the immune system against pathogens and enhance the host defense system against pathogens by increasing the chemiluminescent response and by superoxide anion production (Sakai, 1998), however immunostimulants can usually focus on the pathogen with disadvantage rather than growth of fish.

In recent years, direct application of various herbal levels as a natural feed additive are used in aquaculture (Jung et al., 2002). There are many kinds of herbal plant as a feed additive has been used : terrestrial herbs (Immanuel et al., 2004), sea weed (Nakagawa and Kasahara, 1986), algae (Nakazoe et al., 1986), aloe (Kim et al., 1999), green tea (Park et al., 1999), orange (Song et al., 2002), paprika (Hancz et al., 2003), manda (natural fermented food in Japan) (Ashida and Okimasu, 2005) and so on, to increase the feeding efficiency, production and reduce microbial diseases.

Opuntia ficus-indica (prickly pear), a cactus native of Mexico growing in desert and sub-desert areas, was introduced into Jeju Island in Korea. This plant is widespread in Jeju Island and is cultivated for its sweet fruits, and its fruits production reaching over 3,000 tons/year.

Cactus has been reported to have several important therapeutic properties including anti-tumor, anti-oxidant, anti-viral, anti-ulcer, cholesterol-lowering and anti-bacterial effects in mammals (Ahmad et al., 1996; Chung, 2000; Kuti, 2004; Lee et al., 1998; Palumbo et al., 2003; Shin et al., 1998). In former study, we found for the first time that fermented cactus fruit fluid (FCFF) has significant effect on the growth and feed efficiency of olive flounder (unpublished).

The present was carried out to investigate the effects of cactus powder, a fermented stable by effective microorganism mixed with moist pellet feed, on the growth for olive flounder.

Materials and Methods

Experimental fish and experimental conditions

The experiment was conducted to determine the effect of fermented cactus fruit powder (FCFP) on the growth of flounder for eight months. The juvenile olive flounder, *Paralichthys olivaceus*, obtained from a private fish farm, with a mean body length of 32.4 cm and 394 g mean body weight, were transported to the rearing facilities of the Jeju Province Fisheries Resources Research Institute. There was five treatments (0, 0.02, 0.04, 0.08 and 0.16 % FCFP, respectively), each with three replicates. Cylindrical poly-propylene (PP) tanks (diameter 1.2 m×1.0 m height) were used as experimental tanks. Each tank was stocked with 20 flounder.

The rearing water was changed 15-20 times a day. Length and weight of fish were measured every month. After measuring, all fish were bathed with 100ppm HCL-Oxytetracline (OTC) solution for an hour.

The water quality parameters in the tank was monitored daily using a YSI system (YSI 556 multi-probe system). The ranges were : temperature 15.7-22.4 ℃, salinity 32-33 ppt, DO 4.1-9 ㎎/L, and pH 7.15-8.15.

Manufacturing of fermented cactus fruit powder (FCFP)

The fruits of cactus (*O. ficus-indica*) used for this research were collected from the local market in the Jeju Island. For fermentation, the effective microorganism (EM) product marketed by Korea EM Research Organization (EMRO) was used. The fermentation process involved pulverizing the fruits and mixing them into starch syrup (10 % of the cactus fruit) and EM (5 % of the cactus fruit). After incubation for 2 weeks at room temperature, the substance was freeze-dried and used for the experiment.

Diet preparation and feeding

The assorted diets used for the experiment were prepared in the form of a moist pellet with horse mackerel fish and the diet contained 61.2 % protein, 16.2 % lipid and 17.0 % ash on dry matter basis (Table 1). FCFP were added to the diet at the levels of 0 %, 0.02 %, 0.04 %, 0.08 % and 0.16 %, respectively. The additive method of FCFP was to mix it with the diet at desired concentration before pelleting. The diets were fed to visual satiety twice a day.

Table 1. Proximate compositions of moist pellet (MP) diet and fermented cactus fruit powder (FCFP) (%)

Component	MP (%)	FCFP (%)
Moisture	74.1	14.02
Crude protein	16.0 (61.0***)	6.02
Crude lipid	4.2 (16.2***)	1.32
Crude fiber	4.4 (17.0***)	10.9
Crude ash	* NA	7.61
Ca	* NA	1.85
P	* NA	0.02
Vitamin C	* NA	**
Vitamin E (a-Tocopherol)	* NA	0.01
Carotenoid	* NA	**
Flavonoid	* NA	0.08

*, not analyzed; **, less than 0.01 %; ***, % dry basis

Analytical methods

Proximate compositions, amino acid and fatty acid of FCFP were analyzed according to standard procedures by Science Lab. Center Co., Korea. These results are shown in Table 1, 2, 3 respectively. At the end of the feeding trial, 5 fish from each tank were randomly sampled for whole body proximate composition and blood analyses. Five fish after the fish were starved for 24 h, anesthetized with MS-222 at concentration of 100 ppm and blood samples were collected by disposable syringe from the caudal vein. Blood plasma was separated after centrifugation (5,000 rpm, 15 min) and was analyzed for albumin, total protein, glutamic-oxaloacetic transminase (GOT), glutamic-pyruvate transminase (GPT) and total cholesterol by automatic analyzer (Boehringer Mannheim, Germany).

The feed gain ratio (FGR), specific growth rate (SGR), daily feeding rate (DFR) and condition factor (CF) were calculated by the following formulae and their values were compared with each other.

Feed gain ratio (FGR) = TF/TWG

Specific growth rate (SGR) = $\{(\ln FW - \ln IW)/t\} \times 100$

Daily feeding rate (DFR) = $(TF \times 100)/\{(IW + FW) \times day\ fed/2\}$

Condition factor (CF) = $(BW/TL^3) \times 10^3$

where :

BW : body weight, IW : initial weight, FW : final weight, TF : total feed, TL : total length, t : rearing time (day), TWG : total weight gain

Statistical analysis

The data were calculated using the statistical program for Windows (SPSS ver.7.5, USA) and a significant value (P<0.05) was verified with Duncan's multiple range test after One-way ANOVA test.

Table 2. Amino acid levels fermented cactus fruit powder (FCFP) (%)

Amino acid	FCFP (%)
Aspartic acid	0.40
Threonine	0.17
Serine	0.18
Glutamic acid	0.63
Proline	0.42
Glycine	0.30
Alanine	0.28
Valine	0.21
Isoleucine	0.12
Leucine	0.22
Tyrosine	0.18
Phenylalanine	0.14
Histidine	0.21
Lysine	0.17
Arginine	0.21
Cystine	0.15
Methionine	0.06
Tryptophan	0.04

Table 3. Fatty acid levels of fermented cactus fruit powder (FCFP) (%)

Fatty acid	FCFP (%)
C12:0	0.69
C14:0	1.90
C14:1	0.86
C16:0	17.9
C16:1	2.35
C18:0	3.63
C18:1	13.4
C18:2n-6	49.5
C18:4n-3	7.02
C20:0	0.65
C20:1	0.46
C22:0	0.71
C22:5n-3	0.93
Unknown	0.01

Results

Growth performance

The results of growth performance of flounder fed various experimental diets for 8 month are shown in Table 4. The final weight was lowest with fish fed the diet without FCFP (control), and improved by supplement with FCFP. The fish fed the diet with 0.08 % FCFF showed the best growth. There was no significant difference between weight gain control and other dietary levels of FCFP. However, fish fed 0.08 % FCFP showed the lowest survival of fish which was originated from high mortality in one of the rearing tanks, although there was no significant difference between the survival of fish in 0.08 % FCFP and the control group.

Table 4. Total length, body weight and survival rate of olive flounder fed experimental diets containing different levels of fermented cactus fruit powder (FCFP) for eight months

Treatment	Initial		Final		Survival rate (%)
	TL (cm)	BW (g)	TL (cm)	BW (g)	
Control	32.4±0.08	395±8.03	40.4±0.85^{a}	826±65.6^{a}	91.6^{ab}
0.02 %	32.5±0.35	401±10.7	40.8±0.86^{ab}	844±56.7^{a}	95.0^{ab}
0.04 %	32.2±0.22	394±3.33	40.8±0.66^{ab}	856±52.1^{a}	96.7^{ab}
0.08 %	32.1±0.13	392±6.46	42.0±0.67^{b}	969±40.5^{b}	88.3^{a}
0.16 %	32.3±0.21	400±11.1	40.9±0.87^{ab}	858±70.4^{a}	98.3^{b}

* Values (mean±SD of three replications) in the same column not sharing a common superscript are significantly different (P<0.05)

Table 5 shows the value of feed gain ratio (FGR), specific growth rate (SGR), daily feeding rate (DFR) and condition factor (CF) during the experiment. The best value of the FGR was obtained with fish fed the diet containing 0.08 % FCFP although not statistically significant. SGR of fish fed the diet containing 0.08 % FCFP was significantly ($P<0.05$) higher than those of other groups ($P<0.05$). On the other hand, DFR were lower in the fish fed the diets containing FCFP than that of diet without FCFP although there was no significant difference ($P>0.05$) among the DFR values in different treatments. Fish fed the diet containing 0.08 % FCFP showed significantly ($P<0.05$) highest CF values among all groups. FGR, SGR and CF were increased with increasing dietary FCFP levels, reached a plateau by 0.08 % supplement. On the other hand, DFR was observed opposite tendency of FGR, SGR and CF.

Table 5. Effect of different levels of dietary fermented cactus fruit powder (FCFP) on FGR, SGR, DFR and CF of olive flounder

Treatment	FGR	SGR	DFR	CF
Control	3.86±0.58	3.30±0.03^{a}	2.09±0.18	12.2±0.08^{a}
0.02 %	3.60±0.42	0.30±0.02^{a}	2.01±0.13	12.1±0.16^{a}
0.04 %	3.57±0.33	0.32±0.02^{a}	1.99±0.08	12.2±0.21^{a}
0.08 %	3.55±0.11	0.37±0.02^{b}	1.90±0.07	12.8±0.05^{b}
0.16 %	3.70±0.17	0.31±0.03^{a}	1.97±0.10	12.2±0.35^{a}

* Values (mean±SD of three replications) in the same column not sharing a common superscript are significantly different ($P<0.05$)

Body composition

The proximate composition of whole body is shown in Table 6. Crude protein was higher in fish fed diets containing FCFP than that of diet without FCFP although not statistically different among groups. Fish fed diet containing 0.08 % FCFP showed significantly (P<0.05) higher crude lipid compare to the fish fed diets containing 0 to 0.04 % FCFP. On the other hand, moisture was lowest in fish fed diet containing 0.08 % FCFP. No difference was observed in whole body ash content in fish fed different diets.

Table 6. Proximate body composition of olive flounder fed diets containing different levels of fermented cactus fruit powder (FCFP) for eight months (%)

Treatment	Moisture	Crude Protein	Crude Lipid	Crude Ash
Control	75.6±1.02	18.2±0.83	$2.81±1.04^{a}$	2.12±0.70
0.02 %	74.7±1.15	18.7±0.58	$3.07±0.87^{a}$	2.23±0.81
0.04 %	76.1±1.67	18.6±1.62	$3.14±0.82^{a}$	1.85±0.17
0.08 %	73.9±2.58	18.2±0.41	$4.91±1.73^{b}$	2.18±0.39
0.16 %	74.9±0.57	18.7±0.74	$3.92±0.85^{ab}$	2.24±0.56

[*] Values (mean±SD of three replications) in the same column not sharing a common superscript are significantly different (*P*<0.05)

Blood composition

The hemochemical parameters of fish are shown in Table 7. The value of albumin in plasma was the higher in fish fed diets containing FCFP than that of control group. However, there was no significant difference among groups. There was no significant difference between the total protein in groups fed diets containing different levels of FCFP. Glutamic-oxaloacetic transaminase (GOT) value of fish was decreased in fish fed diets containing FCFP than that of control group, although no clear difference was observed between the fish fed diets containing different levels of FCFP. The lowest value of the glutamic-pyruvate transaminase (GPT) was observed with fish fed diet containing 0.16 % FCFP although not statistically significant. The values of GOT and GPT were apparently improved in fish fed diets containing different levels of FCFP than that of diet without FCFP, although not statistically significant. Total cholesterol was no significantly different among groups. Similar to the trend observed in the GPT and the total cholesterol of fish, decreased with increasing dietary FCFP levels.

