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A THESIS

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Effects of *Ecklonia cava* on the growth of lactic acid bacteria and the improvement of innate immune response of olive flounder (*Paralichthys olivaceus*)

WONWOO LEE

Department of Marine Life Sciences

GRADUATESCHOOL

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2013. 08

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Effects of *Ecklonia cava* on the growth of lactic acid bacteria and the improvement of innate immune response of olive flounder (*Paralichthys olivaceus*)

WONWOO LEE
(Supervised by professor You-Jin Jeon)

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

August, 2013

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국문초록

사람이 생활하고 있는 지구에는 수많은 미생물들이 서식하고 있다. 자구상의 모든 생물들은 이러한 미생물들에게 오염되거나 함께 공존하면서 살아가고 있다. 사람도 예외 없이 태어나기 전 어머니의 뱃속에서부터 미생물과 함께 살아가고 있다. 이러한 미생물들은 대부분 사람과 직접적인 연관없이 살아가지만, 일부의 미생물은 사람에게 질병을 일으키기도 하고, 오히려 건강하게 살수 있도록 도움을 주면서 살아가기도 한다. 그 중에서도 사람이 태어나면서 외부(소화관, 구강, 인후, 코, 머리, 피부, 질 등)에 부착하여 살아가는 정상 미생물균총들은 병원 미생물과 달리 사람의 외부에서 유익균과 유해균으로 잘 균형을 이루어 정착해있다.

만약 병원미생물에 감염되어 유해균의 좋은 성장환경을 만들게 되면 감염증이 발생하기 쉽게되어 사람이 질병에 걸리게 되고, 반대로 우리가 먹는 음식 등을 통해서 유익균의 성장환경이 좋게 되면 병원균의 감염을 억제하고 성장을 방해하게 될 뿐만 아니라 우리 몸의 면역을 활성화시켜 질병의 발생을 억제한다. 그러므로 사람에게 유익균을 잘 정착하게 하고, 잘 정착한 유익균을 관리하면 다양한 질병의 발생을 억제 할 수 있다.

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일찍이 메치니코프는 사람의 소화관에 유익균이 증가하게 되면 사람의 장수에 도움을 줄 수 있다고 주장했으며, 이러한 유익균이 많다는 것은 다르게 표현하면 우리몸에 유산균이 많다는 것을 전제로 한다. 건강에 유익한 유산균은 탄수화물을 이용하여 젖산(lactic acid) 등을 다량 생산하는 세균으로 단백질을 분해해서 부패시키는 능력은 없다. 이러한 유산균의 대부분은 편성혐기성성균(Obligate anaerobe) 또는 통성혐기성균(facultative anaerobe)인 *Lactobacillus, Bifidobacterium* 속 세균들이다.

이렇게 사람의 건강을 증진시키기 위해 장내 유산균을 늘리는 연구는 1800년대 후반부터 시작되었으며, 최근 기술발전과 사람 장내 미생물 군집에 대한 연구가 활발하게 이루어짐에 따라, 유산균 증식에 대한 연구도 다시 수면위로 부상하고 있다. 그 중 프로바이오틱스는 '살아있는 미생물로서 적정량을 섭취할 경우 숙주에 도움이 되어 건강효과를 주는 것'으로 정의 되고 있으며, 섭취 시 장에 정착하여 장내 미생물균총의 일부가 되어 pH와 같은 주요 장내 환경적인 요인을 조절하고 bacteriocins과 같은 항균 물질을 생산하여 장내 미생물균총의 구성에 상당한 영향을 줄 수 있다.

이러한 프로바이오틱스의 증식과 생존에 도움을 주는 요인들을 통틀어 프리바이오틱스라고 부른다. 현재까지 이러한 프리바이오틱스로 알려진 성분으로는 oligosaccharides (Fructo-oligosaccharides, Isomalto-oligosaccharides, Lactilol,

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Lactosucrose, Lactulose, Pyrodextrins, Soy oligosaccharides, Xylo-oligosaccharides 등)와 식이섬유가 있다.이들은 주로 bifidogenic 요인으로서 불리워지며, bifidobacteria의 성장을 자극한다고 알려 있다.

한국의 남해안이나 제주 연안에 서식하고 있는 다시마목(*Laminariales*)의 다시마과(*Alariaceae*)에 속한 감태(*Ecklonia cava*)는 이미 이전 연구들을 통해서 혈압 및 혈중 콜레스테롤의 조정, 항염증, 면역활성 자극효능 및 항균이나 항산화, 항바이러스 같은 생리활성이 검증된 해조류이다. 하지만 추출물의 수율이 낮고, 추출하는 과정에서 많은 유효성분이 버려지고 있으며, 그 성분에 있어 대부분을 차지하는 알긴산이나 후코이단 같은 다당류의 특징상 인체흡수가 용이하지 못하다는 단점을 내포하고 있다. 이러한 문제점의 해결 방안으로 이번 연구에서는 다당류를 다량 함유한 해조류 감태의 유산균증식 효과를 증명하고 그 이차대사산물의 유해균을 억제활성을 평가하였다.

또한, 감태의 유산균 증식과 유해균 억제 효과를 바탕으로 감태가 양식넙치에미치는성장효과, 병원성미생물에대한 넙치 장내에서의프리바이오틱스 및사료첨가제로써의상업적가치를효과를 검증하여 기존에 연구된 단순 항생제 대신 유해 미생물 억제 효과와 생산성 향상을 동시에 갖고 있는 새로운 천연항생제의 가능성을 평가하고자 하였다.

먼저 제주도에 소재한 아쿠아그린텍㈜에서 구매한 총 6가지의 제주산 천연자원

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(감귤박, 당근박, 섬오가피, 우뭇가사리, 파래, 감태)을 1% 첨가한 배지에 프로바이오틱스로 가장 많이 사용되는 3종의 유산균(*Lactobacillus brevis, Lactobacillus pentosus, Lactobacillus plantarum*)을 접종하여 유산균의 증식효과를 확인 하였다. 유산균의 증식 효과를 확인한 결과, 다른 5종의 천연물 자원과 비교하여 감태의 유산균 증식 효과가 3 ~ 3.5 배 이상 증가한 것을 확인 할 수 있었다. 이렇게 높은 증식 효과를 보인 감태의 배지내 최적 첨가농도를 확인하기 위해서 배지 내 첨가 농도별(0, 0.01, 0.1, 1, 10 mg/ml)로 확인한 결과에서도 첨가 농도에 따라 증식효과가 늘어 났으며, 그중 가장 효율적으로 증식효과를 보인 1mg/ml를 최적 첨가 농도로 선정하였다. 더 다양한 유산균의 증식효과를 평가하기 위해 감태 원시료와 감태에서 추출한 4종(물 추출물, 효소 추출물, 50% 에탄올 추출물, 100% 에탄올 추출물)을 첨가한 배지에 프로바이오틱스로 많이 사용되는 3종(*Lactobacillus brevis, Lactobacillus pentosus, Lactobacillus plantarum*)과 3종의 유산균(*lactobacillus casei, lactobacillus delbrueckii ssp. Bulgaricus, lactobacillus reuteri*)을 추가로 접종하여 증식효과를 확인하였다. 그 결과, 감태 원시료와 감태 효소 추출물이 가장 높은 증식 효과를 보여 주었고, 그 다음 감태 물추출물, 감태 50% 에탄올 추출물 순으로 증식효과가 보였으며, 감태 100% 에탄올 추출물은 증식효과가 미비하거나 없는 것으로 평가 되었다. 따라서 감태의 성분들 중 유산균을 증식 시키는 효과는 다당류 또는 단백질로 판단되어, 정확한 성분을 확인하

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기 위하여 5종의 당 분해효소와 5종의 단백질 분해효소를 사용하여 효소추출을 수행하였으며, 6종의 유산균 중, 실제 산업적으로 가장 많이 사용되며, 이미 사료내 프로바이오틱스 제품으로 널리 쓰이는 *Lactobacillus plantarum*을 이용하여 이후, 연구를 수행하였다.

효소추출을 수행한 결과, 수율이 약 20%에서 40%까지 나타났으며, 10종의 효소추출물들이 모두 유산균을 증식시켰으며, 그 중 5종의 당분해효소 추출물들이 단백질 분해효소추출물들 보다 유산균 증식효과가 높다는 것을 확인하였고, 그 중에서도 Celluclast효소추출물이 가장 높은 증식효과를 보여 다음 실험을 위해 사용하였다.

Celluclast효소추출물로부터 유효성분을 좀 더 분리하기 위해 막분리 시스템을 이용하여 분리하였고, 얻어진 30kDa이상/이하의 분획물을 가지고 유산균을 증식을 확인하였다. 그 결과, 두 분획물 모두, 유산균을 증식 시켰으나, 30kDa이상의 분획물이 더 좋은 효과를 보였다. 그래서 30kDa이상의 분획물로부터 에탄올 분획법을 사용하여 다당류 성분을 침전시켜 분리하였다. 그 결과, 분리된 감태 유래 다당류 (*E. cava* Crude Polysaccharide: ECP)는 농도 의존적으로 유산균을 증식시켰다.

또한, 감태 원시료와 감태에서 추출한 4종(물 추출물, 효소 추출물, 50% 에탄올 추출물, 100% 에탄올 추출물)을 첨가한 배지에 어병균 5종(*Edwardsiella tarda,*

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Streptococcus iniae, Vibrio anguillarum, Vibrio harveyi, Streptococcus parauberis)을 접종하여 그 증식률을 확인한 결과, 감태 100% 에탄올 추출물에서 가장 우수한 어병균 증식 억제효과를 보인 것을 확인 할 수 있었다. 그 중 가장 높은 효과를 보인 3종(*Edwardsiella tarda, Streptococcus iniae, Vibrio harveyi*)에서 배지 내 감태 100% 에탄올 추출물의 첨가 농도 별로 확인을 한 결과에서도, 농도의존적으로 상당하게 증식을 억제한 것을 확인 할 수 있었다.

감태로 증식된 유산균의 이차대사산물의 어병균 증식 억제 효과를 평가한 결과에서는 감태 원시료와 효소추출물이 첨가되어 증식된 유산균에서 생성된 이차대사산물이 어병균 3종(*Edwardsiella tarda, Streptococcus iniae, Vibrio harveyi*)에 대해서 높은 증식 억제 효과를 보였다. 따라서 감태의 첨가가 유산균을 증식시키고 증식되는 과정에서 다량으로 생성되는 물질이 bacteriocins과 같은 항균물질인 것으로 확인 되었다.

따라서, 이러한 감태의 첨가 효과를 바탕으로 사료내 감태를 첨가하여 넙치 사양시험을 수행하였다. 사양실험은 표선에 위치한 만해수산에서 300~350 g의 넙치를 이용하여 수행하였으며, 12개의 수조에 각각 1,800마리의 넙치를 순치한 후, 3개의 수조마다 각각 다른 사료(감태 1%첨가, 감태 에탄올 추출물 0.1% 첨가, 감태 에탄올 추출물 0.5% 첨가)를 제작하여 16주간 실험하였다.

사료 내 감태 1% 첨가는넙치성장에유의적인효과를나타내었으며사료에대한적정

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첨가농도는 1%였다. 한편, 감태 에탄올 추출물 0.5% 첨가 사료를섭이한넙치에서는감태 1% 첨가 사료보다낮은성장을보였다. 이러한결과는감태 1% 첨가 사료가일반사료 및 다른 첨가 사료에비해서양식넙치의성장에효과적인것으로보이며따라서감태 1% 첨가 사료는양식넙치의성장및사료효율개선에효과적인사료첨가제로사료된다. 또한감태 1% 첨가 사료를 섭이한 넙치의 혈액을 분석한 결과, GOT, GPT 값은낮아지고 Hematocrit 값은 높아졌으며, NBT활성, 라이소자임 활성, MPO활성은 일반사료를 먹은 넙치에 비해 크게 증가하는 것을 보였다. 이로써, 감태 1% 첨가 사료는 넙치의 면역력개선에효과적으로작용하는것으로생각된다.