Table 7. Blood chemistry of olive flounder fed diets containing different levels of fermented cactus fruit powder (FCFP) for eight months

Treatment	[1]ALB(㎎/㎗)	[2]TP(㎎/㎗)	[3]GOT(IU/ℓ)	[4]GPT(IU/ℓ))	[5]TC(㎎/㎗)
Control	1.74±0.22	4.94±0.62	38.0±16.3	6.25±3.95	290±39.3
0.02 %	1.81±0.40	5.19±0.78	24.0±4.76	4.75±2.06	341±26.4
0.04 %	2.11±0.32	5.36±0.18	25.3±9.74	4.50±1.29	324±85.7
0.08 %	1.89±0.51	4.83±0.5	25.5±16.3	4.50±1.91	309±57.8
0.16 %	1.86±0.30	4.98±0.63	22.5±8.50	3.50±1.29	283±40.7

[1] ALB : albumin
[2] TP : total protein
[3] GOT : glutamic-oxaloacetic transaminase or AST(asparatate aminotranferase)
[4] GPT : glutamic-pyruvate transaminase or ALT(alanine aminotranferase)
[5] TC : total cholesterol

Discussion

Considerable work has been reported on feed additives of cultured flounder, and diverse herbal compounds are thought to be attractants or feeding stimulants.

Various herbal plant including traditional Chinese pharmacies used for human treatment has been reported to enhance the non-specific immunity (Hwang et al., 1999), antibiotic function (Jung et al., 2002), growth (Kim et al., 1998; Park et al., 2003) and fleshy tissues (Lee et al., 1998). In the present study, fish fed diet containing 0.08 % FCFP significantly ($P<0.05$) improved the growth of fish. The feed efficiency values were also better in fish fed diets containing FCFP than that of diet without FCFP. This agrees with our previous results (Go and Satoh, unpublished) that the fish fed diet containing 1 % fermented cactus fruit fluid (FCFF) had significantly ($P<0.05$) highest growth in fingerling and young olive flounder. Feed gain ratio (FGR), specific growth rate (SGR) and condition factor (CF) of *P. olivaceus* fed diet containing 1 % FCFF were better than the other groups receiving different levels of FCFF. However, no difference was observed in survival from fish fed various diets. A similar trend has been observed by Kim et al. (2000) who reported that adding aloe powder to the feed for olive flounder fingerlings would increase their growth after 8weeks, and the proper additive rate was about 0.5 %. Park et al. (2003) reported olive flounder fed on diet containing 0.5 % obosan (medical Chinese herbal mixture)showed the highest values of growth, feed efficiency and condition factor compared to feed additives, such as wasabi and *Undaria*.

Likewise, there is a difference in the fish growth and the feed efficiency depending upon the fish species, days after birth, concentrate level and the feeding span of time (Nakagawa et al., 1984) as well as environmental condition in rearing tank. Particularly, in case of herbal plant, longer application would be more effective (Hwang et al., 1999).

Asida and Okimura (2005) reported manda (natural fermented food in Japan) as a dietary immunostimulant, had enhanced the non-specific immune response of *P. olivaceus* in vitro although not affected the growth. Generally plant extract is known for antimicrobial effect on all kinds of bacteria and fungi (Immanuel et al., 2004). Therefore, using plant extract as immuno-stimulants seems to be an attractive alternative way of controlling fish diseases. Heo et al. (2003) reported fish fed dietary FCFF showed strong activity against Gram-positive bacteria, *Streptococcus* sp. *in vitro*, although the precise antibacterial mechanism remains to be elucidated.

From this present study, it seems that FCFP may have an important role as a feed additive as well as imunostimulant. However, further studies are needed to feeding dose and bacterial infection in applied aquaculture practices.

Among 18 amino acids in FCFP, the contributions of glutamic acid, proline, aspartic acid were higher than other acids (Table 3). Kim and Lall (2000) reported that the major amino acid composition are glutamic acid and aspartic acid in olive flounder. Thus, supplementation of FCFP seems should have positive effect for olive flounder. It is well known mostly amino acid, nucleic acid, organic acid, and sugar act as feeding stimulants, which are constituent with absorptive function less than 1,000 molecular weight (Carr et al., 1996). As to this feeding stimulant substance, many lower amino acid shows an effect differently depending upon the fish species and amino acid (Elias and Joesph, 1999; Fukuda et al., 1989; Kohbara et al., 1989). It seems that in the present study, some of the high molecular weight amino acids might have changed to low molecular weight during fermentation and acted as stimulants. In case of fatty acid (Table 3), of all 13 items analyzed, FCFP mainly composed of linoleic acid, palmitic acid and oleic acid.

The whole body proximate composition of flounder is shown in Table 6. Crude protein and lipid contents were higher in fish fed diets containing dietary FCFP levels than that fed control diet. Particularly, crude lipid content was observed to increase among fish fed diets 0 to 0.08 % FCFP. Moisture was lowest in fish fed diet containing 0.08 % FCFP, which might be due to the high lipid deposition in fish body.

This present result is not consistent with findings of Kim et al. (2002) who found that crude lipid content in whole body decreased with increasing dietary protein levels. Body lipid deposition in fish is affected by numerous factors, but especially by diet. In addition, since fish have the ability to synthesize lipid from amino acids (proteins) and carbohydrates, stored body lipid is not only derived from dietary lipid (Oku and Ogata, 2000). This present study suggest that FCFP has a important role of body lipid deposition of flounder although its contains very low lipid value.

Protein is the most important component in formulated fish diets because it provides the essential amino acids to synthesize protein, and also provides energy for maintenance and growth (Kim et al., 2002). Lipid is also very important source of energy and essential fatty acids for fish (NRC, 1993). Due to its importance, many researchers have conducted experiments concerning dietary protein and lipid requirements of cultured fish. However, there was very few studies done. It has been carried out to estimate the optimum dietary protein level for maximum growth of olive flounder range from 46.4 % to 51.2 % (Kim et al., 2002). The appropriate dietary protein and lipid levels for this species have also been reported by Lee et al. (2000) who have suggested that a diet high in protein (50 %) and low in lipid (7 %) is suitable for the growth of juvenile flounder. On the other hand, Kikuchi et al. (2000) reported feed efficiency and protein efficiency ratio increased with increasing lipid in the diets containing 55 % protein for flounder.

The crude protein and lipid levels in the experimental moist diet were 61

% and 16.2 % dry basis, respectively (Table 1). Moist pellet diet used for this study was composed of higher moisture level (74.1 %), and this value led to increasing FGR value compare with those of above studies. However, since this fish were fed on satiation level, they might have ingested enough feed to fulfill their protein requirement. Difference in dietary protein and lipid levels between studies is considered to be originated from fish size, temperature and duration of feeding experiment.

Fish fed diets containing FCFP had better albumin, GOT and GPT values than that of diet without FCFP. Particularly, GPT was clearly decreased with increasing dietary FCFP levels.

This agrees with the result obtained by Choi et al. (1995) who reported GOT and GPT levels were reduced with increasing dietary seaweed, *E. compressa* and affected physiological activities in common carp. According to Jung et al. (2002) fish fed medical herbal plant improved physiological activities and then, triggered a cascade for anti-bacterial reaction of olive flounder, although not statistically different hematological values.

The results of this study suggests that the FCFP, land herbal resource as a feed additive might have a positive influence on the growth and feed efficiency for olive flounder and the proper supplemental level to a moist feed is assumed to be 0.08 %.

1-3. Effects of EP diet contained fermented cactus fruit (*Opuntia ficus-indica*) fluid on the growth of olive flounder, *Paralichthys olivaceus*

Abstract

This study was conducted to determine the utilization of extruded pellet (EP) diet contained fermented cactus fruit (*opuntia ficus-indica*) fluid (FCFF) on the growth of olive flounder, *Paralichthys olivaceus*. Four experimental EP diets containing different levels of FCFF, 0 % (Control diet), 0.57 % (diet A), 1.0 % (diet B), 2.0 % (diet C) were prepared, respectively. EP diet was designed with 50 % crude protein and 8 % crude lipid mainly. FCFF was made by mixing cactus fruit and commercially available effective microorganisms for 2 weeks at room temperature. Three replicated groups of olive flounder, initially average weight of 3.85 g were fed the experimental diets for 3 months. The growth, weight gain rate, feed gain ratio (FGR) and specific growth rate (SGR) of fish fed diet C were best than the other groups, although not statistically significant. Survival was higher in fish fed diet A, B, C than that of control diet. However, their daily feed rate (DFR) and condition factor (CF) were lower. From the results of the present study, it may concluded that EP diet containing FCFF might be an effective ingredient for the growth and the feed efficiency of olive flounder, and preferable EP diets were assumed to be diet C for olive flounder.

Introduction

Currently, yield of formula feed has been increased to have originated from the culture of fresh water fish every year, however it is performed in the supply food of the raw fish like sardine, horse mackerel, mackerel, anchovy as the main ingredient (protein source) in formulated feeds due to its abundance, low price, high nutritive value and palatability at the sea-aquaculture farms (Kikuchi et al., 2000). Olive flounder, *Paralichthys olivaceus*, farming has rapidly progressed with development of artificial feeding skills in the late 1980's and with the adoption of aquacultural facilities on land.

However, its culture has been still relied on raw fish and supplied by moist pellet feed (Ji et al., 2003). Due to this aquaculture system depends on raw food, new problems have been arising : environmental pollution (Kikuchi et al., 2000), along with many different stresses and diseases (Murata et al., 1996), lower marketing value (Nakagawa and Kasahara, 1986). Particularly, since most aquaculture wastes are ultimately from dietary origin, reduction of waste outputs should first be through improvements of diet formulation (Cho and Bureau, 2001).

Olive flounder is carnivorous and its farming requires feeds containing high levels of fish meal (Forster and Ogata, 1998). This means that the feed used for the flounder requires high prime cost.

It is very important to raise the productivity and to reduce the cost performance with development of low-price formula feed, to led to upgrade for growth in aquaculture fish (Kikuchi et al., 1992). Therefore, finding an alternative protein source for fish meal and reducing protein content in the feeds by improving utilization of dietary protein are required to produce a stable supply of commercial feeds at a reduced price. Recently, It has been carried out to estimate the optimum dietary protein and lipid levels for maximum growth of olive flounder (Kikuchi et al., 2000; Kim et al., 2002; Kim et al., 2004; Lee et al., 2000; Lee et al., 2002).

In former study, we found for the first time fermented cactus fruit fluid (FCFF) has significant influence on the growth and feed efficiency of olive flounder (unpublished).

Therefore, this study was conducted to determine the effects of extruded pellet (EP) diet containing FCFF on the growth of olive flounder.

Materials and Methods

Experimental fish and experimental conditions

The olive flounder, *P. olivaceus*, initially average weight of 3.85 g were kept at the rearing facilities of the Jeju Province Fisheries Resources Research Institute. Experiments were conducted for three months to determine the utilization of extruded pellet (EP) diet contained fermented cactus fruit (*Opuntia ficus-indica*) fluid (FCFF) on the growth of olive flounder.

There were four dietary treatments each Expt-1 and Expt-2, and all the treatments had three replicates. Cylindrical polypropylene (PP) tanks (diameter 1.2 m×1.0 m height) were used as experimental tanks. Each tank was stocked with 100 of olive flounders.

The rearing water was changed 15-20 times a day. Length and weight of fish were measured every month. After measuring, all fish were bathed with 100 ppm HCL-Oxytetracline (OTC) solution for an hour. The water quality parameters in the tank was monitored daily using a YSI system (YSI 556 multi-probe system). The range were : temperature 17.4-18.9 ℃, salinity 31-32 ppt, DO 8.16-9.14 ㎎/L, and pH 7.50-8.10.

Manufacturing of fermented cactus fruit fluid (FCFF)

The fruits of cactus (*O. ficus-indica*) used for this research were collected from the local market in the Jeju Island. For fermentation, the effective microorganism (EM) product marketed by Korea EM Research Organization (EMRO) was used. The fermentation process involved pulverizing the fruits and mixing them into starch syrup (10 % of the cactus fruit) and EM (5 % of the cactus fruit). After anerobic bacteria incubation for 2 weeks at room temperature, the substance was filtered and used for the experiment.

Diet preparation and feeding

The experimental diets were prepared in the form of an extruded pellet (EP) containing different levels of FCFF, 0 % (Control diet), 0.57 % (diet A), 1.0 % (diet B) and 2.0 % (diet C), respectively. EP diet was designed with 50 % crude protein and 8 % crude lipid mainly. Experimental diets were provided from Marubeni-Nissin Feed Co., Japan. Composition of experimental diets is shown Table 1. The diets were fed to visual satiety twice daily.

Analytical methods

The weight gain rate (WG), feed gain ratio (FGR), specific growth rate (SGR), daily feeding rate (DFR) and condition factor (CF) were calculated by the following formulae and their values were compared with each other.

Weight gain rate (WG) = (FW-IW) × 100 / IW

Feed gain ratio (FGR) = TF/TWG

Specific growth rate (SGR) = {(ln FW - ln IW)/t} × 100

Daily feeding rate (DFR) = (TF × 100)/{(IW + FW) × day fed/2}

Condition factor (CF) = $(BW/TL^3) \times 10^3$

where :

BW : body weight, IW : initial weight, FW : final weight, TF : total feed, TL : total length, t : rearing time (day), TWG : total weight gain

Statistical analysis

The data were calculated using the statistical program for Windows (SPSS ver.7.5, USA) and a significant value ($P<0.05$) was verified with Duncan's multiple range test after One-way ANOVA test.