그러므로, 감태 1% 첨가 사료로 섭이한 넙치가 갖는 면역증강 능력을 확인 하기 위해, *Edwardsiella tarda, Streptococcus iniae, Vibrio harveyi*를 접종하여 공격실험을 수행한 하였다. 접종 후 2주간의 폐사율을 측정한 결과, 감태 1% 첨가 사료로 섭이한 넙치가대조구에비해낮은사망률을보였다. 이는감태의 첨가로 인해 증가한 유산균의길항특성(probiotics)으로양식넙치에있어서면역력을증가시키고생존율을높이는것으로판단 된다.

제주에서 다량 발생하는 해조류인 감태는 시판되고있는양식사료첨가제인키토산, 베타글루칸그리고어보산에비해 가공단계가 적고, 건조나 사료 내 첨가에 있어 활용이 원활하여, 다른사료첨가제들에비해양식사료첨가제로써상대적으로효과가좋은것으로사료된다.

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지금까지의 결과를 통해 감태는 프리바이오틱스로서 유산균의 대량 증식을 도우며, 사료 내에 첨가 시 어류의 성장과 면역력을 크게 증가 시킨다는 것을 확인할 수 있었다. 이것은 제주에서 나는 감태가 연구적인 가치뿐만이 아니라 산업적으로도 유용될 수 있다는 사실을 이번 연구를 통해 다시 한번 확인하였다.

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harveyi group; lane 2, kidney in *E.tarda* group; lane 3, kidney in *S. iniae* group; lane 4, Liver in *V. harveyi* group; lane 5, Liver in *E.tarda* group; lane 6, Liver in *S. iniae* group ; lane 7, 8, kidney and Liver of control group; M, 100bp DNA ladder.

Figure 2-8. Cumulated mortality of Olive flounderfed by three different diets (◆1% *E. cava,* × 0.1% EPE,▲ 0.5% EPE) and regular diets (● withoutinfected, ■with infected) for 14 days of after challenge with *Edwardsiella tarda.*

Figure 2-9. Cumulated mortality of Olive flounderfed by three different diets (◆1% *E. cava,* × 0.1% EPE,▲ 0.5% EPE) and regular diets (● withoutinfected, ■with infected) for 14 days of after challenge with *Streptococcus iniae.*

Figure 2-10. Cumulated mortality of Olive flounderfed by three different diets (◆1% *E. cava,* × 0.1% EPE,▲ 0.5% EPE) and regular diets (● withoutinfected, ■with infected) for 14 days of after challenge with *Vibrio harveyi.*

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Part I.

Effect of *Ecklonia cava* on the growth of lactic acid bacteria (LAB) and pathogen bacteria

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ABSTRACT

Prebiotics that stimulate the growth and/or function of beneficial intestinal microorganisms are also so important for confer health beneficial ingredients. The purpose of this study is to investigate whether Jeju natural productssuch as seaweeds can play as beneficial prebiotics via inducing growth of probiotics including lactic acid bacteria(LAB) ((*Lactobacillus brevis*(*L. brevis*), *Lactobacillus casei*(*L. casei*), *Lactobacillus delbrueckii ssp. Bulgaricus*(*L. delbrueckii ssp. Bulgaricus*), *Lactobacillus pentosus* (*L. pentosus*), *Lactobacillus plantarum*(*L. plantarum*),and *Lactobacillus reuteri*(*L. reuteri*)). The original *E. cava* increased the growth of LAB and itsCelluclastextract showed the highest effects on the growth of *L. plantarum*. Also, the >30 kDa fraction from the Celluclastextract of *E. cava* and its crude polysaccharide markedly and dose-dependently increased the growth of *L. plantarum.* Moreover, the original*E. cava*, Celluclast and 100% ethanol extracts showed anti-bacteria activities on pathogen bacteria. Interestingly, the secondary metabolites produced by the original*E. cava* from *L. plantarum*in MRS medium showed the anti-bacterial activities on pathogen bacteria (*Edwardsiella tarda, Streptococcus iniae, Vibrio anguillarum, Vibrio harveyi* and *Streptococcus parauberis*). According to these results, *E. cava*consisted of a polysaccharide ofmight be a natural prebiotic via enhancing LAB and inhibiting pathogen bacteria. Also, our conclusion from this evidence is that *E. cava* can be used and applied as a useful prebiotic.

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1.INTRODUCTION

Seaweeds are a widely available source of biomass, considering that over two million tons are either harvested from the oceans or cultured annually for food or phycocolloid production (Ahn et al, 2008). The three basic types of seaweeds are brown (Phaeophyta), red (Rhodophyta) and green (Chlorophyta), divided according to the dominant pigment, respectively, xanthophylls, fucoxanthin, phycoerythrin and chlorophyll a and b (Ahn et al, 2008). The brown seaweeds abound in polyphenols and polysaccharides that possess many bioactive properties (Ahn et al., 2008, Heo et al., 2005a and 200b, Siriwardhana et al., 2004; Ruperez & Saura-Calixto., 2001). *AlariaceaeEcklonia cava(E. cava)* is rich in xanthopophyll, fucoxathin, vitamins, vitamin precursors such as α-tocopherol, β-carotene, niacin, thiamin and ascorbic acid, and polysaccharides such as fucoidan, alginates, fucans and laminarans, which are water-soluble dietary fibers and phycocolloids(Ahn et al, 2008). In addition, *E. cava* grows plentifully near Jeju Island of Korea (Guiry & Bulunden., 1991) and, reportedly, contains polyphenol and polysaccharide compounds than other brown seaweeds (Heo et al., 2005a). For few years, biological activity studies showed that *E. cava* has anti-oxidant *in vitro,* anti-coagulant, anti-cancer, immunomodulation, anti-inflammation and immune activation effects(Athukorala et al., 2006, Ahn et al., 2007, 2008 and 2010; Kim et al., 2006a). In particular, previous studies have showed *Lactobacillus brevis*-fermented *Ecklonia cava* (*E. cava*) has anti-inflammatory, radio-protective and anti-oxidant effects. In addition, the fermented *E. cava* using bacteria*Lactobacillus brevis* increased the protein contents in comparison with the non-fermented *E. cava* (Lee et al., 2011 and 2013). From these results, we thought that the protein contents increased by fermentation process might be related to the growth of *Lactobacillus brevis* induced during fermentation process. Normally, *Lactobacillus*

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brevis isa kind of lactic acid bacteria (LAB) known as a common type of probiotics that may confer a health benefit on the host. For many years, probiotic bacteria have been known to confer health benefits to the consumer (Blum et al., 2002). Probiotics including LAB was shown to prevent adherence, establishment, and replication of several enterophathogens through antimicrobial mechanism and modulation of the host mucosal immune response (Blum et al., 2002). Therefore, inducing growth of probiotic including LAB is so important. At these points, prebiotics that stimulate the growth and/or function of beneficial intestinal microorganisms are also so important for confer health beneficial ingredients. Indeed, many researchers have reported that plants and seaweed played roles as prebiotics via inducing growth of probiotics such as LAB.

Prebiotics were defined as non-digestible foodingredients that beneficially affect the host by selectively stimulatingthe growth and/or the activity of one or a limited number ofbacteria in the colon, and thus improve host health (Gibson et al., 1995, Teitelbaum and Walker, 2002, Grimoud et al., 2010). Most ofthe probiotic strains belong to the Lactobacillus and Bifidobacteriumgenera, which are health promoting bacteria forming part of thebalanced intestinal microbiota (Ventura et al., 2009, Grimoud et al., 2010). Recent studies indicated that the main prebiotics are fibres and carbohydrates, such as resistant starch, wheat bran,inulin or oligosaccharide, which are short polymers of glycosidicresidues such as fructose in fructooligosaccharides or galactose ingalactooligosaccharides and have been demonstrated to selectively (Grimoud et al., 2010, Boucher et al., 2003, Rousseau et al., 2005). As indicated in above, *E. cava* can be a source for fibres and carbohydrates known as main prebiotics. However, various studies related with E. cava have reported, there is no report about the possibility of *E. cava* as a prebiotic.

Therefore, the present study investigated whether *E. cava* play as beneficial prebiotics via inducing growth of probiotics including LAB such as *Lactobacillus brevis, Lactobacillus*

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casei, Lactobacillus delbrueckii ssp. Bulgaricus, Lactobacillus pentosus, Lactobacillus plantarum and *Lactobacillus reuteri* and inhibiting growth of pathogen bacteria such as *Edwardsiella tarda, Streptococcus iniae, Vibrio anguillarum, Vibrio harveyi* and *Streptococcus parauberis*.

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2. MATERIALS AND METHODS

2.1. Preparation of samples

Citrus by-products, Carrot by-products, *Acanthopanax koreanum, Gelidium amansii*, and *Enteromorpha* were kindly provided from Aqua Green Technology Co., LTD and *Ecklonia cava*(*E. cava*) was collected from the coast of Jeju Island, South Korea. Then, they were respectively washed with fresh water, freeze-dried and pulverized into powder with a grinder. The freeze-dried samples were used for next experiments.

2.2. Preparation of ethanol, aqueous, and enzymatic extracts from *E. cava*

Here, aqueous and 50% and 100% ethanol extracts were prepared from *E. cava*. After freeze-dry, the*E. cava*powder (1 g) was respectivelyhomogenized in 50% and 100% ethanol and distilled waters (100 ml). After 24 h, the samples were obtained and kept at -20℃ for further experiments.

To prepare an enzymatic extract of *E. cava*,*E. cava*powderwas used for the enzymatic extraction technique using followed previously reported method (Heo et al., 2005). Each one gram of the powdered *E. cava* was homogenized with 100ml of distilled water (from pH 4.5 and pH 8.0)and 100μg or 100 μl of carbohydrases (AMG, celluclast, Ultraflo, Termamyl and Viscozyme) or proteases (Neutrase, Alcalase, Flavourzyme, Protamex andKojizyme) (Novo Nordisk, Bagsvaerd, Denmark). The reactant was adjusted to the optimum pH and temperature range of the respective enzyme and enzymatic reaction was performed for 24 h(Heo et al., 2005a). Following digestion, the digest was boiled for 10 min at 100℃ to

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inactivate the enzymes. After centrifugation (3000 rpm, for 20 min at 4℃), the supernatants were adjusted to pH 7.0 hereafter and designated to enzymatic extracts. The samples were kept at -20℃and used for further experiments.

2.3. Preparation of molecular weight fractionsfrom theCelluclast extract of *E. cava*

To preparedifferent molecular weight fractions,the Celluclast extract of *E. cava* was applied to Lab scale TFF system (PHILOS) using micro-filtration membrane (30 kDa). Then, all the fractions (whole extract, >30 kDa fraction, and <30 kDa fraction) were freeze-dried. The freeze-dried samples were separately evaluated the growth effects on LAB.

2.4. Isolation of a polysaccharide from > 30 kDa fraction of *E. cava*Celluclast extract

A polysaccharide was isolated from > 30 kDa fraction of the *E. cava*Celluclast extract by ethanol precipitation according to slightly revised method indicated in previous study (Athukorala et al., 2009). The > 30 kDa fraction of *E. cava*Celluclast extract (1 L) was mixed with 2 L of 99.5% ethanol for 24 h at 4℃. After centrifugation at 10000 rpm for 20 min at 4℃, the crude polysaccharide was collected from its precipitant. Then, the crude polysaccharide isolated from the > 30 kDa fraction of *E. cava*Celluclast extract was freeze-dried and used for next experiments.

2.5. Lactic acid bacteria (LAB) culture

Six kinds of lactic acid bacteria(LAB) (*Lactobacillus brevis*(*L. brevis*), *Lactobacillus*

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casei(*L. casei*), *Lactobacillus delbrueckii ssp. Bulgaricus*(*L. delbrueckii ssp. Bulgaricus*), *Lactobacillus pentosus* (*L. pentosus*), *Lactobacillus plantarum*(*L. plantarum*),and *Lactobacillus reuteri*(*L. reuteri*)) were obtained from the KCCM (Korean Culture Center of Microorganisms, Seoul, Korea) (Table 1-1). These bacteria were incubated in MRS broth or MRS agar at 30℃ until use.

2.6. Pathogen bacteria culture

Five kinds of pathogen bacteria such as *Edwardsiella tarda* (*E. tarda), Streptococcus iniae* (*S. iniae*), *Vibrio anguillarum* (*V. anguillarum*), *Vibrio harveyi* (*V. harveyi*) and *Streptococcus parauberis* (*S. parauberis*) were obtained from the KCTC (Korean Collection for Type Culture, Daejeon, Korea) (Table 1-2). These bacteria were incubated in BHIA broth or agar for *E. tarda, S. iniae, V. anguillarum,* and *S. parauberis* and TSA broth or agar for *V. harveyi*at 25℃ until use.

2.7. Colony count assay

To evaluate the effects of the samples on the growth of LAB, colony count assay was performed. First, the freeze-dried samples(10 mg) were respectively added to the MRS broth (1 ml) for LAB cultureand autoclaved. Then, LAB(10^7 colony forming units, CFU) were incubated at 30℃ in MRS broth containing each samples prepared in above for 24 h. Then, the LAB and pathogen bacteria were respectively diluted and incubated on MRS agar at 30℃for 24 h. The formed CFUs were counted. The relative growth rates were calculated as % based on the non-added MRS broth.

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Next, to investigate the anti-bacterial effects of the samples onsix pathogen bacteria, colony count assay was performed. Each pathogen bacteria (10^3 CFU) were incubated at 25℃ in BHA broth containing each samples prepared in above for 24 h. Then, the pathogen bacteria were respectively diluted and incubated on BHIA or TSA agar at 25℃ for 24 h. The formed CFUs were counted as described earlier. The relative growth rates were calculated as % based onnon-added BHIA or TSA broth.

2.8. Statistical analysis

Data was analyzed using the statistical package for the social science (SPSS) package for Windows (Version 10). Values were expressed as means ± standard error (SE). A *p*-value of less than 0.05 was considered significant.

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Table 1-1.List of strains used for experiment.

	Strains	Number
Lactic acid Bacteria (LAB)	*Lactobacillus brevis*	KCCM 40399
	Lactobacillus casei	KCCM 12452
	Lactobacillus delbrueckii ssp. Bulgaricus	KCCM 35463
	Lactobacillus pentosus	KCCM 40997
	Lactobacillus plantarum	KCCM 12116
	Lactobacillus reuteri	KCCM 40717

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Table 1-2. List of strains used of antibacterial experiment.

	Strains	Number
Pathogen bacteria	***Edwardsiella tarda***	**KCTC 12267**
	Streptococcus iniae	**KCTC 3657**
	Vibrio anguillarum	**KCTC 2711**
	Vibrio harveyi	**KCTC 12724**
	Streptococcus parauberis	**KCTC 3651**

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2. RESULTS

3.1. The original *E. cava* showed the highest growth effect on the growth of LAB

To evaluate the effects of Jeju natural products on the growth of LAB, colony count assay was performed. First, six kinds of Jeju natural products (Citrus by-products, Carrot by-products, *Acanthopanax koreanum, Gelidium amansii*, *Enteromorpha,* and *E. cava*) were added to the culture medium and respectively incubated with 3 kinds of LAB such as *L. pentosus*, *L. brevis,* and *L. plantarum* for 24 h. Among the samples, the original *E. cava*showed the highest effect on the growth of the LAB, compared to non-treated LAB, whereas the other samples did not show (Fig. 1-1). In addition, the addition of the original *E. cava* dose-dependentlyincreased the growth of three kinds of LAB and showed the higher growth of *L. brevis* and *L. plantarum* than that of *L. pentosus* (Fig. 1-2).This result indicated that the original *E. cava* might be a potential prebiotic via stimulating the growth of LAB. To more evaluate whether the original *E. cava* has the potential effect on the growth of the other strains of LAB, colony forming assay was performed. As indicated in table 1-1, the six kinds of LAB were used in this study. As indicated in Table 1-3, the original *E. cava*enhanced the growth of all LAB including *L. brevis,L. casei*, *L. delbrueckii ssp. Bulgaricus*, *L. pentosus*, *L. plantarum,*and *L. reuteri*, in comparison with non-treated control. In particular, the original *E.* cava showed the higher growth effects on *L. brevis* and *L. plantarum* and respectively were about 352±15.3% and 339±9.6%, compared to non-treated control (100%).

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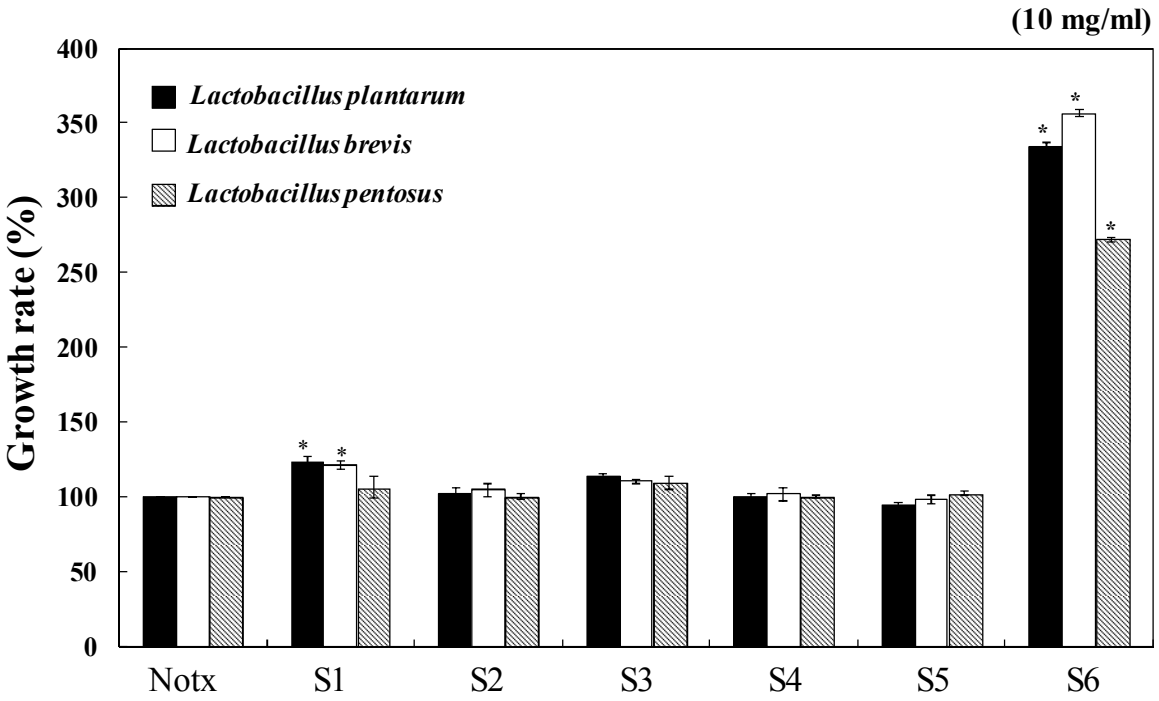

Figure.1-1.

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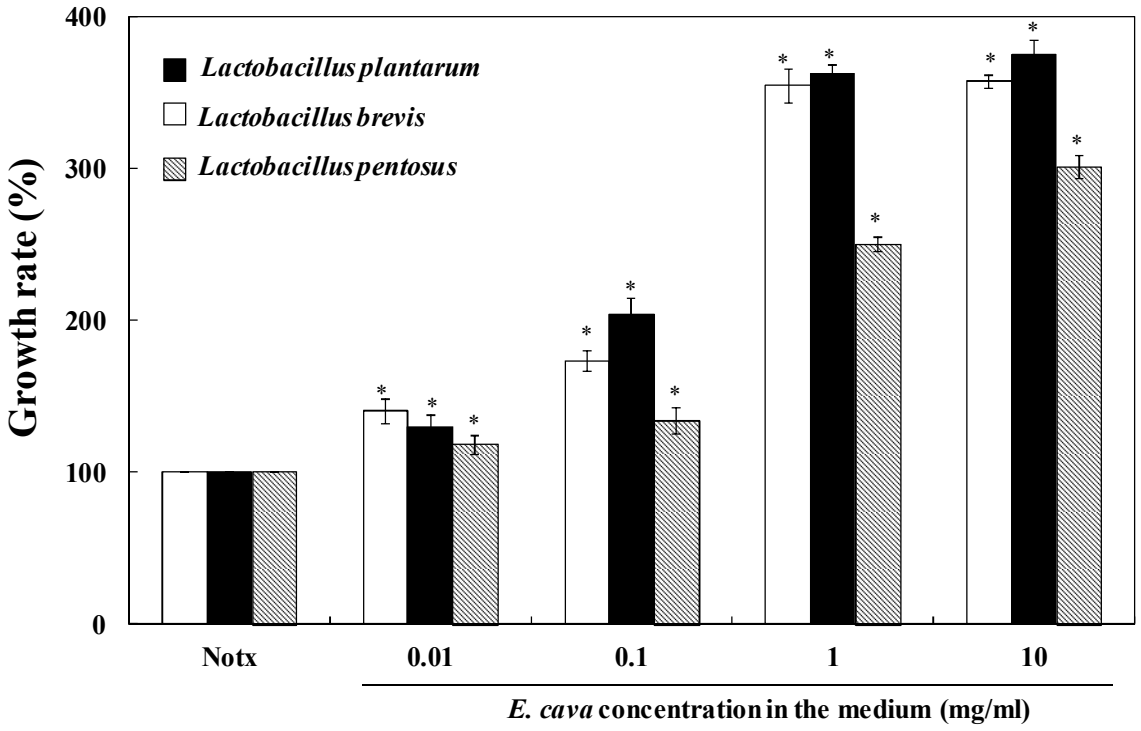

Figure 1-2.

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3.2. The Celluclastextract of *E. cava* showed the highest effect on the growth of *L. plantarum*

To identify what component play as an important role in the growth of LAB in the original *E. cava*, four type extracts were prepared by using distilled water, Celluclast enzyme and 50% and 100% ethanol. In Table 1-3, the three extracts of *E. cava*including aqueous extract, Celluclast extract, and 50% ethanol extract markedly increased the growth of all LAB used in this study, whereas the 100% ethanol extract did not show, compared to non-treated control. Interestingly, among the four extracts, the aqueous and Celluclast extracts showed the higher effects on the growth of the six LAB, compared to the other organic solvent extracts (50% and 100% ethanol extracts). Also, among them, the Celluclast extract of *E. cava* showed the highest growth effect on all LAB used in this study, compared to those of the others. In addition, the Celluclast extract of *E. cava* especially led to the higher growth of *L. brevis* and *L. plantarum.* These results indicate that the major components for the growth of LAB might be water-soluble components such as polysaccharides.

Next, the effects of the other 9 enzymatic extracts prepared by carbohydrases (AMG, Ultraflo, Termamyl and Viscozyme) or proteases (Neutrase, Alcalase, Flavourzyme, Protamex andKojizyme) on the growth of *L. plantarum*were evaluated.As shown in Fig. 1-3, the enzymatic extraction technique improved the extraction yields and among them, the AMG and Celluclast extracts showed the higher extraction efficiency (41.52% and 40.66%, respectively) than the others. Also, the 10 kinds of enzymatic extracts markedly increased the growth of *L. brevis*, compared to the non-treated control (Fig. 1-3). In particular, the Celluclast extract of *E. cava* showed the highest growth effect on *L. plantarum* compared to those of the others. Therefore, the Celluclast extract was selected for the next experiments.

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Table 1-3. The lactic acid bacteria growth effect in accordance with the extraction method.