Table 1. Composition of the experimental diets (%)

Component	Control	Diet A	Diet B	Diet C
jack mackerel meal	40	40	40	40
white fish meal	30	30	30	30
krill oil	5	5	5	5
fish oil	2	2	2	2
fermented cactus fruit fluid	0	0.57	1	2
potato starch	7	7	7	7
corn starch	1	1	1	1
wheat flour	6.96	6.96	6.96	6.96
vitamin premixture[1)]	3	3	3	3
ascorbly-2-phosphate	0.04	0.04	0.04	0.04
mineral premixture[2)]	5	5	5	5
Total	100.0	100.6	101.0	102.0

Remark : cactus fluid was addition respectively in the identical combination percentage. total feed combination was not struck 100 %, but this was considered to be ignore.

[1)] vitamin A oil (675,000 I.U), vitamin D3 oil (60,000 I.U), DL-α-Tochopherol acetate (29,333 mg), menadion sodium bisulfite (2,566 mg), thiamin mononitrate (800 mg), riboflavin (1,466 mg), pyridoxal hydrochloride (800 mg), niacin (2,400 mg), calcii pantothenate (4,667 mg), inositol (56,333 mg), biotin (47 mg), folic acid (800 mg), choline chloride (194,666 mg), cyanocobalamin (10,667 mg)

[2)] (%) $MgSO_4 \cdot 7H_2O$(15.0), $NaH_2PO_4 \cdot 2H_2O$(25.0), KH_2PO_4(32.0), $Ca(H_2PO_4)_2 \cdot H_2O$(14.0), Fe-citrate(2.5), $ZnSO_4 \cdot 7H_2O$(6.0), $MnSO_4 \cdot 4H_2O$(1.0), Ca-lactate(3.5), Trace element mixture*(1.0)

* (%) ; $CuSO_4 \cdot 5H_2O$(15.0), $CoCl_2 \cdot 6H_2O$(0.1), KIO_3(0.3), $Ca(H_2PO_4)_2 \cdot H_2O$(84.6)

Results

Growth and Survival rate

The results of growth performance of olive flounder fed various experimental diets for 3 month are shown in Table 2. The growth was lowest in fish fed the control diet, and was improved by supplementing with FCFF, and the highest in fish on the diet C. Survival of fish in each diets were over 67 % and there was no significant difference among treatments. Fish fed diets containing different levels of FCFF showed higher growth and survival than those of control diet.

Table 2. Total length, body weight and survival rate of olive flounder fed experimental EP diets containing different levels of fermented cactus fruit fluid (FCFF) for three months

Treatment	Initial		Final			Survival rate (%)
	TL (cm)	BW (g)	TL (cm)	BW (g)	W.gain (%)	
Control	7.50±0.09	3.73±0.07	16.1±0.63	44.1±7.76	1079	67.0
A	7.57±0.17	3.97±0.31	17.3±0.31	52.0±6.65	1217	86.3
B	7.56±0.06	3.85±0.07	17.1±1.05	51.7±12.3	1242	95.3
C	7.64±0.06	3.85±0.20	17.4±0.77	52.5±7.80	1268	78.7

[*] Values (mean±SD of three replications) in the same column not sharing a common superscript are no significantly different (P<0.05)

A, B and C were EP diets containing 0.57 %, 1 % and 2 % FCFF, respectively.

FGR, SGR, DFR and CF

Table 3 shows the value of feed gain ratio (FGR), specific growth rate (SGR), daily feeding rate (DFR) and condition factor (CF) during the experiment 1. The best value of the FGR was obtained with fish fed diet C. SGR of fish fed diet C was higher than those of other groups.

FGR and SGR were better with increasing FCFF levels. On the other hand, DFR was lower in the fish fed diet containing different levels of FCFF than that of control group. Fish fed diet containing different levels of FCFF show slower CF values than that of diet without FCFF. However, there was no significant difference (P>0.05) among the all parameters in different treatments.

Table 3. Effect of EP diets containing different levels of fermented cactus fruit fluid (FCFF) on FGR, SGR, DFR and CF of olive flounder

Treatment	FGR	SGR	DFR	CF
Control	0.74±0.13	2.73±0.21	4.74±1.53	10.3±0.88
A	0.70±0.02	2.85±0.18	3.60±0.87	9.87±0.79
B	0.67±0.11	2.86±0.28	3.48±0.96	9.88±0.79
C	0.66±0.05	2.89±0.21	3.70±0.94	9.79±0.72

[*] Values (mean±SD of three replications) in the same column not sharing a common superscript are no significantly different ($P<0.05$)

A, B and C were EP diets containing 0.57 %, 1 % and 2 % FCFF, respectively.

Discussion

The rapid expansion of aquaculture, along with improvements in fish culture techniques have increased the demand for fish culture techniques have increased the demand for fish feeds that depend on fish meal (protein source) as the major dietary components (Watanabe, 2002). Protein is the most important component in formulated fish diets because it provides the essential amino acids to synthesize protein, and provides energy for maintenance and growth (Kim et al., 2002). Lipid is also very important source of energy and essential fatty acids for fish (NRC, 1993). Due to its importance, many researchers have conducted experiments concerning dietary protein and lipid requirements of cultured fish. However, there was very few studies done in optimum dietary protein and lipid levels for olive flounder and parrot fish.

It has been carried out to estimate the optimum dietary protein level for maximum growth of olive flounder range from 46.4 % to 51.2 % (Kim et al., 2002). The appropriate dietary protein and lipid levels for this species have also been reported by Lee et al. (2000), who have suggested that a diet high in protein (50 %) and low in lipid (7 %) is suitable for the growth of juvenile flounder. Kikuchi et al. (2000) reported feed efficiency and protein efficiency ratio increased with increasing lipid in the diets containing 55 % protein for flounder.

In present study, we designed in the form of an extruded pellet (EP) containing different levels of fermented cactus fruit fluid (FCFF) such as 0 % (Control diet), 0.57 % (diet A), 1.0 % (diet B) and 2.0 % (diet C) FCFF, respectively. EP diet was formulated with 50 % crude protein and 8 % crude lipid mainly. In this study, fish fed experimental diet C showed highest in final growth, weight gain, specific growth rate (SGR) of olive flounder among all groups although not statistically significant ($P>0.05$). In addition, survival and feed gain ratio (FGR) were better in fish fed diets containing FCFF than that of control diet. However, their daily feed rate (DFR) was slightly lower. This present study suggested that this levels of protein (50 %) and lipid (8 %) in the diet seems to be adequate for growth and nutrition of olive flounder. However, in a study by Saitoh et al. (2003) of extruded soybean meal for olive flounder, final body weight and weight gain tended to be greater with an increase of extruded soybean meal content in the diet, reached a plateau by 44.5 % protein and 14 % lipid, but not statistically significant. Difference in protein and lipid levels reported by Cho and Kaushik (1990), who have suggested that adequate levels of non-protein energy sources, such as lipids and carbohydrates, in the diet can minimize the use of protein as a source of energy.

The tendency of growth performance and feed efficiency in this study were consistent with findings of former study. In former study fish fed diet containing 1 % FCFF were significantly ($P<0.05$) improved the growth olive flounder. The feed efficiency values were also better in fish fed diets containing FCFF than that of diet without FCFF (unpublished).

Difference in FCFF levels between present and previous studies is considered to be changed which originated from extruded pellet processing.

The results of the present study suggests that the extruded pellet contained FCFF might be an effective influence on the growth and the feed efficiency of olive flounder, so it is preferred to use that for a aquaculture, and preferable EP diets were assumed to be diet C for olive flounder.

Chapter Ⅱ.

Effects of dietary microorganism on the growth and resistance against pathogen bacteria of olive flounder, *Paralichthys olivaceus*

2-1. Effects of dietary lactic acid bacteria (LAB), *Lactobacillus plantarum* (CNU001) isolated from cactus fruit, *Opuntia ficus-indica* on the growth and pathogen resistance of fingerling olive flounder, *Paralichthys olivaceus*

Abstract

The objective of this study was to determine the effect of *Lactobacillus plantarum* on the growth and resistance against pathogenic bacteria of fingerling olive flounder. Triplicate groups of cultured fish were fed an experimental diet supplemented with 1, 5 and 10 % *L. plantarum* culture (about 10^9 colony-forming units mL^{-1}) by volume for 3 months. Growth rate, survival rate, feed coefficient, and serum components were monitored. *Lactobacillus plantarum* induced higher growth and survival rates. Blood plasma (albumin, total protein, GOT, GPT and total cholesterol) of fish fed a diet containing 10 % *L. plantarum* culture had better hemochemical parameters, although there was no significant difference between the groups fed diets with lower concentrations of *L. plantarum*. *In vitro* tests found that this lactic acid bacteria effectively inhibited the growth of *Streptococcus* sp., *Edwardsiella tarda* and *Vibrio* sp. The mortality of fish fed a diet supplemented with *L. plantarum* was lower than that of the control group. These results suggest that *L. plantarum* may have antibacterial properties that increase the immunity of olive flounder and result in increased growth and survival rates.

Introduction

Probiotics are cultured products or live microbial feed supplements used in aquaculture that benefit hosts by improving intestinal (microbial) balance; they can be used as supplementary disease control agents and supplementary or replacement antimicrobial compounds (Irianto and Austin, 2002; Vazquez et al., 2005). Probiotics are known to reduce mortality (Moriarty, 1998) and to improve disease resistance (Gatesoupe, 1991), the ability to adhere to and colonize the gut (Joborn et al., 1997), the ability to reduce the number of bacterial cells in kidneys (Park et al., 2000), the production of polyamines and digestive enzyme activity (Tovar et al., 2002), the development of the non-specific immune system by means of cellular systems (Irianto and Austin, 2002), the preservation of various products such as milk, meat, poultry, fruits, vegetables, cereals (Lerol et al., 1996) and fish products (Gelman et al., 2001), and to have anti-microbial (Nuku-Paavola et al., 1999) and cinogenic effects (Fermandes and Shahani, 1990) on cultured aquatic animals. Of course, probiotics must not harm hosts (Salminen et al., 1999) and need to be effective over a range of temperatures and salinities. As the restricted use of antibiotics and other chemotherapeutics is encouraged in aquaculture to avoid the development of antibiotic-resistant strains and other environmental complications, probiotics are encouraged as an alternative health management tool.

The facultative anaerobic Gram-positive nonsporulating bacterium, *Lactobacillus plantarum*, a natural inhabitant of the human gastrointestinal tract whose complete genome sequence has recently become available (Vries et al., 2004), has been evaluated as a probiotic for such uses as the improvement of water quality (Austin et al., 1995). *Lactobacillus plantarum* is a versatile, fermentative bacterium found in many food fermentations. *Lactobacillus* *plantarum* is thought to antagonize potential pathogens in the aquatic environment. Direct benefits of using *L. plantarum* would be the reduction in the use of chemicals and antibiotics in the aquatic environment, and enhanced growth of the farmed species. While the involvement of probiotics in aquaculture is comparatively new, they are rapidly gaining recognition as an important means for disease control. However, the effects of *L. plantarum* CNU001, isolated from the fruit of the cactus, *Opuntia ficus-indica,* on the growth and antibacterial properties of fish have not been examined.

Olive flounder, *Paralichthys olivaceus*, is considered a main aquaculture species in Korea, with production levels reaching 34,533 tons in 2003 (Statistical Year Book of Maritime Affairs and Fisheries, 2003). In a previous study, we found that fermented cactus fruit fluid (FCFF) had potential antibacterial properties against Gram-positive *Streptococcus* sp. (Heo et al., 2003) and Gram-negative *Edwardsiella tarda* and *Vibrio* sp. (unpublished data) in cultured olive flounder. In the present study, we examined the effect of the lactic acid bacteria (LAB), *L. plantarum* CNU 001, one of the important microorganisms found in FCFF, on the growth characteristics of fingerling olive flounder and its resistance against the Gram-positive bacteria, *Streptococcus* sp. and Gram-negative bacteria, *E. tarda* and *Vibrio* sp.

Materials and Methods

Isolation of the lactic acid bacterium *Lactobacillus plantarum* CNU 001

Several lactic acid bacterial strains were isolated from slightly fermented cactus (*Opuntia ficus-indica*) fruit using MRS supplemented with $CaCO_3$ or BCP as the isolation medium. The strain identified as *L. plantarum* by 16S rDNA sequencing, biology, and other data, and designated *L. plantarum* CNU 001, was selected for this experiment. *Lactobacillus plantarum* CNU 001 reached 10^9 colony-forming units (CFU) mL^{-1} after 3 days of growth at 25 °C on MRS (with $CaCO_3$) medium. This 3-day-old culture of *L. plantarum* CNU 001 (10^9 CFU mL^{-1}) was used as a dietary supplement.