(%)

10^7 CFU/ml	Notx	*E. cava* (1 mg/ml)				
		Original	Water extract	Celluclast extract	50% ethanol extract	100% ethanol extract
Lactobacillus brevis	100	352±15.3*	293±3.1*	345±6.2*	214±13.6*	115±9.4*
Lactobacillus casei	100	273±8.9*	225±4.4*	274±8.9*	181±7.9*	109±11.2
Lactobacillus delbrueckii ssp. Bulgaricus	100	195±3.2*	177±6.6*	204±11.7*	136±6.4*	104±6.7
Lactobacillus pentosus	100	265±7.9*	213±2.8*	247±3.6*	166±3.6*	102±9.9
Lactobacillus plantarum	100	339±9.6*	268±5.6*	337±10.5*	224±4.9*	103±7.7
Lactobacillus reuteri	100	276±4.5*	225±2.3*	259±5.5*	185±2.3*	98±7.8

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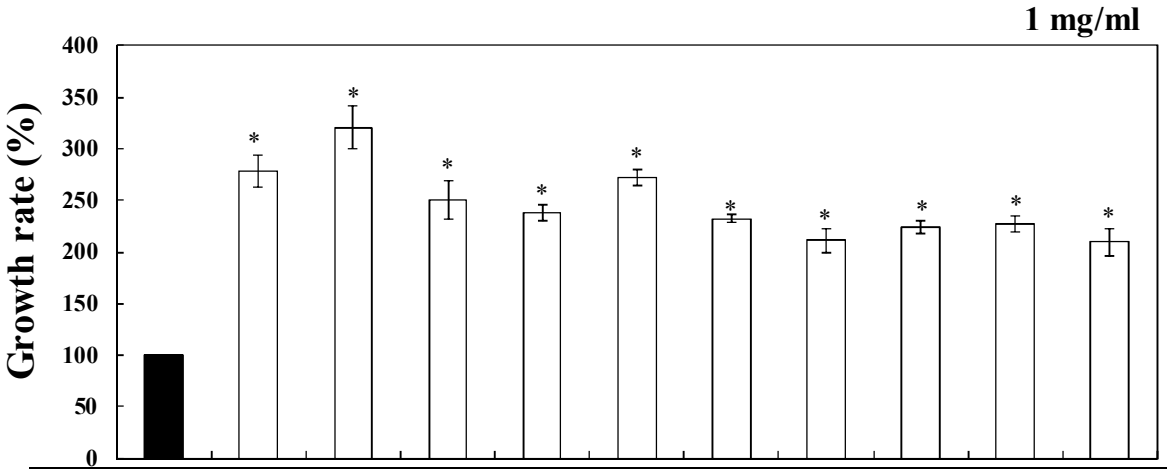

Yield	Notx	AMG	Ce	Ul	Te	Vi	Ne	Al	Fl	Pro	Ko
(%)	-	41.52	40.66	28.26	23.29	39.56	26.69	32.72	32.81	28.12	20.94

Figure 1-3.

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3.3. The >30 kDa fraction from the Celluclastextract of *E. cava* markedly increased the growth of *L. plantarum*

To identify what component plays as an important role in the growth of LAB in the Celluclastextract of*E. cava*, two molecular weight fractions (>30 kDa and <30 kDa fractions) were prepared by an ultra-membrane filtration system using 30 kDa filter membrane (Fig. 1-4). Figure 1-5 indicated that two molecular weight fractions of Celluclast extracts of *E. cava*enhanced the growth of *L. plantarum*in dose-dependent manner, compared to the non-treated control. In particular, the >30 kDa fraction of the Celluclast extract of *E. cava* showed the higher growth effect on *L. plantarum*at all used concentrations than those of the <30 kDa fraction. This result indicates that any hydrophiliccomponent with the high molecular weight might be an important factor for the growth of *L. plantarum.*

3.4. The crude polysaccharide isolated from the >30 kDa fraction of the *E. cava* Celluclastextract markedly and dose-dependently increased the growth of *L. plantarum*

Normally, major components of *E. cava* are polyphenols of hydrophobic low molecular weight components and polysaccharides of hydrophilic high molecular weight components (Ahn et al., 2007 and 2011). At these points, the major component of the >30 kDa fraction might be a polysaccharide. Therefore, a crude polysaccharide was isolated from the>30 kDa fraction of the *E. cava* Celluclast extract by ethanol precipitation technique indicated in previous study (Fig. 1-4) (Ahn et al. 2007 and 2013).

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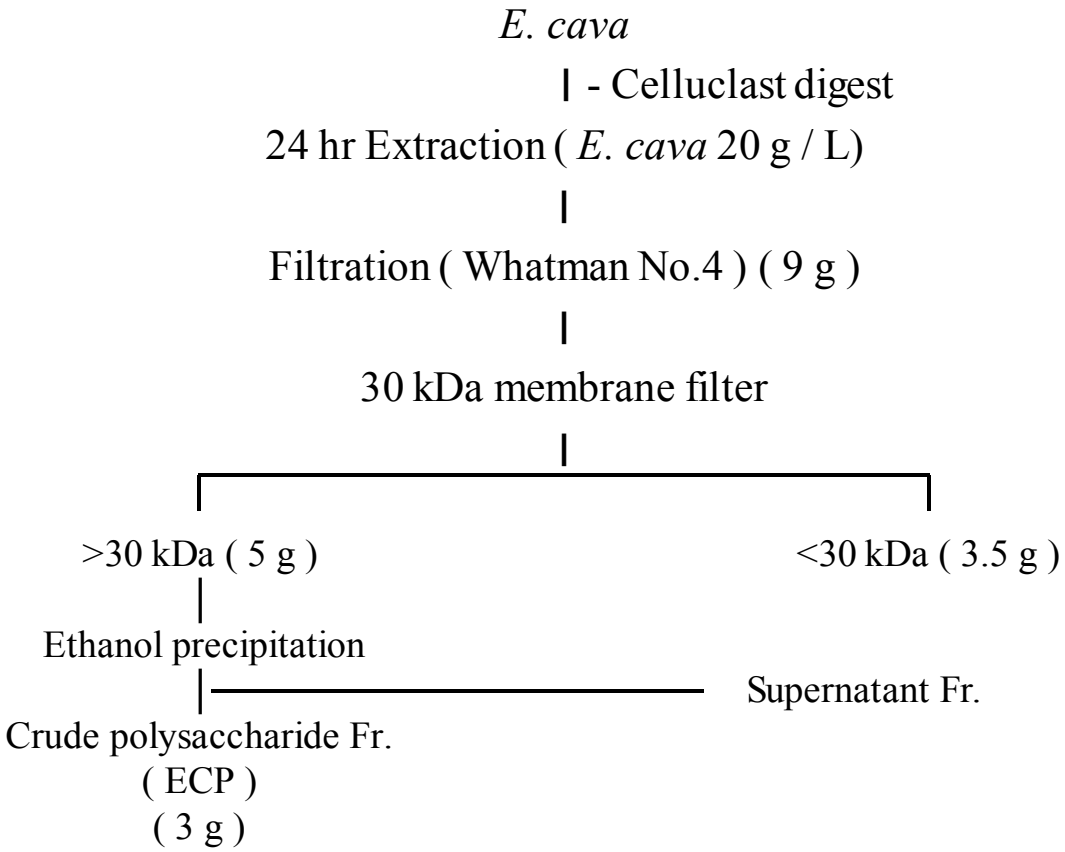

Figure 1-4.

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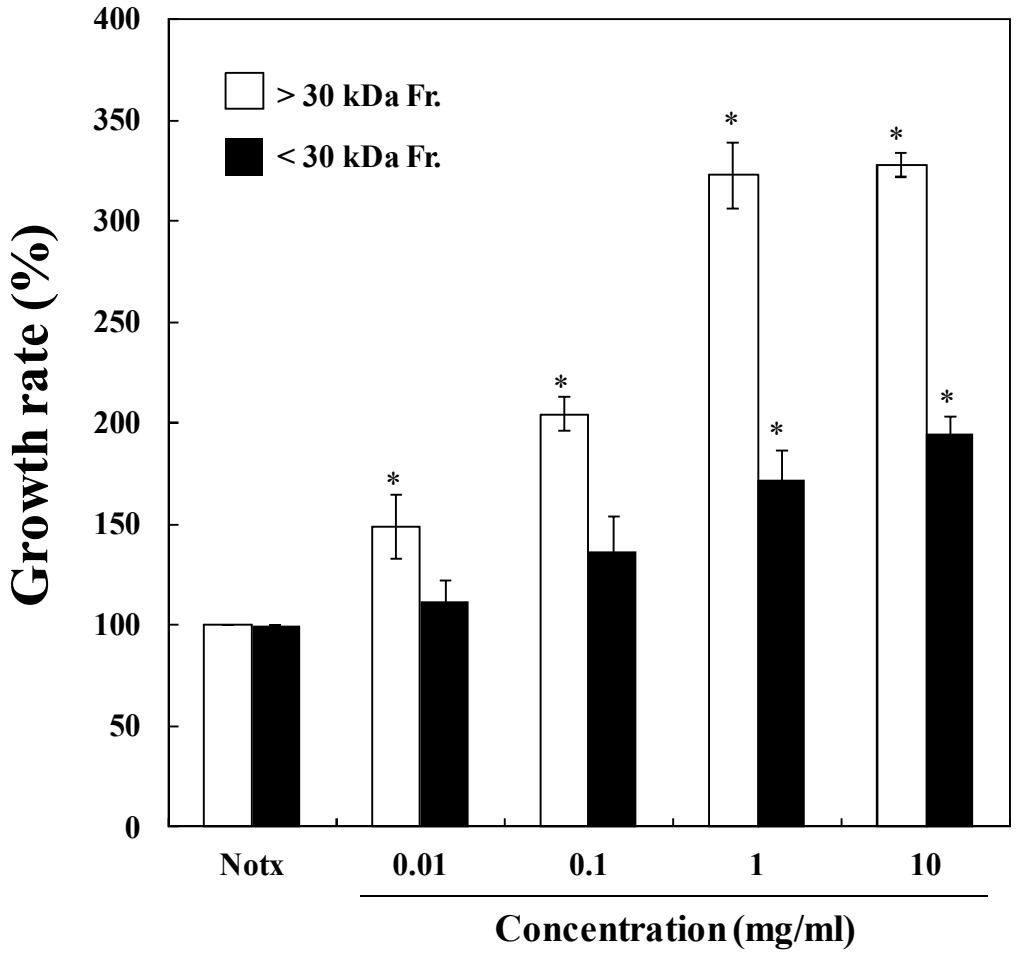

Figure 1-5.

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The effect of the crude polysaccharide isolated from >30 kDa fraction of the *E. cava* Celluclast extract on the growth of *L. plantarum* was examined by colony count assay.Figure 1-6 showed that the crude polysaccharide isolated from the >30 kDa fraction of the *E. cava* Celluclast extract dose-dependently increased the growth of *L. plantarum* at the concentrations from 0.01 mg/ml to 1 mg/ml. In addition, the 1 mg/ml and 10 mg/ml of polysaccharides showed the similar growth effect on *L. plantarum*. This result indicates the major active compound for the growth of LAB is a polysaccharide component.

3.5. The original*E. cava*, Celluclast and 100% ethanol extracts showed the anti-bacterial activity on five pathogen bacteria

Next, to access whether the samples have anti-bacterial activities on the five kinds of pathogen bacteria, colony count assay was performed. As indicated in Table 1-4, the addition of the original *E. cava* and its four extracts (the original *E. cava*, aqueous, Celluclast, and 50% and 100% ethanol extracts) (1 mg/ml) effectively inhibited the growth of the four pathogen bacteria (*E. tarda, S. iniae,V. harveyi, and V. anguillarum*), but, did not show significant change in*S. parauber*. Especially, the 50% and 100% ethanol extracts of *E. cava* showed the higher anti-bacteria effects than those of the other samples. Also, as shown in Figure 1-7, the original *E. cava* and its 100% ethanol and Celluclast extractsdose-dependently and markedly decreased the growth of the pathogen bacteria (*E. tarda, S. iniae,* and *V. harveyi*), compared to non-treated control. These results indicate that both hydrophobic and hydrophilic compounds of the original *E. cava* have the beneficial anti-bacterial capacity via inhibiting the growth of the pathogen bacteria.