Experimental fish and experimental conditions

Fingerling olive flounder, *Paralichthys olivaceus*, 5.8 cm long and 1.86 g in weight on average, were obtained from a commercial hatchery and transported to the rearing facilities of the Jeju Province Fisheries Resources Research Institute. Three experiments were conducted to determine the most effective dietary supplement of *L. plantarum* for the olive flounder. In the first experiment, the effect of four dietary treatments of *L. plantarum* was evaluated over 3 months. Blood was analyzed in the second experiment, and the antibacterial effects of the dietary supplements were evaluated in the third experiment. Cylindrical polypropylene (PP) tanks (0.56 m in diameter, 0.6 m in height, 3 replicates per treatment) were each stocked with 30 juvenile flounder. The water was changed 15-20 times per day. Fish length and weight were measured monthly. After measurements, all fish were bathed with 100 ppm HCL-Oxytetracycline (OTC) solution for 1 h. Water temperature in the rearing tanks fluctuated between 16.0 and 20.4 °C during the experimental period.

Diet preparation and feeding

The feed used for the experiment was in the form of a commercial extruded pellet (Marubeni-Nissin formula feed, Japan). *Lactobacillus plantarum* cultures (10^9 CFU mL^{-1}) were added to the diet at 0, 1, 5 and 10 % (by volume) by soaking the feed in the cultures. This diet was fed to the fish twice daily. The proximate composition of the commercial diet was analyzed following standard procedures by Science Lab Center Co., Korea (Table 1). Weight gain, feed gain ratio (FGR), specific growth rate (SGR), daily feeding rate (DFR), and condition factor (CF) were calculated for the fish.

Table 1. Approximate compositions of commercial diet (%, dry matter basis)

Composition	Commercial Diet (%)
Moisture	3.36
Crude protein	52.0
Crude lipid	12.6
Crude fiber	1.86
Crude ash	12.0
Ca	2.56
P	1.90
Vitamin C	0.01
Vitamin E (α-Tocopherol)	0.06

Blood analysis

At the end of the diet experiment, blood was sampled from the caudal veins of five fish from each treatment group using disposable syringes. The blood serum was separated by centrifugation (5,000 rpm for 15 min) and examined using an automatic analyzer (Boehringer Mannheim, Mannheim, Germany).

Antibacterial experiment

Bacterial stock

We isolated *Streptococcus* sp., *Edwardsiella tarda* and *Vibrio* sp. from cultured olive flounder bought at a local farm on Jeju Island, Korea. The bacterial agents were stored at -20 °C in glycerol solutions. Bacterial colonies were developed by inoculation in a xylose lysine desoxycholate (XLD) medium and incubation at 30 °C for 24 h. Subcultures from these colonies were placed on brain-heart infusion agar (BHI; Difco Laboratories, Livonia, MI, USA) with 2 % NaCl.

In vitro procedure

Bacterial multiplication rates were analyzed on liquid plates to determine the resistance properties of *L. plantarum* to *Streptococcus* sp., *E. tarda*, and *Vibrio* sp. First, *L. plantarum*was mixed at 2 % (w/v) density in BHI for 30 min at room temperature, and then the pH was adjusted to 7.0. This compound mixture was placed in an ice box and subjected to an ultrasonic wave process that was repeated eight times for 15 s. After the ultrasonic treatment, the insoluble constituents of the mixture were removed by filtration and the mixture was then strained through a rough sterilized syringe filtering system (0.45-㎛ pore size). *Lactobacillus plantarum*-free BHI served as a control. Bacteria were inoculated onto BHI plates at a rate of 1.2×10^4 CFU mL^{-1} and incubated at 22 °C. The number of bacteria after 0, 2, 4, 8, 12 and 24 h of incubation were measured on tryptic soy agar (TSA; Difco) plates supplemented with 2 % NaCl and 100 ㎕ sterilized physiological saline solution for the experimental and control groups, respectively. Each result was measured twice.

In vivo procedure

At the conclusion of the rearing experiment, fish from the experimental groups that were fed a diet supplemented with 1, 5 and 10 % *L. plantarum*and those from the control groups were infected via intraperitoneal injection with 9.8, 9.8×10^2, 9.8×10^4, 9.8×10^6 and 9.8×10^8 viable cells of *Streptococcus* sp., *E. tarda*, and *Vibrio* sp. Four fish were injected for each concentration of pathogenic bacteria, and the mortality rate and presence of infectious symptoms were noted regularly for up to 30 days. Dead fish and those displaying signs of infection were dissected to establish cause of death, and to examine both the occurrence of symptoms and bacteria in the liver and kidney.

Statistical analysis

Data were analyzed using SPSS version 7.5 for Windows (SPSS Science, Chicago, IL, USA); significant differences (at $P<0.05$) were verified using one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests.

Results

Growth Performance

The growth performance of fingerling flounder fed various experimental diets for 3 months is shown in Table 2. The final weight of fish fed diets containing various levels of *L. plantarum* was higher than that of fish fed a diet lacking *L. plantarum*. There was no significant difference ($P>0.05$) in the final weight of fish fed diets supplemented with 1, 5 and 10 % *L. plantarum* culture, although weight did increase with dietary *L. plantarum* levels. Survival was highest in fish fed a diet containing 10 % *L. plantarum* culture. Table 3 shows the feed gain ratio (FGR), specific growth rate (SGR), daily feeding rate (DFR), and condition factor (CF) during the experiment. The FGR of fish fed a diet containing 10 % *L. plantarum* culture was higher than that of fish fed a diet lacking *L. plantarum* ($P<0.05$). The FGR increased with increasing dietary *L. plantarum* culture levels. The highest SGR was found in fish fed the 10 % *L. plantarum* culture, although this result was not statistically significant. On the other hand, the DFR was lowest in the fish fed the 10 % *L. plantarum* culture diet, and decreased with increasing dietary *L. plantarum* culture levels. Fish fed the diet containing 1 % *L. plantarum* culture had the highest CF values, although there was no significant difference among the different treatments for the CF ($P>0.05$).

Table 2. Total length, body weight and survival rate of olive flounder fed on experimental diets containing different levels of *Lactobacillus plantarum* culture (10^9 CFU/mL) for three months

Treatment	Initial		Final		Survival rate (%)
	TL (cm)	BW (g)	TL (cm)	BW (g)	
Control	5.82±0.08	1.75±0.10	15.6±0.42	38.0±1.35	96.7
1 %	5.83±0.07	1.80±0.06	15.5±0.21	38.9±1.16	98.9
5 %	5.80±0.06	1.76±0.07	15.7±0.17	40.0±0.73	96.7
10 %	5.80±0.04	1.73±0.00	15.6±0.41	40.2±2.56	98.9

Table 3. Effect of different levels of dietary *Lactobacillus plantarum* culture (10^9 CFU/mL) on FGR, SGR, DFR and CF of olive flounder

Treatment	FGR[1)]	SGR[2)]	DFR[3)]	CF[4)]
Control	0.79±0.01	3.27±0.04	16.1±0.79	10.3±0.40
1 %	0.75±0.01	3.27±0.07	15.5±0.28	10.5±0.19
5 %	0.74±0.01	3.33±0.02	15.2±0.38	10.2±0.17
10 %	0.71±0.03	3.34±0.07	15.1±1.04	10.3±0.33

[*] Values (mean±SD of three replications) in the same column not sharing a common superscript are significantly different ($P<0.05$).

[1)] FGR = TF/TWG

TF : total feed, TWG : total weight gain

[2)] SGR = {(ln FW - ln IW)/t} × 100

FW : final weight, IW : inital weight, t : rearing time (day)

[3)] SFR = (TF × 100)/{(IW + FW) × day fed/2}

TF : total feed, IW : inital weight, FW : final weight

[4)] CF = $(BW/TL^3) \times 10^3$

BW : body weight, TL : total length

Blood constituents

The level of albumin in plasma was higher in fish fed the 5 and 10 % *L. plantarum* culture diets than in the control group, while the lowest levels were found in fish fed the 1 % *L. plantarum* culture diet (Table 4); however, there were no significant differences among the groups (P>0.05). Total protein was highest in fish fed the 10 % *L. plantarum* culture diet. GOT and GPT were lowest in fish fed the 10 % *L. plantarum* culture diet, but this difference was also not statistically significant. Total cholesterol (TC) was lowest in fish fed the 10 % *L. plantarum* culture, and reached a plateau at the 5 % *L. plantarum* culture level, but the differences among groups were not significant (P>0.05).

Table 4. Levels of ALB, TP, GOT, GPT and TC of the blood plasma of fingerling olive flounder fed diets containing different levels of *Lactobacillus plantarum* culture (10^9 CFU/mL)

Treatment	1)ALB(㎎/dℓ)	2)TP(㎎/dℓ)	3)GOT(IU/ℓ)	4)GPT(IU/ℓ))	5)TC(㎎/dℓ)
control	1.56±0.13	4.74±0.40	101±42.9	12.4±5.18	326±82.7
1 %	1.39±0.12	4.46±0.38	87.6±26.2	12.6±2.70	355±153
5 %	1.61±0.08	4.89±0.30	118±79.0	13.4±7.23	419±72.9
10 %	1.64±0.25	5.20±0.69	83.0±31.2	5.20±1.64	282±93.6

1) ALB : albumin
2) TP : total protein
3) GOT : glutamic-oxaloacetic transaminase or AST (asparatate aminotranferase)
4) GPT : glutamic-pyruvate transaminase or ALT (alanine aminotranferase)
5) TC : total cholesterol

Antibacterial Effects

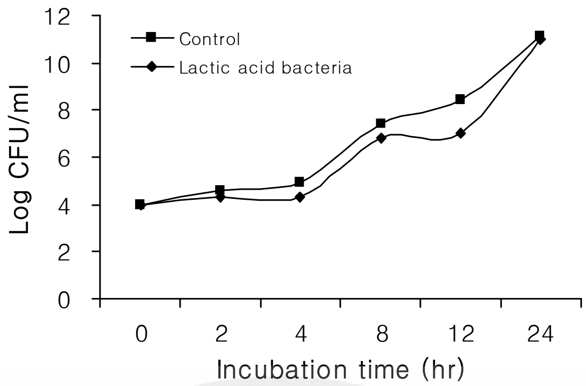
In vitro effect

Fig. 1 shows the inhibitory effects of *L. plantarum* on the growth rates of *Streptococcus* sp., *E. tarda* and *Vibrio* sp. on plates. *Lactobacillus plantarum* inhibited the growth of these bacteria for at least 12 h. The values were 8.4 log CFU mL^{-1} at 12 h and 11.1 log CFU mL^{-1} at 24 h in the control group, and 7.0 log CFU mL^{-1} at 12 h and 11.0 log CFU mL^{-1} at 24 h in the *Streptococcus*sp. group (Fig. 1A). In the *E. tarda* group, inhibition increased steadily in the control, i.e., 8.4 log CFU mL^{-1} at 12 h and 11.0 log CFU mL^{-1} at 24 h, while remaining low for the first 12 h in the experimental trial, i.e., 4.5 log CFU mL^{-1} at 12 h and 9.6 log CFU mL^{-1} at 24 h (Fig. 1B). There was strong resistance to *E. tarda* proliferation for the first 12 h. The effect of *L. plantarum* on *Vibrio* sp. was similar to the effect on *Streptococcus* sp. and showed a resistance tendency for 12 h, with 6.9 log CFU mL^{-1} in the experimental trial compared with the control trial result of 9.2 log CFU mL^{-1}. However, the increase in the experimental trial reached a plateau at 12 h (Fig. 1C).

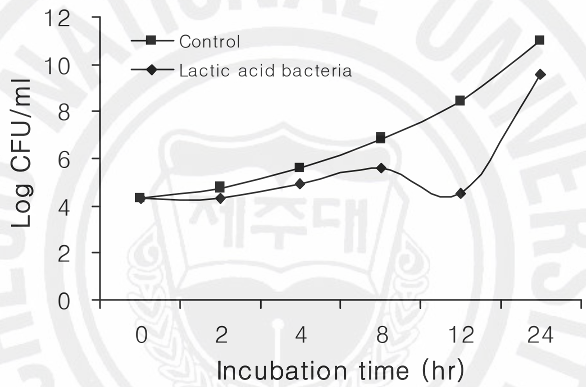
In vivo effect

In the *in vivo* trial, mortality from *Streptococcus* sp. in fish fed a control diet lacking *L. plantarum* was 50, 100, and 25 % for inoculations with 1×10^9, 1×10^7 and 1×10^5 viable cells, respectively. However, fish fed *L. plantarum* did not show much more resistance, i.e., 100, 25, 50, and 25 % mortality when injected with 1×10^9, 1×10^7, 1×10^5, and 1×10^3 viable cells, respectively (Table 5A). Dead fish infected with *Streptococcus* sp. were characterized by skin ulcers confined to scale-covered parts of the body surface, and often diffuse or petechial hemorrhage in internal organs. Fish infected with 1×10^9 and 1×10^7

(A) *Streptococcus* sp.



(B) *Edwardsiella tarda*



(C) *Vibrio* sp.

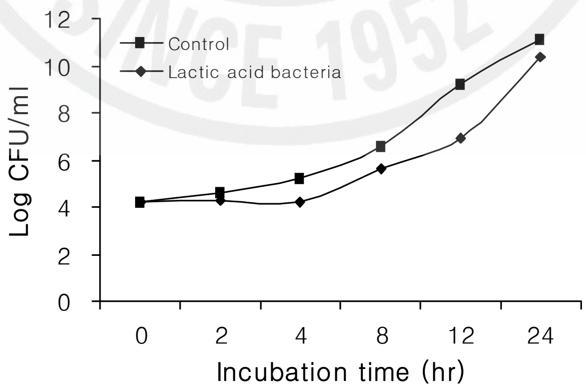


Fig. 1. Effect of *Lactobacillus plantarum* CNU 001 on the growth rate of fish pathogenic bacteria, *Streptococcus* sp., *Edwardsiella tarda* and *Vibrio* sp.

viable cells of *E. tarda* and fed a control diet experienced 100 and 75 % mortality, respectively, while the mortality rates of fish fed *L. plantarum* were lower (Table 5B). Flounder infected with *E. tarda* were found to have heavy viscous liquid in the body, liver congestion, and abnormal kidney and spleen growth, i.e., similar anatomical symptoms as naturally infected olive flounder. For *Vibrio* sp. infection, the mortality rate of fish fed *L. plantarum* was lower than that of the control group (Table 5C). Fish affected by *Vibrio* sp. showed typical signs of generalized septicemia with hemorrhaging on the base of fins, exophthalmia, corneal opacity, and edematous lesions predominantly centered on the hypodermis.

Table 5. Antibacterial effect of *Lactobacillus plantarum* culture (10^9 CFU/mL) against *Streptococcus* sp., *Edwardsiella tarda* and *Vibrio* sp. in cultured olive flounder

(A) *Streptococcus* sp.

Treatment	Inoculum (cell/㎖)	Control		Experimental	
		Average died (day)	mortality (%)	Average died (day)	mortality (%)
1	1×10^1	-	0	-	0
2	1×10^3	-	0	11	25
3	1×10^5	30	25	18.5	50
4	1×10^7	16	100	14	25
5	1×10^9	8	50	17	100

(B) *Edwardsiella tarda*

Treatment	Inoculum (cell/㎖)	Control		Experimental	
		Average died (day)	mortality (%)	Average died (day)	mortality (%)
1	1×10^1	-	0	-	0
2	1×10^3	-	0	-	0
3	1×10^5	-	0	-	0
4	1×10^7	10	75	-	0
5	1×10^9	5.5	100	5.5	100

(C) *Vibrio* sp.

Treatment	Inoculum (cell/㎖)	Control		Experimental	
		Average died (day)	mortality (%)	Average died (day)	mortality (%)
1	1×10^1	-	0	-	0
2	1×10^3	-	0	-	0
3	1×10^5	8	25	-	0
4	1×10^7	2.5	75	3.5	50
5	1×10^9	1	100	1	100

Discussion

LAB probiotics are characterized as Gram-positive, usually non-motile, nonsporulating bacteria that produce lactic acid as the major or sole product of fermentative metabolism (Ringo and Gatesoupe, 1998). To date, LAB have been used in both artificial feed and live feed (Robertson et al., 2000), such as Artemia and rotifers (Harzevili et al., 1998; Planas et al., 2004). Certainly, many studies have used whole or components of microbial cells as immunostimulants, specifically to stimulate the immune system against pathogens. Immunostimulants are known to enhance host defense systems against pathogens by increasing the chemiluminescent response, and by superoxide anion production (Sakai, 1998). Although several reports have indicated the antibacterial activity of some LAB, there is very little information available on the effects on growth and antibacterial properties in fish. In the present study, a diet containing live *L. plantarum* was shown to have some positive effects on growth and feeding efficiency, as well as survival rate, in olive flounder, although there was no significant difference between groups fed a diet containing *L. plantarum* and the control.

Cai et al. (1998), Gatesoupe (1991) and Refstie et al. (2005) reported that the beneficial effects of *Lactobacillus* as a probiotic included higher growth and feed efficiency, as well as the prevention of intestinal disorders and the pre-digestion of anti-nutritional factors present in some fish. Similarly, in crustaceans, Venkat et al. (2004) reported significantly higher growth in probiotic-fed groups than in a control group in a 60-day feeding experiment with *Lactobacillus*-based probiotics in the post-larval *Macrobrachium rosenbergii*. Weight gain, specific growth rate, feed efficiency ratio, protein efficiency ratio, and protein gain were also significantly higher in the group fed probiotics than in the control. Chang and Liu (2002), Moriarty (1998) and Queiroz and

Boyd (1998) suggested that probiotics improved the survival of larvae, increased food absorption by enhancing protease levels, and resulted in better growth. However, the optimum concentration of probiotics in the diet needs to be determined. The optimal dose of probiotic bacteria may depend on the size and species of fish and the strain of probiotic, as well as environmental conditions in the rearing tanks.

In this study, the FGR and SGR were higher in fish fed a diet containing *L. plantarum* than in the control group, although the DFR showed the opposite trend. Irianto and Austin (2002) suggested that probiotics may stimulate appetite and improve nutrition by the production of vitamins and the detoxification of dietary compounds. This improved appetite and/or growth performance in farmed species was not found in the present study, and the inference about improved appetite and growth is difficult to reconcile. In particular, it is important to determine whether the probiotic actually tastes good or if it modifies the feed, thereby improving digestibility and taste (Irianto and Austin, 2002). In addition, the evaluation of serum parameters, such as albumin, total protein, total cholesterol, GOT, and GPT, suggested that *L. plantarum* in the fish may improve antibacterial activity; fish fed a diet containing 10 % *L. plantarum* had higher levels of albumin and TP and lower levels of GOT, GPT and TC than did the control group, although there were no significant differences among the levels of *L. plantarum*. The cholesterol-lowering effect of *Lactobacillus* has also been reported in humans (Sindhu and Khetarpaul, 2003).

A growing number of diseases have appeared with the worldwide development of aquaculture, and may be attributed to distinct bacteria, such as *Streptococcus* sp., *E. tarda* and *Vibrio* sp. These bacteria are also responsible for most bacterial diseases found in the aquaculture of olive flounder. Streptococcal infection of fish is considered a reemerging disease affecting a variety of wild and cultured fish throughout the world, and should be regarded as a complex of similar diseases caused by different genera and species capable of inducing central nervous damage characterized by suppurative exophthalmia and meningoencephalitis (Toranzo et al., 2005). *Edwardsiella tarda* is the etiological agent of several diseases of freshwater and marine fish, causing septicemia with extensive skin lesions and affecting muscles and internal organs, such as the liver, kidney and spleen (Plumb, 1993). Pathogenic *Vibrio* sp., the etiological agent of classical Vibriosis, is widely distributed and causes a typical hemorrhagic septicemia in a wide variety of warm- and cold-water species of economic importance (Toranzo et al., 2005). The seriousness of the infection damage caused by *Streptococcus* sp., *E. tarda* and *Vibrio* sp. in olive flounder farming has been recognized (Bang et al., 1992; Kwon et al., 1999; Lim et al., 2003; Park and Kim, 1994); treatment and relief using antibiotics or chemotherapeutics are under evaluation, and results vary widely in different situations (Immanuel et al., 2004; Kim et al., 2002; Kwon et al., 2002; Sivaram et al., 2004).

One of the most promising and urgent applications of LAB is as a probiotic to fight pathogens and as a source of immunostimulants. The effect of *Lactobacillus* on the specific and innate immunity of fish has been reported elsewhere (Herias et al., 1999; Nikoskelanien et al., 2003; Panigrahi et al., 2004, 2005; Salinas et al., 2005). However, there has been a tendency toward laboratory-based experiments, rather than field studies. Consequently, the information produced is often of limited value for aquaculture. This study provides evidence that *L. plantarum* has antibacterial effects. In particular, *L. plantarum* appears to be effective against Gram-positive bacteria, i.e., *Streptococcus* sp., and Gram-negative bacteria, i.e., *E. tarda* and *Vibrio* sp., as also found by Vaarala (2003) and Vazquez et al.(2005). Vaarala (2003) reported that the beneficial effect of microbial load in the maturation of the immune system and in the prevention of allergic diseases was not restricted to intestinal Gram-positive bacteria, such as bifidobacteria and lactobacilli.

Vazquez et al. (2005) described the inhibitory effects of lactic and acetic acids on the growth of *Vibrio* sp., as well as the total lack of response of the bacteriocins on these Gram-negative bacteria.

Although the exact mechanism by which *L. plantarum* inhibits bacterial proliferation in fish remains to be elucidated, our observations indicate that a diet supplemented with *L. plantarum* may positively affect the fish immune system, has the potential to reduce the frequency of bacterial diseases, and can induce higher growth and survival rates in fingerling olive flounder.

Chapter Ⅲ.

Evaluations of fermented cactus fruit (*Opuntia ficus-indica*) fluid as a feed additive as well as a feeding stimulant

3-1. Effects of dietary fermented cactus fruit (*Opuntia ficus-indica*) fluid, chitosan, β-glucan and obosan on the growth of fingerling olive flounder, *Paralichthys olivaceus*

Abstract

The aim of this study was conducted to evaluate the effects of fermented cactus fruit (*Opuntia indica-ficus*) fluid (FCFF), chitosan, β-glucan and obosan as a feed additive on the growth of fingerling olive flounder, *Paralichthys olivaceus*, for 3 months. The growth of fish fed diet containing FCFF and chitosan were significantly higher ($P<0.05$) than that of control group. Weight gain and survival were higher in fish fed various additives than that of diet without additive, although not statistically different. Feed gain ratio (FGR) was better in fish diets containing FCFF, chiosan and β-glucan. On the other hand, daily feed rate (DFR) of fish fed diet containing FCFF was significantly lowest ($P<0.05$) than that of control diet. Condition factor (CF) was best value in fish fed diet containing chitosan. However, there was no significantly difference among groups. The results indicate that FCFF might be recommended as a feed additive compared to another additives for the growth and the feed efficiency of fingerling olive flounder.

Introduction

In recent years, cultivation of olive flounder has become one of the most important aquaculture activities in Korea, reached a production of 34,533 tons in 2003 (Statistical Year Book of Ministry of Maritime Affairs and Fisheries, 2003).

However, there are several problems further considering of this industry like environmental pollution by raw feed (Kikuch et al., 2000), along with many different stresses and diseases (Murata et al., 1996), lower marketing value (Nakagawa and Kasahara, 1986). In addition, the feed used for the olive flounder requires more than half of the prime cost, and therefore, it renders the greatest burden to the fish farms (Lee et al., 1995).

A number of approaches have been applied in an attempt to address these problems including adopt with natural herbs as a feed additives to upgrade the feeding efficiency and production (Immanuel et al., 2004; Karunasagar et al., 1994; Sahul Hameed and Balasubramanian, 2000; Song et al., 2002), adopt with natural immunostimulants, such as Chitosan and Glucan to reduce microbial diseases instead of chemotherapy with a particular emphasis on the use ofantibiotics (Bagni et al., 2005; Matsuo and Miyazano, 1993; Robertsen et al., 1990; Tsai and Hwang, 2004). Recently, by direct application of medicinal supplements at traditional Chinese herbal pharmacies like obosan, into the aquaculture, many different effects such as enhancement of immunity (Jung et al., 2002), growth (Kim et al., 1998b; Park et al., 2003) and fleshy tissues (Lee et al., 1998) have been shown.

Cactus (*Opuntia ficus-indica*), land herbal resource has been reported to have several important useful properties including anti-tumor qualities (Shin et al., 1998), anti-oxidant effect (Baik et al., 1999), anti-viral effect (Ahmad et al., 1996), anti-ulcer effect (Lee et al., 1998), Cholesterol-lowering properties

(Palumbo et. al., 1999) and anti-bacterial effect (Chung, 2000) in mammals.

In former study, we found for the first time fermented cactus fruit fluid (FCFF) has significant influence on the growth and feed efficiency of olive flounder, red sea bream and parrot fish (unpublished). Therefore, the aim of this study was to determine the commercial value of fermented cactus fruit as a feed additive compare with chitosan, β-glucan, and obosan which have been widely used in olive flounder fish farms.