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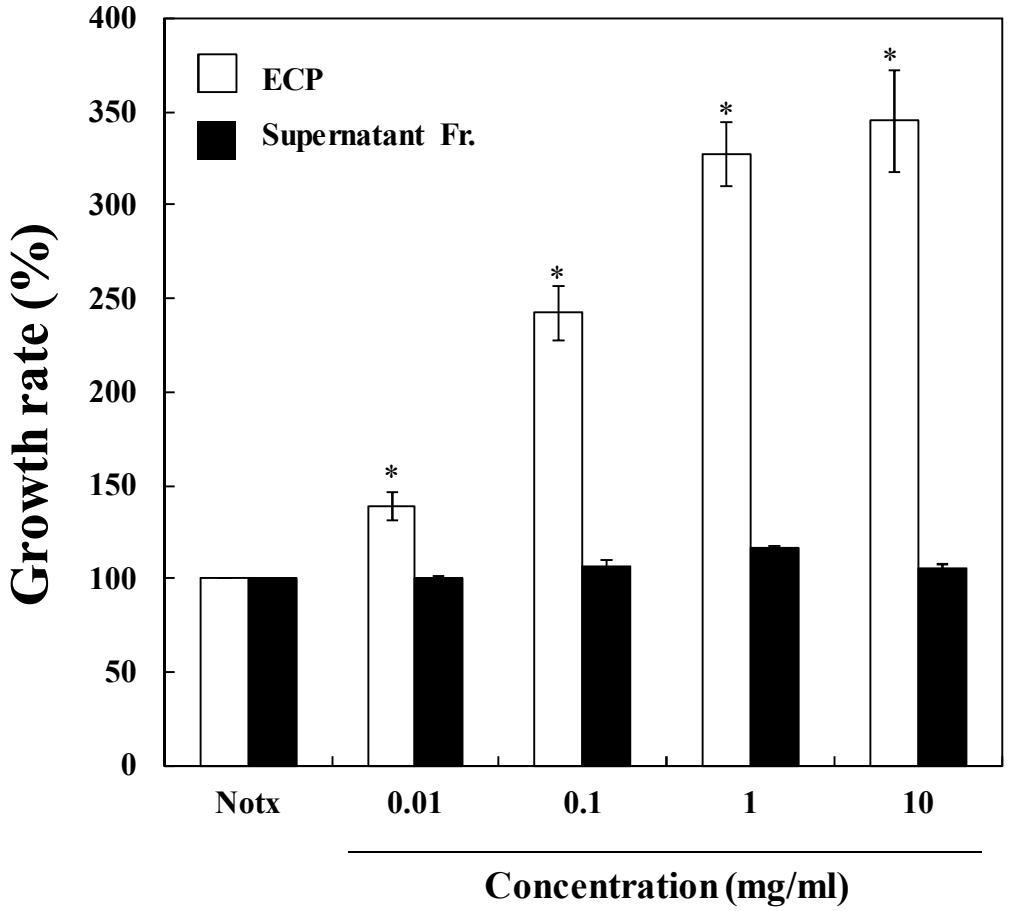

Figure 1-6.

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Table 1-4.The antibacterial effect in accordance with the extraction method.

(%)

10^3 CFU/ml	Notx	*E. cava* (1 mg/ml)				
		Original	Water extract	Celluclast extract	50% ethanol extract	100% ethanol extract
Edwardsiella tarda	100	78±9.4*	72±8.5*	64±5.0*	54±13.6*	37±3.2*
Streptococcus iniae	100	81±2.7*	69±8.8*	70±2.6*	46±3.3*	43±5.6*
Vibrio anguillarum	100	79±4.5*	79±1.9*	75±8.1*	63±9.4*	69±11.2*
Vibrio harveyi	100	61±9.0*	40±10.1*	43±8.3*	33±5.2*	29±5.8*
Streptococcus parauberis	100	99±9.6	93±7.7	90±15.6	87±4.4*	86±8.1

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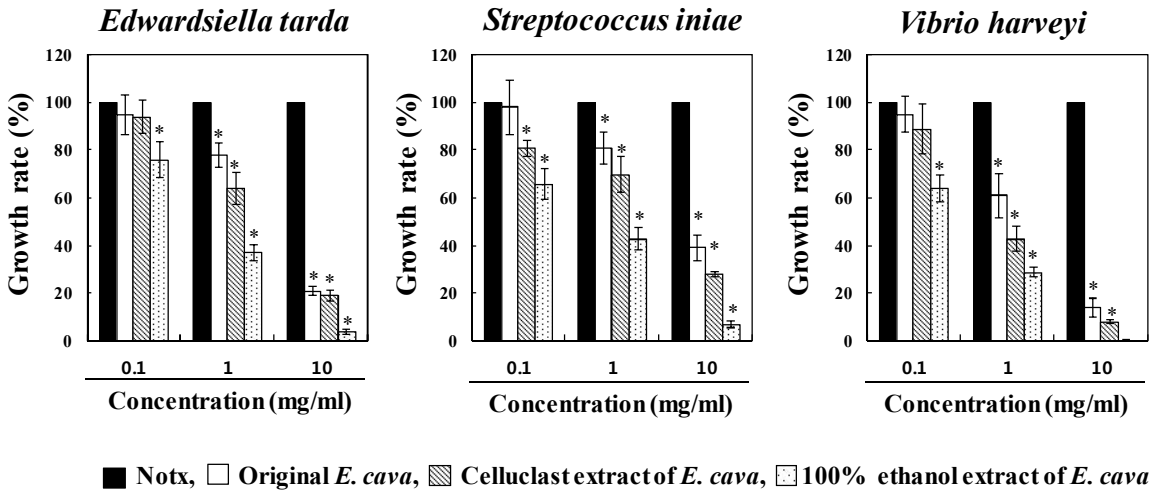

Figure 1-7.

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3.6. The original*E. cava* improved the production of the secondary metabolites from *L. plantarum* in MRS medium

Normally, prebiotics can produce secondary metabolites with the increased growth of LAB and the secondary metabolites can have biological activities such as the anti-bacterial activity. Therefore, here, the effects of the original *E. cava* and 100% ethanol extract on production of secondary metabolites from *L. plantarum* were investigated. The result was indicated in Figure 1-8. The original *E. cava*and its 100% ethanol extract did not induce the production of secondary metabolites in only MRS medium (Fig. 1-8 and Table 1-5). However, the addition of *L. plantarum* produced the 324.5±15.5 mg of the secondary metabolitesin MRS medium. Interestingly, the addition of the original *E. cava* increased the amount of the secondary metabolites from the*L. plantarum*contained MRS medium(422.2 ± 11.4 mg), whereas the addition of the 100% ethanol extract did not affect(313.8±28.4 mg), compared to that of the *L. plantarum*contained MRS medium.

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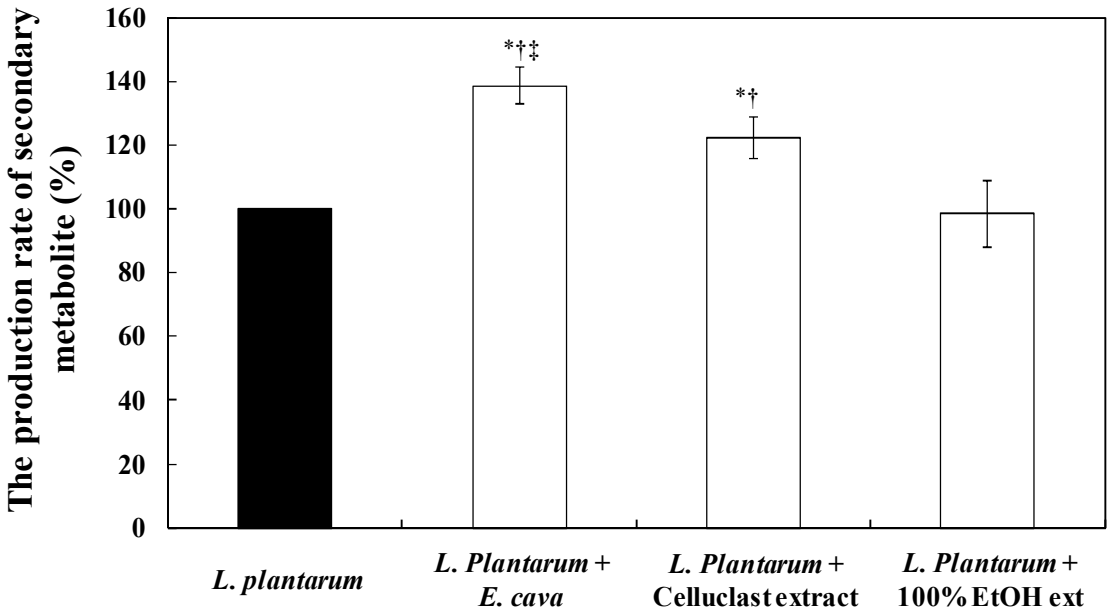

Figure 1-8.

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Table 1-5. The production rate of secondary metabolite in accordance with the extraction method.

	L. plantarum	*L. Plantarum* + *E. cava*	*L. Plantarum* + Celluclast extract	*L. Plantarum* + 100% EtOH ext
Secondary metabolite (mg)	**324.5±15.5**	**422.2±11.4**	**376.5±11.2**	**313.8±28.4**

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3.7. The secondary metabolites produced by the original*E. cava* from the*L. plantarum*-containedMRS mediumshowed the anti-bacterial activity on the pathogen bacteria

Next, the anti-bacterial effect of the secondary metabolites produced from *L. plantarum*in MRS medium with/without the original *E. cava*, its Celluclast and 100% ethanol extracts were examined. In Table 1-6, the secondary metabolites produced by the addition of the original *E. cava* and Celluclast extract markedly inhibited the growth of pathogen bacteria (*E. tarda, S. iniae* and *V. harveyi*). In addition, the secondary metabolite produced by the original *E. cava* considerably inhibited the growth of three pathogen bacteria and they were dose-dependent (Fig. 1-9).

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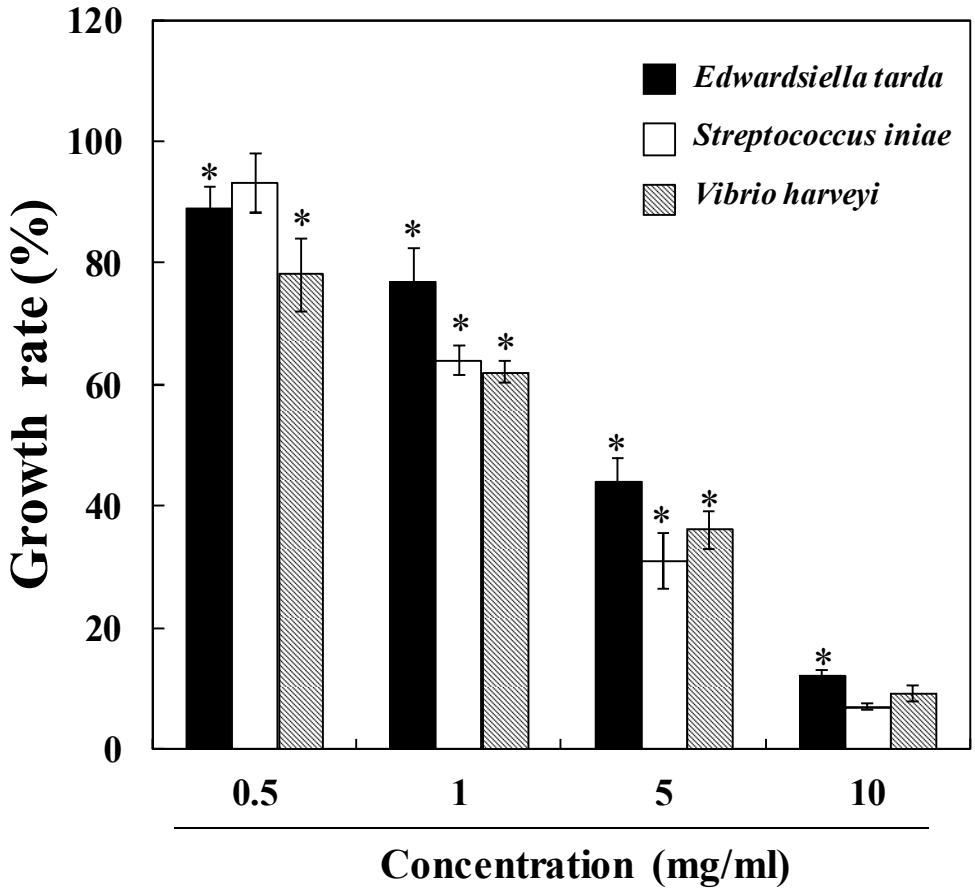

Figure 1-9.

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Table 1-6. The antibacterial effect of secondary metabolite in accordance with the extraction method.

(%)

10^3 CFU/ml	Notx	Secondary metabolites (5 mg/ml)		
		Original	Celluclast extract	100% ethanol extract
Edwardsiella tarda	100	44±3.4*	37±4.7*	87±6.7*
Streptococcus iniae	100	31±5.5*	29±6.4*	92±4.6*
Vibrio harveyi	100	36±3.3*	22±4.1*	89±8.0*

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3. Discussion

The present study first demonstrates that the polysaccharide component of *E. cava* is a potential prebiotic via enhancing the growth of probiotics such as LAB and inhibiting the growth of pathogen bacteria.

E. cava fermented by LAB probiotics including namely *Lactobacillus brevis*, *Saccharomyces cerevisiae*, and *Candida utilis* have biological capacities on immune response and oxidative stress such as anti-inflammatory and anti-radio-protective effect (Lee et al.). In addition, the previous our studies showed that the fermentation processwithLAB probiotics increased the extraction yields and the protein contents in *E. cava* (Lee et al.). We also indicated the increased protein contents might be increased LBA products (Lee et al.). Interestingly, here, we showed that the original *E. cava* enhanced the growth of all LAB including *L. brevis, L. casei*, *L. delbrueckii ssp. Bulgaricus*, *L. pentosus*, *L. plantarum,* and *L. reuteri*, in comparison with non-treated control. In particular, the original *E.* cava highly enhanced the growth of *L. brevis* and *L. plantarum*. These results indicate the protein contents increased by the fermentation process were corresponded to the increased growth of LBA products. Also, the application of enzymatic extraction technique markedly improved the extraction yields in all the enzymatic extracts of *E. cava*, compared to its aqueous extract (Lee et al.). In this study, the aqueous and enzymatic extracts showed the higher effects on the growth of LBA products, compared to the ethanol extracts. Moreover, the Celluclast extract of *E. cava* showed the highest growth effect on *L. plantarum* compared to those of the others. At these points, this suggests that the extraction yields improved by the enzymatic extraction process in the fermented *E. cava* might be due to the increased growth of LBA products. This

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also indicates the possibility of any hydrophilic component with the high molecular weight might be an important factor for the growth of *L. plantarum* as supported by the higher growth effect of the *E. cava* enzymatic extract on LBA products than its ethanol extracts.

Normally, seaweed fibre is a common constituent of the diet in Southeast Asia, whereas the Western world eaten raw or incorporated in food as emulsifiers and thickeners (Warrand et al., 2006, Ramnani et al., 2012). Most in vitro and in vivo studies suggest its inert nature and low ferment ability by gut microbiota (Ramnani et al., 2012, Dierick et al., 2008, Michel et al., 1996, Salyers et al., 1977). Recent papers reported that natural seaweed fibres are predominantly high molecular weight polymers that pass through the gut too rapidly to allow bacterial utilization (Deville et al., 2007, Warrand, 2006, Ramnani et al., 2012). Owing to the structural complexity, seaweed polysaccharides are resistant to degradation by gut bacteria (Ramnani et al., 2012). With these points, various saccharide compounds of seaweeds have potential possibilities as prebiotics (Ramnani et al., 2012, Grimoud et al., 2010, Gibson and Roberfroid, 1995, Teitelbaum and Walker, 2002, Ventura et al., 2009, Gibson, 1998). Our results showed the > 30 kDa fraction of the *E. cava* Celluclast extract enhanced the growth effect on *L. plantarum* at all used concentrations than those of the < 30 kDa fraction. Also, the crude polysaccharide isolated from the > 30 kDa fraction markedly and dose-dependently increased the growth of *L. plantarum*.This result indicates the major active compound for the growth of LAB is a polysaccharide component. As indicated above, the crude polysaccharide might be a natural seaweed fibre as a high molecular weight polymer that passes through the gut too rapidly to allow bacterial utilization.

Here, we showed that the original *E. cava*, its Celluclast extract, and/or 100% ethanol extracts have the anti-bacterial activities on five pathogen bacteria including *E. tarda, S. iniae, V. anguillarum, S. parauberis*, and *V. harveyi*. The pathogen bacteria used in this study are known as representative bacteria causing the aquaculture outbreaks of infectious diseases.

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Recently, the aquaculture outbreaks of infectious diseases caused by viruses, bacteria, and parasites are considered to be a major economic problem, inflicting severe loss on fish production in Southeast Asia (Go, 2006). Among them, the use of antibiotics orchemotherapeutics for therapy of microbial diseases in fish is costly, difficult, not particularly effective and may createenvironmental hazards (Immanuel et al., 2004; Kim et al.,2002; Kwon et al., 2002; Sivaram et al., 2004). When we considered the problems, *E. cava* might be a beneficial prebiotic via inducing the growth of LBA products and inhibiting the growth of the pathogen bacteria without side effects. In addition, the secondary metabolites produced by the original *E. cava* from the *L. plantarum*-contained MRS medium showed the anti-bacterial activity on the pathogen bacteria. Recent studies reported that LAB havebeen used in both artificial feed and live feed (Robertson et al., 2000), such asArtemia and rotifers (Harzevili et al., 1998; Planas et al., 2004).In the previous study, a diet containing live *L. plantarum* was shown tohave some positive effects on growth and feeding efficiency, as well assurvival rate, in olive flounder (Go, 2006). This suggests that the enhancement of probiotics and the production of the secondary metabolites induced by *E. cava* and their anti-bacterial activities supported to be the possibility as a prebiotic. Also, previous our study showed that *E. cava* led to the immune activation and proliferation/differentiation of immune cells (Ahn et al., 2013). With these results,*E. cava* might be a potential prebiotic showing some positive effects on growth and feeding efficiency, as well assurvival rate, in olive flounder.

Taken together, this study suggests that *E. cava* consisted of a polysaccharide ofmight be a natural prebiotic via enhancing LAB and inhibiting pathogen bacteria. Also, our conclusion from this evidence is that *E. cava* can be used and applied as a useful prebiotic.

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Part II

Effect of *Ecklonia cava* on the growth and host immune response in *in vivo* system

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ABSTRACT

Olive flounder also known as Japanese flounder in Japan is one of the commercial important marine finfish species cultured inKorea and Japan. The feed used for the flounder requires high prime cost and arisesenvironmental pollution and many differentstresses and diseases. The purpose of this study is to evaluate whether *Ecklonia cava* (*E. cava*) as a prebiotic via inducing the growth and production of secondary metabolites of lactic acid bacteria leads to the growth and host immune response of olive flounder. The result showed that fish fed a diet supplemented with 1 % of the original *E. cava*increased the growth and the body weight of Olive flounder. Also, the supplements of the 1% original *E. cava* and the 0.5% EPE decreased the mortality of oliveflounder, whereas did not show a significant difference in the biochemical profile. In addition, the supplements of the 1% original *E. cava* showed the highest respiratory burst, lysozyme, and myeloperoxidase activity in oliveflounder. Further experiment showed the supplements of the 1% original *E. cava* and 0.1% and/or 0.5% EPE markedly decreased the accumulative mortality of oliveflounder infected by pathogen bacteria (*Edwardsiella tarda, Streptococcus iniae, Vibrio anguillarum*). Taken together, these results suggest that *E. cava* as a prebiotic via the enhancing the growthof probiotics including LAB and inhibiting the growth of pathogen bacteria led to the host immune response in fish injected by pathogen bacteria.

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1. Introduction

During the last 15 years, cultured fish production has become more than double and marine aquaculture industries in South Korea produced a number of fish species (Cha, 2010, Harikrishnan et al., 2010).Among the cultured fish, olive flounder also known as Japanese flounder in Japan, *Paralichthysolivaceus* is one of the commercial important marine finfish species cultured inKorea and Japan and its culture production has increased rapidly since thelate 1980's in Korea, reached to 34,533 tons in 2003 (Statistical Year Book ofMinistry of Maritime Affairs and Fisheries, 2003). Recently,in the aquaculture system, many new problems have been arising:environmental pollution (Kikuchi et al, 2000), along with many differentstresses and diseases (Murata et al., 1996), lower marketing value (Nakagawaand Kasahara, 1986). In particular, the aquaculture outbreaks of infectious diseases caused by viruses,bacteria, and parasites are considered to be a major economicproblem, inflicting severe loss on fish production in Southeast Asia (Harikrishnan et al., 2010).Among these infectious diseases, bacteria diseases of the cultured fishhave frequently affected to economic losses (Toranzo et al., 2005). In the aquaculture of olive flounder, most bacterial diseases are caused by distinct pathogen bacteria, such as *E. tarda, Streptococcus* sp. and *Vibrio* sp. (Go, 2006). *Edwardsiella tarda* is the etiological agent of several diseases of freshwater andmarine fish, causing septicemia with extensive skin lesions and affectingmuscles and internal organs, such as the liver, kidney and spleen (Plumb,1993). Streptococcal infection of fish is considered a reemerging disease affecting avariety of wild and cultured fish throughout the world, and should beregarded as a complex of similar diseases caused by different genera andspecies capable of inducing central nervous damage characterized bysuppurative exophthalmia and meningoencephalitis (Toranzo et al., 2005). Pathogenic *Vibrio* sp., the etiological agent of classical Vibriosis, iswidely distributed and causes a typical hemorrhagic

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septicemia in a widevariety of warm- and cold-water species of economic importance (Toranzo etal., 2005). At these points, many researchers have studied about the treatmentand relief using antibiotics or chemotherapeutics on the serious disease caused by the bacteria (Immanuel et al., 2004; Kim et al.,2002; Kwon et al., 2002; Sivaram et al., 2004), but antibiotic use in aquaculture may be detrimentalto the environment and human health, and involves thedevelopment and transfer of resistance to other aquatic bacteria Petersen, fish pathogens, human pathogens, and theaccumulation of residual antibiotics in aquaculture products (Petersen et al., 2002, Alcaide et al., 2005, Schmidt et al., 1999, WHO, 1999, Huys et al, 2007, Cabello et al., 2006, WHO, 2006).

Brown seaweed *AlariaceaeEcklonia cava(E. cava)* is rich in xanthopophyll, fucoxathin, vitamins, vitamin precursors such as α-tocopherol, β-carotene, niacin, thiamin and ascorbic acid, and polysaccharides such as fucoidan, alginates, fucans and laminarans, which are water-soluble dietary fibers and phycocolloids. *E. cava* grows plentifully near Jeju Island of Korea (Guiry & Bulunden., 1991) and, reportedly, contains polyphenol and polysaccharide compounds than other brown seaweeds (Heo et al., 2005a). A number of studies have been evaluated in an attempt tobiological activities of *E. cava*(Ahn et al., 2007, 2008a,b and 2010; Kim et al., 2006a; Athukorala et al., 2006 and 2009; Kim et al. 2006b). In particular, previous studies have showed *Lactobacillus brevis*-fermented *Ecklonia cava* (*E. cava*) has anti-inflammatory, radio-protective and anti-oxidant effects. In addition, the fermented *E. cava* using bacteria*Lactobacillus brevis* increased the protein contents in comparison with the non-fermented *E. cava* (Lee et al., 2011, 2013). From these results, we thought that the protein contents increased by fermentation process might be related to the growth of *Lactobacillus brevis* induced during fermentation process. Normally, *Lactobacillus brevis* isa kind of lactic acid bacteria (LAB) known as a common type of probiotics that may confer a health benefit on the host. In the last decade, probiotics, especially lactic acid bacteria (LAB), as a dietary

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supplement, have been widely employed to protect fish from various infectious diseases (VerschuereGatesoupe, 2000).Many researchers have reported that LAB probiotics have been shown to be effective against edwardsiellosis, furunculosis, and vibriosis (Gatesoupe, 1999, Gildberg and Mikkelsen, 1998, Nikoskelainen et al., 2001). In addition, LAB probiotics havebeen considered safe for food fish and their immunological response against harmful pathogens ininhibiting the growth of Aeromonas salmonicida, Vibrio anguillarum,and Flavobacterium psychrophilum has been proven in rainbow trout (Gildberg and Mikkelsen, 1998, Nikoskelainen et al., 2001).With these reports, recently, the application of probiotics is increasinglyused in disease control against fish pathogens (Brunt and Austin, 2005, Kim and Austin, 2006, Brunt et al., 2007, Pieters et al., 2008). The materials enhancing the growth of LAB probiotics are known as prebiotics. This means applying the LAB probiotic as an additive of feeds for olive flounder with pathogen bacteria diseases and evaluating the possibility of *E. cava* as a beneficial prebiotic on the growth of LAB are so valuable.