Materials and Methods

Experimental fish and experimental conditions

The fingerling olive flounder, *Paralichthys olivaceus*, obtained from commercial hatchery, average 6.7 cm long and 2.6 g weight, were transported to the rearing facilities of the Jeju Province Fisheries Resources Research Institute. The experiments were conducted to evaluate the effective dietary fermented cactus fruit fluid (FCFF) in comparison with dietary chitosan, glucan and obosan in fingerling olive flounder for three months. All the treatments had three replicates.

Cylindrical polypropylene (PP) tanks (diameter 0.56 m×0.6 m height) were used as experimental tanks. Each tank was stocked with 40 of fingerling flounders.

The rearing water was changed 15-20 times a day. Length and weight of fish were measured every month. After measuring, all fish were bathed with 100 ppm HCL-Oxytetracline (OTC) solution for an hour. The water quality parameters in the tank was monitored daily using a YSI system (YSI 556 multi-probe system). The ranges were : temperature 17.4-21.6 ℃, DO 5.80-7.21 ㎎/L and pH 7.57-8.04.

Manufacturing of fermented cactus fruit fluid (FCFF)

The fruits of cactus (*O. ficus-indica*) used for this study were collected from the local market in the Jeju Island. For fermentation, the effective microorganism (EM) product marketed by Korea EMRO (EM Research Organization) was used. The fermentation process involved pulverizing the fruits and mixing them into starch syrup (10 % of the cactus fruit) and EM (5 % of the cactus fruit). After incubation for 2 weeks at room temperature, the substance was filtered and used for the experiment.

Diet preparation and feeding

The assorted diet used for the experiment was in the form of a commercial extruded pellet (Marubeni-Nissin formula feed, Japan). FCFF were added at the level of 1 % to basal diet. chitosan, β-glucan and obosan were followed by the methods of each company. Diets were fed to visual satiety twice a day.

Proximate composition of the commercial diet used for this study was analyzed according to standard procedures by Science Lab. Center Co., Korea. This result is shown in Table 1.

Analytical methods

The weight gain rate (WG), feed gain ratio (FGR), specific growth rate (SGR), daily feeding rate (DFR) and condition factor (CF) were calculated by the following formulae and their values were compared with each other.

Weight gain rate (WG) = (FW-IW) × 100 / IW

Feed gain ratio (FGR) = TF/TWG

Specific growth rate (SGR) = {(ln FW - ln IW)/t} × 100

Daily feeding rate (DFR) = (TF × 100)/{(IW + FW) × day fed/2}

Condition factor (CF) = $(BW/TL^3) \times 10^3$

where :

BW : body weight, IW : initial weight, FW : final weight, TF : total feed, TL : total length, t : rearing time (day), TWG : total weight gain

Statistical analysis

The data were calculated using the statistical program for Windows (SPSS ver.7.5, USA) and a significant value (P<0.05) was verified with Duncan's multiple range tests after One-way ANOVA test.

Table 1. A proximate compositions of commercial diet and fermented cactus fruit fluid (FCFF) (%, dry matter basis)

Composition	Diet (%)	FCFF (%)
Moisture	3.36	85.6
Crude protein	52.0	1.08
Crude lipid	12.6	0.60
Crude fiber	1.86	0.67
Crude ash	12.0	1.34
Ca	2.56	*
P	1.90	0.11
Vitamin C	0.01	0.01
Vitamin E (α-Tocopherol)	0.06	*

*, less than 0.01 % dry matter basis

Results

Growth performance

The results of growth performance of fingerling flounder fed various experimental diets for 3 months are shown in Table 2. The final growth of fish fed diets containing FCFF and β-glucan were significantly (P<0.05) higher than that of control group. However, there was no significantly (P>0.05) different among in fish fed diets containing additives. Fish fed diets containing additives showed higher weight gain rate than that of control group, but not statistically significant.

Survival of fish in each dietary group was over 50 % and there was no significant difference among treatments. Fish fed diet containing FCFF showed highest survival than that of control diet. The growth and survival of fish were higher in fish fed various additives that of diet without additive.

Table 2. Total length, final body weight and survival rate of olive flounder fed diets containing fermented cactus fruit fluid (FCFF), chitosan, β-glucan and obosan for three months

Treatment	Initial		Final		WG	Survival
	TL (cm)	BW (g)	TL (cm)	BW (g)	(%)	rate (%)
Control	6.60±0.22	2.36±0.32	13.6±0.52^{a}	27.4±4.22^{a}	1081^{a}	50.8^{a}
FCFF	6.53±0.18	2.56±0.17	15.0±0.09^{b}	35.9±1.34^{b}	1309^{a}	71.5^{a}
chitosan	6.85±0.40	2.80±0.79	14.4±0.80^{ab}	32.4±3.40^{ab}	1155^{a}	57.5^{a}
β-glucan	6.70±0.11	2.28±0.21	14.7±0.40^{b}	34.0±2.66^{b}	1405^{a}	62.5^{a}
obosan	6.88±0.33	2.84±0.55	14.6±0.38^{b}	32.5±2.65^{ab}	1090^{a}	65.8^{a}

[*] Values (mean±SD of three replications) in the same column not sharing a common superscript are significantly different (*P*<0.05)

FGR, SGR, DFR and CF

Table 3 shows the value of feed gain ratio (FGR), specific growth rate (SGR), daily feeding rate (DFR) and condition factor (CF) during the experiment. The groups fed diets containing FCFF, chitosan and β-glucan showed better FGR value than that of control group, however no significantly different. The best value of the SGR was obtained with fish fed diet containing chitosan, but not statistically significant. On the other hand, DFR was significantly lowest ($P<0.05$) in fish fed diet containing FCFF than that of control diet.

Fish fed diets containing various additives were higher CF than that of control diet although there was no significant difference ($P>0.05$) among the CF values in different treatments .

Table 3. Effect of dietary fermented cactus fruit fluid (FCFF), chitosan, β-glucan and obosan on FGR, SGR, DFR and CF of olive flounder

Treatment	FGR	SGR	DFR	CF
Control	0.83±0.14	2.47±0.19	$23.6±1.49^{b}$	10.5±0.36
FCFF	0.74±0.13	2.67±0.04	$14.8±0.41^{a}$	10.5±0.38
chitosan	0.79±0.05	2.50±0.23	$20.1±4.40^{ab}$	10.6±0.62
β-glucan	0.67±0.10	2.73±0.13	$19.8±6.08^{ab}$	10.4±0.03
obosan	0.85±0.01	2.47±0.13	$17.2±1.23^{ab}$	10.4±0.10

[*] Values (mean±SD of three replications) in the same column not sharing a common superscript are no significantly different (*P*<0.05).

Discussion

Many researchers are urgently studying to develop the most efficient feed and maximize the feed efficiency, reduce microbial diseases, decrease environmental pollution, and bring forth a guide to profitable growth by using the natural herbalplant or natural immunostimulants as a feed additives. In former study we found for the first time that fish fed diet containing 1 % fermented cactus fruit fluid (FCFF) were significantly ($P<0.05$) improved the growth of olive flounder, Red sea bream and Parrot fish. In addition the feed efficiency values were also better in fish fed diets containing FCFF than that of diet without FCFF (unpublished). Subsequently, this study carried out to determine the evaluate the commercial value of FCFF as a feed additive in comparison with chitosan, β-glucan, and obosan which have been widely used in presence at the olive flounder fish farms.

In this study, fish fed dietary FCFF and chitosan showed significantly ($P<0.05$) highest final weight than that of control diet. Weight gain and survival were better in fish fed various additives than that of non-fed additives. The groups fed diets containing FCFF and Chitosan were obtained with higher values in feed gain ratio (FGR), specific growth rate (SGR) and condition factor (CF) than that of control group. Particularly, daily feeding rate (DFR) value was significantly lowest ($P<0.05$) in fish diet containing FCFF than that of control diet which was consistent with findings of former study. This might be related to the taste of the FCFF used which probably decreased the feed intake by fish.

It is known that use of immunostimulants increases the non-specific immunity and valuable for the control of fish disease and may be useful in fish culture. The immune stimulatory effects of immunostimulants like glucan, chitosan for fish and shrimp have been reported.

Chitosan is a natural product derived from chitin that is found in the exoskeletons of shellfish like shrimp or crabs (Gomez-Guillen et al., 2005). Chitosan have various applications in biochemical, phamaceutical, agricultural, food, and biotechnological fields that decrease the cholesterol levels (Lehoux et al., 1993), enhance the strong antimicrobial activity against various microorganisms (Tsai and Hwang, 2004; Wang and Chen, 2005), increase macrophage activity (Esteban et al., 2000), accelerate wound healing (Diegelmann et al., 1996), prohibit parasite (Dautremepuits et al., 2004) and extend fish preservation (Tsai et al., 2002). On the other hand, β-glucan is polymer of glucose, giving structural integrity to the cell walls of bacteria, fungi and plants (Robertsen et al., 1990). Glucan has been reported to have a number of functional properties concerned with usually resistance to bacterial pathogens (Sahoo and Mukherjee, 2002) and increase immune activity (Bagni et al., 2005).

Several authors have reported relationships between immunostimulation and growth promoting activity. Shiau and Yu (1999) suggested that both dietary Chitin and chitosan supplementation depresses tilapia growth regardless of the supplementation level in tilapia. They assumed that the dilution with chin or chitosan allowed for constancy of the protein/calorie ratio, which controled by fiber led to the growth. However, Kono et al. (1987) reported that no growth depression was observed in fish fed diets supplemented with chitin when red sea bream, olive eel and yellowtail were fed diets containing 10 % chitin or chitosan. Cook et al. (2003) showed that snapper fish fed glucan increased in growth rate compared to the control fish, although no difference was seen between the groups.

These results indicate that chitosan and glucan generally has a stronger activity against bacteria rather than growth in fish, although glucan reached the highest growth rate in this study. However, for the effective use of immunostimulants, the timing, dosages, method of administration and the physiological condition of fish need to be taken into consideration (Sakai, 1999).

It is known oral administration is the most practical method for delivery of immunostimulants; however, the effects of long-term oral administration of immunostimulants are still unclear (Sakai, 1999). Matsuo and Miyazano (1993) reported that rainbow trout treated with peptidoglucan orally for 56 days did not show protection after challenge with *Vibrio anguillarum*, although fish treated for 28 days showed increased protection. However, in this study, there was not disadvantages of long-term oral administration of chitosan and β-glucan. Traditional Chinese herbal medicines, which are mixtures of more than one active ingredient, has been increasingly used throughout the world, as they are considered to be effective and to have few side-effects such as treatments of chronic liver disease (Wang, 2000), psoriasis (Tse, 2003), diabetes (Qin et al., 2004) and anaesthesia (Kam and Liew, 2002). obosan as such a Chinese medical herbal mixture have been widely used in olive flounder fish farms. There are a few papers concerned with obosan (Kim et al., 1998a, b; Kim et al., 2003; Lee et al., 1998; Lee et al., 1999; Lee et al., 2001; Park et al., 2003).

This present study found that the group fed diet containing obosan showed lower in FGR, SGR, DFR than those of control, although their growth and survival rate were higher. These results are not consistent tendency with those of Kim et al. (1988), who found that the dietary 0.3 % obosan for 48 weeks showed significantly higher survival rate than control ($P<0.05$), and also improved yields in weight gain, specific growth rate, feed conversion ratio and condition factor, significantly ($P<0.05$) in olive flounder.

Park et al. (2003) reported olive flounder fed on diet containing 0.5 % obosan showed the most highest values of growth, feed efficiency and condition factor compare to feed additives, such as wasabi, *Undaria* and obosan. Likewise, there is a difference in the fish growth and the feed efficiency depending upon the fish species, days after birth, concentrate level and the feeding span of time (Nagakawa et al., 1984) as well as environmental condition in rearing tank.

From the proximate compositions of FCFF (Table 1), crude fiber was one of the main of FCFF, although its role for fish remains to be elucidated.

The results of this study suggests that the FCFF, land herbal resource as a feed additive as well as a natural immunostimulant might be an effective influence on the growth and feed efficiency for olive flounder, so it is preferred to use that for a aquaculture fish farms.

3-2. Effects of essential amino acids (EAAss), taurine and sucrose as a feeding stimulants on the growth of fingerling olive flounder, *Paralichthys olivaceus*

Abstract

The aim of this study was conducted to determine the effects of essential amino acids (EAAs), taurine and sucrose as a feeding stimulants on the growth of fingerling olive flounder, *Paralichthys olivaceus*, for 3 months. Triplicate groups of fish were fed diets 0.3 % EAAss, taurine and sucrose to a commercial diet. The final growth of fish fed diets supplemented lysine, valine, tryptophan and methionine were significantly improved ($P<0.05$) among EAAss groups. Taurine and sucrose were also better. Weight gain (WG) and specific growth rate (SGR) were further improved by the supplementary taurine, although not statistically different ($P>0.05$). On the other hand, fish fed supplementary leucine was lowest growth, survival rate and SGR while highest in daily feeding rate (DFR). The best value of feed gain ratio (FGR) was observed in supplementary tryptophan among all groups. However, there was no significantly difference compare with other groups. Valine in condition factor (CF) was highest among all groups, although not statistically different. The results in the present study suggest that lysine, methionine, tryptophan, valine as a EAAss, taurine and sucrose requirements of olive flounder changes with growth and feed efficiency.