Therefore, the present study investigated whether the capacities of *E. cava* as a prebiotic via the enhancing the growth of probiotics including LAB and inhibiting the growth of pathogen bacteria such as *Edwardsiella tarda, Streptococcus iniae* and *Vibrio harveyi* affect to the host immune response in fish injected by pathogen bacteria.

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2. MATERIALS AND METHODS

2.1. Preparation of samples

Ecklonia cava(*E. cava*) was collected from the coast of Jeju Island, South Korea. Then, they were respectively washed with fresh water, freeze-dried and pulverized into powder with a grinder. The freeze-dried samples were used for next experiments.

2.2. Preparation of ethanol extracts from *E. cava* (EPE)

Here, 100% ethanol extracts (EPE) were prepared from *E. cava*. After freeze-dry, the*E. cava*powders (1 g) were respectivelyhomogenized in 100% ethanol and aqueous (100 ml). After 24 h, the samples were obtained and kept at -20°C for further experiments.

2.3. Pathogen bacteria culture

Six kinds of pathogen bacteria such as*Edwardsiella tarda, Streptococcus iniae, Vibrio anguillarum, Vibrio harveyi* and *Streptococcus parauberis* was obtained from the KCTC (Korean Collection for Type Culture, Daejeon, Korea) (Table 2-1). These bacteria were incubated in BHIA broth or agar for *Edwardsiella tarda, Streptococcus iniae, Vibrio anguillarum,* and *Streptococcus parauberis*and TSA broth or agar for *Vibrio harveyi*at 25°C until use.

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Table 2-1 List of strains used of antibacterial experiment.

	Strains	Number
Bacteria	*Edwardsiella tarda*	KCTC 12267
	Streptococcus iniae	KCTC 3657
	Vibrio harveyi	KCTC 12724

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2.4. Preparation of the original *E. cava*, EPS, and *L. plantarum*supplemented diets

The basic experimental diet was in the form of a commercial extrudedpellet (Daebong, Jeju, Kore). The original *E. cava* and EPE were added to the diet at 0.1, 0.5 or 1 %. The commercial diet wassoaked in the desired concentrations of the original *E. cava* and EPE to absorb the fluid. The 1% of*L. plantarum* was mixed to the prepared all feed before feeding and the diets with the probiotic were then fed to all fish twice daily for 16 wks.

2.5. Fish

Olive flounder (mean body weight 300~350 g) were purchased from a private hatchery (Geumdeung, Jeju Island, South Korea), transferred into the laboratory and acclimated for one week. Cylindricalpolypropylene (PP) tanks (8 m in diameter, 1.5 m in height)were used asexperimental tanks. Each tank was stocked with 1,800 olive flounder, andthe water was changed 10-15 times a day before an initiation of the samples supplemented feeding trial. Seawater temperature (16~18°C), dissolved oxygen content (8.23~9.37 mg/l), and salinity (27.68~31.36 ppt), pH (7.5~8.7) during the experimental feeding period were maintained, respectively. During the experimental period (16 wks), fish were fed with commercially extruded pellet (Suhyup Feed Co. Ltd., Korea) twice a day (06:00 and 17:00 h).

Experimental group were divided into four groups following; control group was fed containing the only 1% *L. plantarum*,group I was fed containing the 1% *L. plantarum* with 1% original *E. cava*, group II was fed containing the 1% *L. plantarum* with 0.1% EPE, and group III was fed containing the 1% *L. plantarum* with 0.5% EPE.

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2.6. Body weight and mortality measurement

At the conclusion of the rearing experiment, fish from the experimentalgroups were fed a diet supplemented with 1 % of the original *E. cava* and 0.1 and 0.5% of EPE for 16 wks. Then, the body weight and mortality rate were checked at every 4 wks for 16 wks.

2.7. Blood sampling

At the conclusion of the rearing experiment, fish from the experimentalgroups were fed a diet supplemented with 1 % of the original *E. cava* and 0.1 and 0.5% of EPE. At 16 wks, blood samples (0.5 ml, n ¼ 6) were drawn via the caudal vessels,punctured using a heparin-coated needle and syringe. Heparin was used as an anticoagulant. The collected blood was allowed to clot at a room temperature for 30 min and then kept at 4°C for 3 hrs. The clotted sample was centrifuged at 3000 rpm for 10 min at 4°Cto collect the serum, which was analyzed within 16 hrs. Individual fish weresampled only once to avoid affecting the assays with multiplebleeds and handling stress in the fish.

2.8. Physiological and chemical analysis of blood

To identify the physiological and chemical analysis in serum, total protein (TP), tryglyceride (TRIG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose (GLU), phosphorus (PHOS),cholesterol (CHOL), and hematocrit (HT) were examined using an automatic analyzer (Boehringer Mannheim, Mannheim,Germany).

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2.9. Nitroblue tetrazolium reduction analysis (NBT)

To evaluate efects of the original *E. cava* and EPEon the production of intracellular oxidative radicalby neutrophils in blood during respiratory burst, nitroblue tetrazolium (NBT)was evaluated according to Anderson and Siwicki (1994) with a slight modification. At the conclusion of the rearing experiment, fish from the experimentalgroups were fed a diet supplemented with 1 % of the original *E. cava* and 0.1 and 0.5% of EPE. At 16 wks, blood samples were collected. Briefly, blood samples were mixed with0.2% NBTcontaining 1 μg/ml PMA in equal proportion (1:1), and incubated for 30 min at a room temperature. Then 50 μlof the mixture was taken out and dispensed in ependorff tubes. For solubilisation of reduced formazen product, 1 ml of dimethyl formamide (DMSO, Sigma, USA) was added and centrifuged at 2000 rpm for 5 min. Finally, the supernatant was collected and the extent of NBT reduction was measured at an optical density of 540 nm using a micro-reader (Packard spectrocount^{TM}, Austria). DMSO was used as the blank.

2.9. Lysozyme activity

At the conclusion of the rearing experiment, fish from the experimentalgroups were fed a diet supplemented with 1 % of the original *E. cava* and 0.1 and 0.5% of EPE. The lysozyme functions by attacking peptidoglycans (found in the cell walls of bacteria, especially gram-positive bacteria) and hydrolyzing the bond that connects N-acetylmuramic acid with the fourth carbon atom of N-acetylglucosamine. Lysozyme can act to as an innate opsonin, or as an activity of lytic enzyme. At 16 wks, a turbidometric assay utilizing lyophilized *Micrococcus lysodeikticus*(*M. lysodeikticus*) cells (Sigma, USA) was used to determine

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lysozyme activity with a slight modification of previously suggested methods(Kumari and Sahoo, 2005, Cha, 2010). *M. lysodeikticus*suspended in 0.02 M sodium citrate buffer (pH 5.5) at a concentration of 0.2 mg/ml was added to 15 μl of serum in a 96-well microtitre plate. Immediately after adding 150 μl of*M. lysodeikticus*, optical density was taken. The absorbance was measured at every 5 min intervals for 60 min at 450 nm. A unit of lysozyme activity was defined as the amount of sample causing a decrease in absorbance of 0.001/min.

2.10. Myeloperoxidase (MPO) assay

At the conclusion of the rearing experiment, fish from the experimentalgroups were fed a diet supplemented with 1 % of the original *E. cava* and 0.1 and 0.5% of EPE. At 16 wks, plasma was obtained from prechilled heparin anticoagulant tubes. MPO analysis was carried out using a Bioxytech enzyme-linked immunoassay for fish MPO. Samples were incubated in the each well of a microtiter plate coated with a monoclonal antibody against MPO. A 100μl of the sample (1:15 dilution of plasma) or standard was incubated at 37°C for 2 hrs. Then, the wells were aspirated and washed, and MPO polyclonal antibody (100 μl) was added to each well. The plate was again incubated at 37°C for 2 hrs. The wash procedure was repeatedly performed, avidin-coupled alkaline phosphatase solution (100 μl) was added, and the plates were further incubated for 2 hrs. The wash procedure was repeated a third time, and *p*-nitrophenyl phosphate (100 μl) was added to each well. The plate was reacted at 37°C until the absorbance of the highest standard reached approximately 2.5. Finally, stop solution (50 μl) was added to each well, and the absorbance was measured at 405 nm using amicro-reader (Packard spectrocount^{TM}, Austria).

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2.11. Challenge test

Fish were divided into four groups of 30 each in three replicate groups. The groups were given dietary supplementation with the 1.0% of original*E. cava* and the 0.1% and 0.5% of EPE. On 16 wks of feeding, all groups were injected intraperitoneally (i.p.) with 100 μl PBS containing *Edwardsiella tarda, Streptococcus iniae, andVibrio harveyi*at 3×10^5 ciliates mL^{-1}. Another group of 30 fish were used for uninfected control fed with basal diet (C). The respective diets were continued at end of the experiment. On day 1post-challenge, six fish randomly collected from each tank and anaesthetized with MS-222 ($NaHCO_3$ and tricaine methanesulphonate; Sigma Chemicals) 1:4000 in dechlorinated water for 2 min. The cumulative mortality was calculated by the following formula of Amend (1981) at every day.

2.12. Polymerase ChainReaction (PCR)

Kidney and liver tissue of pathogen bacteria-injected oliveflounder were used for PCR. To identify of specific regions of *Vibrio harveyi,Edwardsiella tarda* and *Streptococcus iniae*, PCR of this cDNA purified from kidney and liver and the primer (Bioneer, Daejeon, South Korea),shown in Table 2-2, was performed for 40 cycles witha 5-min denaturing step at 94°C, a 1min annealingstep at 55 to 60°C, and a 20min extension phase at72°C using the TaKaRa PCR machine (Takara Bio,Shiga, Japan). PCR products were run on a 1.5% EtBr/agarose gel and visualized by UV transillumination.

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Table 2-2 Primer sets used in the present study for the detection of three bacteria.

Primer	Oligonucleotide sequence (5'-3')	Expected sizes	Target gene	Reference
VH-K1	CAAGGCACACTTGACGAGCT	638bp	*rpoB*	Oh(2008)
VH-K2	AAACCGCCTTCTTCAGTTAA			
etfD-F	GGTAACCTGATTTGGCGTTC	445bp	etfD	Castro et al. (2010)
etfD-R	CCTAATTCTGTAATCGCTCC			
LOX-1	AAGGGGAAATCGCAAGTGCC	870bp	*lctO*	Mata et al. (2004)
LOX-2	ATATCTGATTGGGCCGTCTAA			

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2.13. Statistical analysis

Data was analyzed using the statistical package for the social science (SPSS) package for Windows (Version 10). Values were expressed as means ± standard error (SE). A *p*-value of less than 0.05 was considered significant.

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3. RESULTS

3.1. The original *E. cava* induced the growth of oliveflounder as increasing the body weight

The growth performance of fingerling flounder fed various experimentaldiets for 16 wks is shown in figure 2-1.The body weight (g) of fish was measured every 4 wks for 16 wks. The final weight of fish fed dietscontaining the 1% original *E. cava*was higher than that of fish fed adiet only*L. plantarum*-treated control (Fig. 2-1). However, fish fed dietscontaining with the 0.1% and 0.5% EPEs have no significant difference compared with non-treated control (Fig. 2-1). These results showed that the supplement of original *E. cava* effectively improved the growth of oliveflounder.

3.2. The supplements of the 1% original *E. cava* and the 0.5% EPE decreased the mortality of oliveflounder

To access the effects of the 1% original *E. cava*and its 0.1% and 0.5% EPE on oliveflounder, the number of mortality of fish was measured at every 4 wks for 16 wks. The number of mortality in fish fed a control diet was markedly increased up to 400% at 4 wks and gradually decreased up to 16 wks (Fig. 2-2). Also, the supplementations of the 1% original *E. cava* and the 0.1% EPEshowed the similar pattern to fish fed a control diet, but the number of mortality of fish feddiet supplemented with the 1% of original *E. cava*and the 0.1% EPEdecreased the number of mortality in fish fed a control diet. However, the supplementations of the 0.5% EPEdid not show significant changes.