Introduction

Many studies show that four types of common, low molecular weight metabolites, acting either alone or as components of mixtures, serve as potent attractions or as stimulants of feeding behavior (Carr et al., 1996). These feeding substances are mostly amino acid, nucleic acid, organic acid, and sucrose as a level with absorptive function less than 1,000 molecular weight (Carr, 1982).

Olive flounder, *Paralichthys olivaceus* is the main species for culture and its farming requires feeds containing high levels of fish meal (Forster and Ogata, 1998). This means that the feed used for the flounder requires high prime cost. It is very important to raise the productivity and to reduce the cost performance in aquaculture fish. A number of approaches have been applied in an attempt to address these problems including adopt with natural components as a feeding stimulants to upgrade the feeding efficiency and production.

There are several publications that show the importance of introducing feeding stimulants into aquaculture : inosine-5′-monophosphate and lactic acid were largely responsible for yellowtail (Hidaka et al., 2000), mixtures of L-alanine, L-serine, inosine-5′-monophosphate and betaine for striped bass (Papatryphon and Soares, 2000), amino acids for rainbow trout (Adron and Mackie, 1978), glutamic acid, aspartic acid, lysine, citric acid and malic acid for tilapia (Adams et al., 1988), mixture of L-lysine and L-alanine for oriental weatherfish (Harada, 1992), lysine for red sea bream (Forster and Ogata, 1998).

Concerning with feeding stimulants for olive flounder, there is little information available on the effects of supplementary essential amino acids and sucrose in domestic as well as overseas.

Recently, Several investigators (Kim et al., 2003; Park and Takeuchi, 2000; Park et al., 2002; Park et al., 2001) suggested that taurine in the diet improves the growth of fingerling and juvenile olive flounder, *Paralichthys olivaceus*.

Therefore, this study was conducted to determine the effects of essential amino acid (EAAss), taurine, and sucrose components as a feeding stimulants on the growth of olive flounder.

Materials and Methods

Experimental fish and experimental conditions

The fingerling olive flounder, *P. olivaceus*, obtained from commercial hatchery, average 11.0 cm long and 12.5 g weight, were transported to the rearing facilities of the Jeju Province Fisheries Resources Research Institute. The experiments were conducted to determine the effect of each of essential amino acids (EAAs), taurine and sucrose supplementation on the growth of fingerling olive flounder for three months. All the treatments had two replicates.

Cylindrical polypropylene (PP) tanks (diameter 1.2 m×1.0 m height) were used as experimental tanks. Each tank was stocked with 40 of fingerling flounders.

The rearing water was changed 15-20 times a day. Length and weight of fish were measured every month. After measuring, all fish were bathed with 100 ppm HCL-Oxytetracline (OTC) soln for an hour. The water quality parameters in the tank was monitored daily using a YSI system (YSI 556 multi-probe system). The range were : temperature 15.9-19.9 ℃ (ave. 17.2 ℃), DO 4.30-7.58 ㎎/L (ave. 6.67 ㎎/L), and pH 8.05-8.41 (ave. 8.31), respectively.

Diet preparation and feeding

The assorted diet used for the experiment was in the form of a commercial extruded pellet (Nisshin formula feed, Japan). Each EAAs, taurine and sucrose were added at the level of 1 % to basal diet. Each EAAs, taurine and sucrose were in the form of a commercial reagents (Sigma, St. Louis, MO, USA). Diets were fed to visual satiety twice daily.

Proximate and amino acid compositions of the commercial diet used for this study were analyzed according to standard procedures by Science Lab. Center Co., Korea. These results are shown in Table 1, 2.

Table 1. A proximate compositions of commercial diet used for this study (%, dry matter basis)

Composition	Diet (%)
Moisture	3.36
Crude protein	52.0
Crude lipid	12.6
Crude fiber	1.86
Crude ash	12.0
Ca	2.56
P	1.90
Vitamin C	0.01
Vitamin E (α-Tocopherol)	0.06

*, less than 0.01 % dry matter basis

Table 2. A amino acid levels of commercial diet (%, dry matter basis)

Amino acid	Diet (%)
Aspartic acid	4.84
Theronine	2.27
Serine	2.18
Glutamic acid	8.16
Proline	2.53
Glycine	3.46
Alanine	3.48
Valine	2.59
Isoleucine	2.19
Leucine	4.07
Tyrosine	1.74
Phenylalanine	2.27
Histidine	1.89
Lysine	4.22
Arginine	2.97
Cystine	0.48
Methionine	1.26
Tryptophan	0.48

Analytical methods

The weight gain (WG), feed gain ratio (FGR), specific growth rate (SGR), daily feeding rate (DFR) and condition factor (CF) were calculated by the following formulae and their values were compared with each other.

Weight gain (WG) = (FW-IW) × 100 / IW

Feed gain ratio (FGR) = TF/TWG

Specific growth rate (SGR) = {(ln FW - ln IW)/t} × 100

Daily feeding rate (DFR) = (TF × 100)/{(IW + FW) × day fed/2}

Condition factor (CF) = $(BW/TL^3) \times 10^3$

where :

BW : body weight, IW : initial weight, FW : final weight, TF : total feed, TL : total length, t : rearing time (day), TWG : total weight gain

Statistical analysis

The data were calculated using the statistical program for Windows (SPSS ver.7.5, USA) and a significant value (P<0.05) was verified with Duncan's multiple range test after One-way ANOVA test.

Results

Growth and Survival rate

The results of growth performance of olive flounder fed diets supplemented EAAs, taurine and sucrose for 3 month are shown in Table 3. The final growth of fish fed diets supplemented lysine, valine, tryptophan and methionine were significantly higher (P<0.05) among EAAs groups. Taurine

Table 3. Total length, body weight, weight gain (WG) and survival rate of olive flounder fed diets supplemented essential amino acids (EAAs), taurine and sucrose for three months

Treatment	Initial		Final		WG	Survival
	TL (cm)	BW (g)	TL (cm)	BW (g)	(%)	rate (%)
Control	11.0±0.16	12.7±0.59	$19.7±0.59^{abc}$	$81.7±9.21^{ab}$	546^{ab}	85.0
Arginine	11.0±0.06	12.5±0.36	$19.4±0.28^{ab}$	$78.7±3.46^{ab}$	532^{ab}	100
Aistidine	10.9±0.15	12.2±0.13	$20.1±0.18^{abc}$	$85.7±3.34^{ab}$	608^{ab}	97.5
Isoleucine	11.0±0.14	12.4±0.60	$19.9±0.35^{abc}$	$80.9±2.89^{ab}$	553^{ab}	92.5
Leucine	10.9±0.04	12.5±0.20	$19.2±0.58^{a}$	$73.4±9.63^{a}$	490^{a}	70.0
Lysine	11.0±0.08	12.7±0.23	$20.7±0.21^{c}$	$95.7±0.21^{b}$	646^{b}	95.0
Methionine	11.1±0.05	13.0±0.01	$20.6±0.66^{bc}$	$94.1±7.91^{b}$	624^{b}	97.5
Phenylalanine	11.0±0.05	12.4±0.08	$20.1±1.20^{abc}$	$89.1±17.9^{ab}$	617^{b}	97.5
Threonine	10.9±0.20	12.3±0.54	$20.8±0.11^{abc}$	$91.7±0.21^{ab}$	648^{b}	97.5
Tryptophan	11.1±0.19	12.7±0.62	$20.5±0.62^{bc}$	$94.2±11.4^{b}$	641^{b}	100
Valine	11.1±0.08	12.8±0.45	$20.3±0.13^{abc}$	$94.7±8.59^{b}$	644^{b}	100
Taurine	10.9±0.08	12.1±0.21	$20.41±0.10^{abc}$	$91.3±0.35^{ab}$	658^{b}	95.0
Sucrose	11.0±0.11	12.6±0.18	$19.9±0.31^{abc}$	$87.0±5.54^{ab}$	592^{b}	97.5

[*] Values (mean±SD of three replications) in the same column not sharing a common superscript are significantly different (*P*<0.05)

and sucrose were also better growth than that of control group, although not statistically different. The lowest weight gain (WG) (490.0 %) was observed in fish fed diet supplemented luecine, but the value was statistically identical to the group fed control diet (545.8 %). Although the highest WG (658.0 %) was recorded by the group fed the diet supplemented taurine, no statistical differences (P>0.05) were found compare with other groups. Survival of fish in each diets were over 70 % and observed similar tendency with that of weight gain rate, the lowest was in fish fed supplementary leucine, which was originated from high mortality in one of the rearing tanks. However, there was no significant difference among treatments.

FGR, SGR, DFR and CF

Fig. 1 shows the value of feed gain ratio (FGR), specific growth rate (SGR), daily feeding rate (DFR) and condition factor (CF) during the experiment.

The group fed diet supplemented tryptophan was best FGR among all groups, the value was significantly different (P<0.05) compare with supplementary leucine. However, supplementary taurine and sucrose in FGR were no difference compare with control group.

SGR was observed to show similar tendency with that of WG, lower value in fish fed diets supplemented leucine, arginine than that of control diet and highest in supplementary taurine.

On the other hand, DFR was highest in fish fed diet supplementary leucine among all groups. No difference in DFR observed in between supplementary leucine and control, although statistical difference (P<0.05) compare with other group (lysine, methionine, threonine, tryptophan, valine and taurine). Fish fed diets supplementary isoleucine and leucine were lower CF than that of control diet, but the values statistically different (P<0.05) compare with valine. Whereas, the CF values of taurine and sucrose were higher than that of control group, although not statistically different.

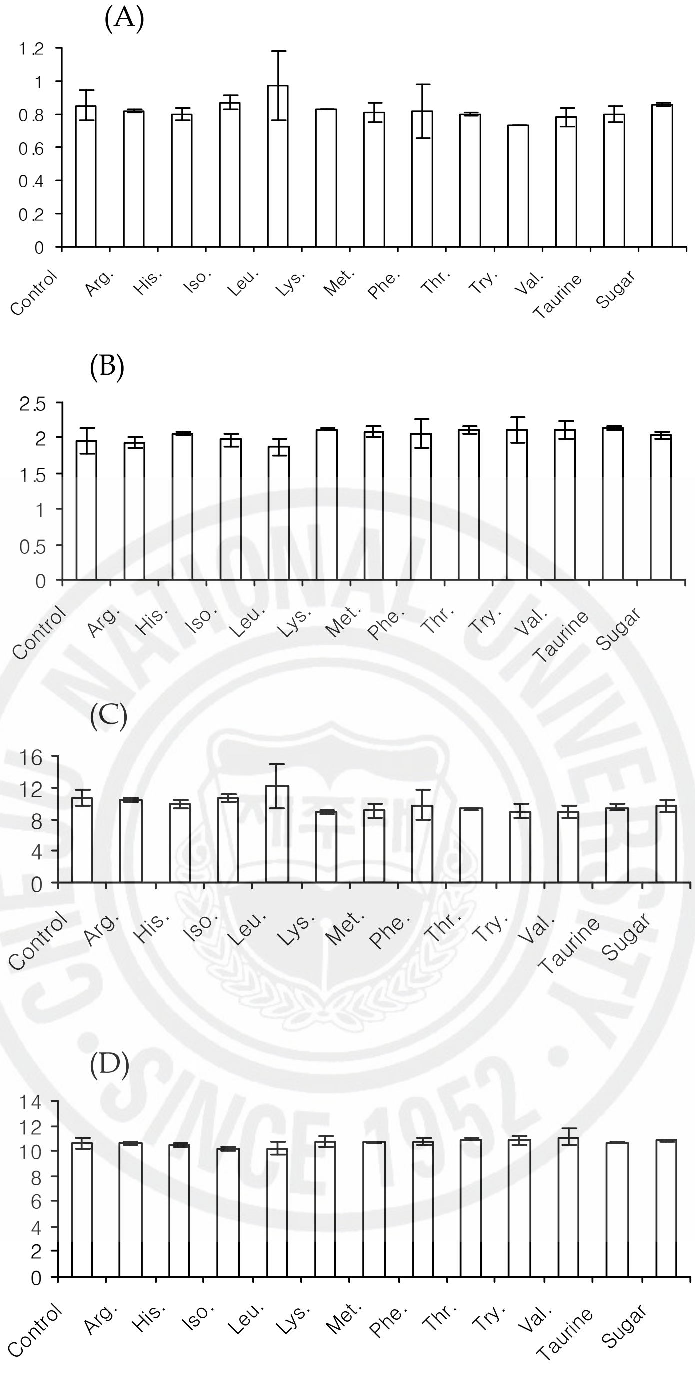


Fig. 1. Effects of necessary amino acids (EAAs), taurine and sugar on growth of of fingerling olive flounder, *Paralichthys olivaceus*

(A) Feed gain ratio (FGR) (B) Specific growth rate (SGR)

(C) Daily feeding rate (DFR) (D) Condition factor (CF)

Discussion

Considerable work has been reported on feeding stimulants for fish, and diverse compounds are thought to be attractants or feeding stimulants. As to this feeding stimulants, many lower amino acid shows an effect differently each other depending upon the fish species and amino acid (Fukuda et al., 1989; Hidaka et al., 2000; Papatryphon and Soares, 2000; Park et al., 2000). Most amino acid requirement studies involve measuring the growth and feed efficiency response of fish fed a series of diets containing graded levels of the test amino acid (Cowey, 1992). Inclusion of practical feed stuffs, like high quality fish meal, can lead to more distinct patterns of response to increasing levels of amino acid (Forster and Ogata, 1998). In this study, lysine, methionine, tryptophan and valine supplemented diets showed significantly higher ($P<0.05$) growth among in essential amino acids (EAAs). In addition their feed gain ratio (FGR), specific growth rate (SGR) and condition factor (CF) were better.

This present study suggested that lysine in the diet seems to be adequate for growth and feed intake of olive flounder. Lysine is often the limiting amino acid in the ingredients used to prepare fish feed. Several investigators have been conducted to determine the requirements of various fish species for this amino acid. Harada (1990) reviewed that stimulant activity of L-lysine showed for yellowtail, cichlid and oriental weather fish. On the other hand, Forster and Ogata (1998) reported that a deficiency in dietary lysine results in poor growth, high mortality in the red sea bream, whereas an excessive supply of lysine depresses weight gain for the tiger prawn (Millamena et al., 1998). Therefore, the effects of lysine on feed intake in fish may depend on fish species, proper level of dose, level of dietary protein or other factors.

The present study found that methionine has influence on the growth for olive flounder, in similar with findings of Kim et al. (2002), who have suggested that proline, threonine, methionine supplemented diet showed significantly higher ($P<0.05$) weight gain and faster specific growth rate in Korean rock fish. Methionine is essential for normal growth as it cannot be synthesised in the body. It is required for protein synthesis and various other metabolic processes. Ruchimat et al. (1997) reported that the methionine requirement of juvenile yellowtail was estimated to be 1.11 % of the diet. Methionine supplementation increased fat digestibility in Atlantic salmon (Nordrum et al., 2000) and improved the dietary quality in juvenile red sea bream (Takagi et al., 2001).

Some feeding stimulants present in very low concentrations, such as tryptophan and valine in food or baits are either major stimulants of fish species or, as is more often the case, make significant contributions to the activity of mixtures. In this study tryptophan and valine were also observed in higher growth performance and feed efficiency as major stimulants for olive flounder. A similar trend was observed in jack mackerel (Ikeda et al., 1988) and carp (Saglio et al., 1990).

On the other hand, supplementary leucine results in poor growth, high mortality in this study, may be considered by Forster and Ogata (1998) that the lower values obtained from SGR data, indicate the greater sensitively of the latter two parameters to reduced protein quality of diets containing marginally deficient levels of an essential amino acids. The reason maybe more reliable as estimates of the actual requirement. Particularly, DFR value was lower in fish diets containing lysine, methionine, tryptophan, and valine than that of control group. This might be related to the taste of the these amino acids used which probably decreased the feed intake by fish.

From the proximate and amino acid compositions of basal diet (Table 1and 2), glutamic acid, aspartic acid and lysine acid showed greater value based on 50 % crude protein and 12.62 crude lipid mainly. This seems that experimental diet composed 50 % protein and 12.62 % lipid maybe adequate for growth and nutrition of olive flounder and lysine has a important role of growth as a EAAs in flounder.

Mixtures of several amino acids have been reported to be more effective than single or combinations of a few amino acids. Papatryphon and Soares (2000) reported combinations tested of L-alanine, L-serine, inosine-5'-monophosphate and betaine stimulated feed intake for carnivorous fish, striped bass, the maximum response being attained when all four compounds were included together in the feed. However, the requirement of all the essential amino acids (EAAs) are known only for a limited number of fishes such as rainbow trout (Yamamoto et al., 2005), catla (Ravi and Devaraj, 1991). Further studies are needed to some combinations or all of EAAs dose in applied flounder aquaculture practices.

Taurine is considered to have a variety of physiological functions in mammals, such as reducing the toxic (Hwang et al., 2000), anti-hypertensive (McCarty, 2004), anti-oxidation (Nakamura et al., 1993), osmoregulation (Thurston et al., 1980), and neuromodulation (Kyriyama, 1980). However, there is little information available on the effects of dietary taurine in flatfish.

In the present study dietary taurine was obtained with highest weight gain and specific growth rate (SGR) among all treatments. This agrees with the observations of Park et al. (2002), who have reported that approximately 1.4 % taurine content in the diet was required for optimum growth of juvenile flounder. Otherwise, Kim et al. (2003) showed that only supplementation of 1 % taurine in the diet of juvenile flounder improved significantly their growth performance, however it was not weak in the fingerling flounder. They suggest that there is a great requirement for taurine for the growth of juvenile flounder (0.4 g at initiation) than fingerling olive flounder (15 g at initiation). Their suggestion of fish size may be not consistent with findings of this study. The present study was conducted with an average body weight of 12.5 g at initiation as fingerling flounder of experiment. However, dietary taurine might be have influence on the growth of fingerling flounder, although further studies are needed to feeding dose levels and fish size in applied aquaculture practices.

It is well known sucrose, which is the least expensive form of dietary energy is the most effective substance stabilizing ATPase activity (Uresti et al., 2005) which lead to a search for cryoprotectants (Cabrita et al., 2003; Dinnyes et al., 1998; Herrera and Mackie, 2004), effect on growth rate for fish (Shiau and Chuang, 1995). Cabrita et al. (2003) demonstrated that turbot embryos can be subject to this cryoprotectant protocol without deleterious effect on the hatching rate.

In this study sucrose have a positive influence on the growth and feed efficiency for olive flounder. A similar trend has been reported by Shiau and Chuang (1995), who have suggested carbohydrates such as sucrose improve weight gain in juvenile tilapia.

As a result, it is important that studies on each species are conducted to identify the most potent feeding stimulants, the concentrations at which they are most effective, as well as any multi-factorial effects or interactions that might exist. Nevertheless, it should be noted that some of the experimental differences might be a result of using different carriers rather than actual biological responses.

Therefore, the results of the present study suggests that lysine, methionine, tryptophan and valine as a EAAs, taurine and sucrose supplementation as a feeding stimulants might be an effective influence on the growth and the feed efficiency of fingerling olive flounder.

CONCLUDING REMARKS

Paralichthys olivaceus, the olive flounder, is a popular food fish in Korea, with more than 40,075 tons produced in 2005. It has been a representative culture species on Jeju Island since the late 1980s; 17,815 tons were produced in 2005. With the adoption of seedling production and feeding in olive flounder aquaculture, production per area has increased and aquaculture has become a high-density, mass-production process. However, new problems have arisen due to this intensification of the culture system, as it has brought about environmental degradation, along with many different stressors and diseases. Moreover, fish farming requires either minced or whole fish as feed, or artificial feeds containing high levels of fish meal. Thus, olive flounder feed poses a high primary cost and is a great economic burden to aquaculture farms. Hence, many researchers are seeking to develop the most efficient feed and to maximize feed efficiency, reduce feeding costs, decrease environmental pollution, and create guidelines for profitable growth by using natural herbal plants as a feed additive.

The cactus *Opuntia indica-ficus* is an herbal resource with several useful properties, including antitumor, cholesterol-lowering, antiulcer, antioxidant, antibacterial, and antiviral effects in mammals. However, the effects of cactus on the growth and bacterial resistance of fish are unknown. In this thesis, we describe a series of studies that were performed to determine the utility of cactus as a feed additive for olive flounder.

In Chapter Ⅰ, we formulated fermented cactus fruit fluid (FCFF) and fermented cactus fruit powder (FCFP) using microorganisms and investigated the effects of dietary FCFF and FCFP on the growth and feeding efficiency of olive flounder. Adding FCFF to a commercial diet had a significant ($P<0.05$)

effect on the growth of olive flounder. In addition, feeding efficiency was higher in fish fed diets containing FCFF, and the optimal concentration was found to be around 1.0%. FCFF resulted in significantly faster growth (at the 1% supplementation level) when compared to the control diet. In this study, we designed extruded pellets containing 0, 0.57, 1, or 2% FCFF based on 50% crude protein and 8% crude lipid. Fish fed the 2% FCFF diet had the highest final size, highest weight gain, and highest specific growth rate (SGR). However, no significant differences ($P>0.05$) were found among the different treatments. This difference in FCFF levels is thought to be attributable to the extruded pellet production process. The adequate formulation of crude protein and lipid based on fish size was not examined, and thus further work is needed in this area.

In a previous study, we found that FCFF significantly ($P<0.05$) influenced the growth and feeding efficiency of red sea bream and parrot fish, and the preferred supplemental level in commercial feed is thought to be 1.0% (unpublished data). Therefore, FCFF may be an effective feed additive for improving the growth and feeding efficiency of aquaculture fish. Moreover, olive flounder fed a diet containing 0.08% FCFP in a moist pellet feed had significantly ($P<0.05$) improved growth. The feeding efficiency values were also higher in olive flounder fed diets containing FCFP than in those fed a diet without FCFP. This agrees with previous results that 0.08% FCFP results from the freeze-drying of 1% FCFF. These results also indicate that supplementation with FCFP for olive flounder requires a higher primary cost than supplementation with FCFF, even though it has a longer shelf life.

Thus, the results from Chapter 1 suggest that FCFF and FCFP maybe effective for enhancing the growth and feed efficiency of olive flounder and that the proper supplemental levels are 1.0 and 0.08%, respectively. Furthermore, FCFF is more cost-effective than FCFP as a feed additive in aquaculture.

In Chapter Ⅱ, our objective was to determine what effect, if any, microorganisms had on the growth and antibacterial activity of olive flounder. Several lactic acid bacterial strains were isolated from slightly fermented cactus fruit using MRS supplemented with CaCO3 or BCP as the isolation medium. A strain identified as *Lactobacillus plantarum* by 16S rDNA sequencing and other data, and designated *L. plantarum* CNU001, was selected for this experiment. *Lactobacillus plantarum* CNU001 appeared to slightly affect the growth and survival rates of olive flounder. The blood plasma of fish fed a diet containing 10% *L. plantarum* CNU001 culture had better hemochemical parameters (albumin, total protein, GOT, GPT, and total cholesterol) than fish fed a control diet, although no significant difference was observed among groups fed diets with lower concentrations of *L. plantarum* CNU001. An in vitro test revealed that *L. plantarum* CNU001 effectively inhibited the growth of the pathogens *Streptococcus* sp., *Edwardsiella tarda,* and *Vibrio* sp. and a pathogenicity test indicated a lower mortality rate in cultured fish fed *L. plantarum* CNU001 compared to the control group. In a previous study, we found that dietary FCFF supplementation enhanced the disease resistance of cultured olive flounder against the pathogenic bacteria *Streptococcus* sp., *Edwardsiella tarda*, and *Vibrio* sp. (unpublished data).

From these data, we conclude that FCFF, which contains active microorganisms, may help protect the immune system and reduce the incidence of bacterial diseases in fish.

In Chapter Ⅲ, we evaluated the commercial value of FCFF as a feed additive and its role as a feeding stimulant in olive flounder compared to the widely used additives chitosan, glucan, and Obosan. Although these additives have different roles as immunostimulants, their effectiveness depends on the timing, dosage, and method of administration, as well as the physiological conditions of the fish. Fish fed diets containing FCFF were larger and had a higher survival rate than fish fed diets with other additives. Microorganismal fermentation using sugar resulted in higher levels of amino acids, and most amino acids, nucleic acids, organic acids, and sugars are well-known feeding stimulants. We investigated the effects of essential amino acids (EAAs), sucrose, and taurine as feeding stimulants on the growth of olive flounder. Requirements of the EAAs lysine, methionine, tryptophan, and valine, and those of sucrose and taurine change with the growth and feed efficiency of olive flounder.

Our findings indicate that FCFF may be a good feed additive and feeding stimulant in olive flounder aquaculture. In particular, FCFF is of great economic benefit because it is more cheaply produced than the other additives.

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