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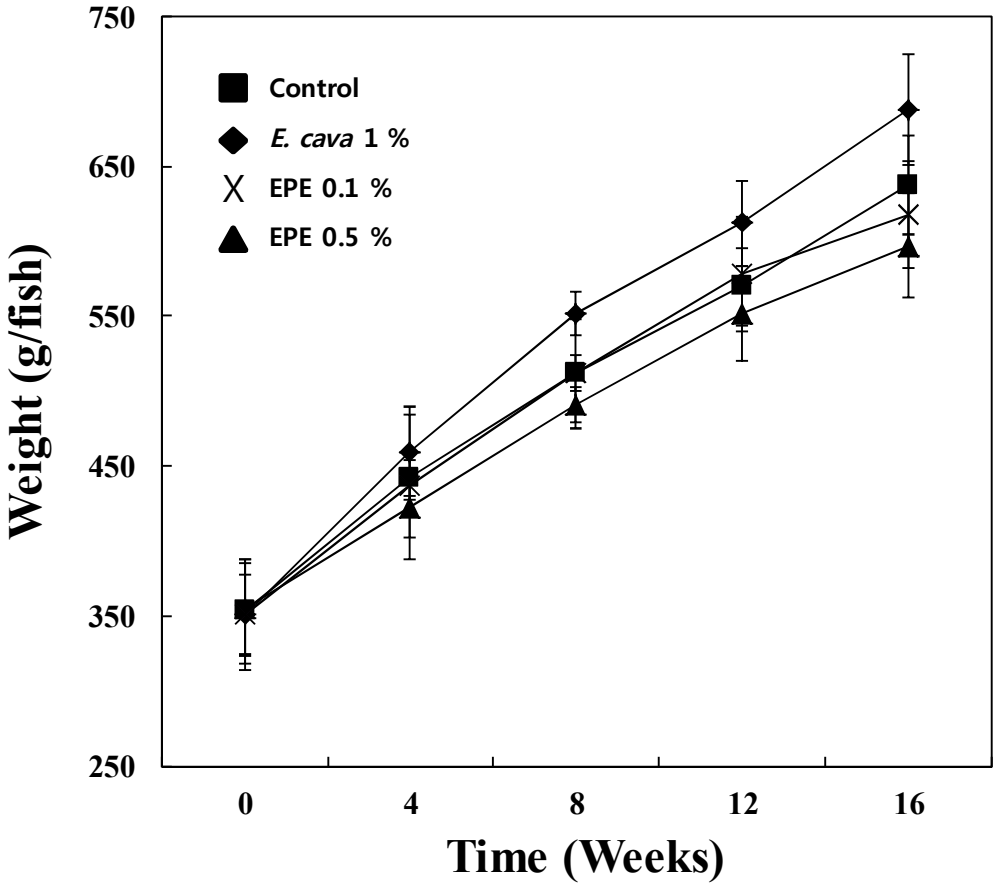

Figure.2-1.

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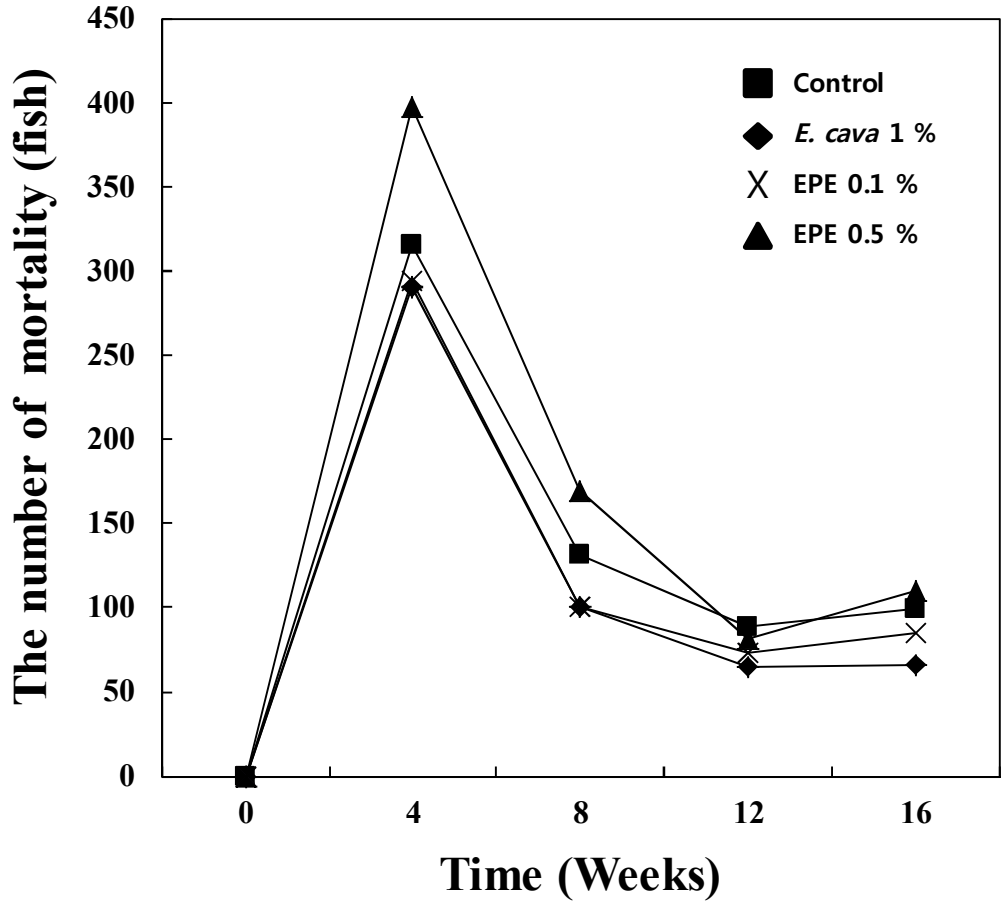

Figure.2-2.

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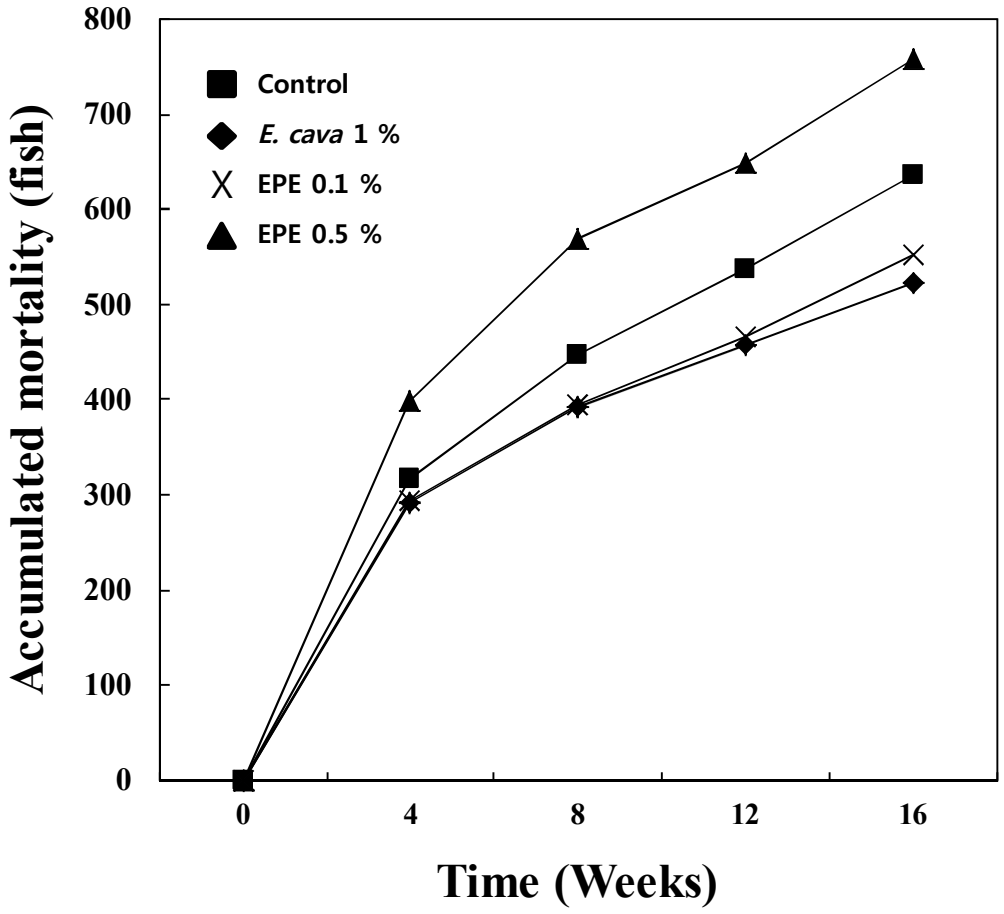

Figure.2-3.

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Moreover, the accumulated mortality of fish fed a control diet was markedly increased up to approximately 750% at 16 wks, whereas the supplement of the 1% original *E. cava*and the 0.1% EPE markedly decreased the mortality of fish (Fig. 2-3).

3.3. The supplements of the 1% original *E. cava* and the 0.1% and 0.5% EPE did not significant difference in the biochemical profilein oliveflounder

To access the biochemical profileof the 1% original *E. cava*and its 0.1% and 0.5% EPE on oliveflounder, the serumof fish was used at 16 wks. To identify the physiological and chemical analysis in serum, total protein (TP), tryglyceride (TRIG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose (GLU), phosphorus (PHOS), cholesterol (CHOL), and hematocrit (HT) were examined.The biochemical profile such as TP, TRIG, AST, ALT, GLU, PHOS,CHOL, and HT levels did not significantly change on 16 wks in fish fed with any diet (Table 2-3).

3.4. The supplements of the 1% original *E. cava* showed the highest respiratory activityin oliveflounder

At the conclusion of the rearing experiment, fish from the experimentalgroups were fed a diet supplemented with 1 % of the original *E. cava* and 0.1 and 0.5% of EPE. At 16 wks, the superoxide anion production of respiratory activity was measured.

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Table 2-3 2-3 The physiological and chemical analysis in blood.

Diet	TP (g/dL)	TRIG (mg/dL)	AST (U/L)	ALT (U/L)	GLU (mg/dL)	PHOS (mg/dL)	CHOL (mg/dL)	HT (%)
Control	5.18 ±0.26	111.67 ±31.79	59.83 ±15.02	16.33 ±5.85	22.33 ±3.61	9.72± 0.66	175.17±34.44	17.95 ±2.39
E. cava 1 %	5.32 ±0.22	130.83 ±21.30	52.00 ±16.47	16.00 ±5.22	18.33 ±7.33	9.72 ±1.52	189.20 ±28.32	23.02 ±2.01
EPE 0.5 %	5.05 ±0.29	100.50 ±41.07	55.67 ±11.13	19.17 ±6.77	16.33 ±4.84	6.33 ±0.63	146.83 ±40.06	22.17 ±2.81
EPE 0.1 %	5.12 ±0.33	128.75 ±38.15	51.60 ±9.71	15.80 ±12.97	20.00 ±3.03	10.45 ±1.64	183.00 ±25.37	21.75 ±1.94

TP - Total protein, TRIG - Tryglyceride, AST - Aspartate aminotransferase, ALT - Alanine aminotransferase, GLU - Glucose PHOS - Phosphorus, CHOL - Cholesterol, HT - Hematocrit

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As shown in figure 2-4, the supplementation with the 1.0% of original *E. cava* and the 0.1% and 0.5% of EPE considerably increased the superoxide anion production, compared to the fish fed a control diet (Fig. 2-4). In particular, the supplementation of 1% original *E. cava* led to the highest respiratory burst activity with the enhanced superoxide formation in olive flounder. These results indicated that the supplements of the 1% original *E. cava* and the 0.1% and 0.5% EPE enhanced the superoxide anion production of respiratory activity in olive flounder.

3.5. The supplements of the 1% original *E. cava* showed the highest lysozyme activityin oliveflounder

At the conclusion of the rearing experiment, fish from the experimentalgroups were fed a diet supplemented with 1.0% of the original *E. cava* and 0.1 and 0.5% of EPE. At 16 wks, the lysozyme activity was measured. The lysozyme activity was significantly enhanced in fish fedwith 1.0% of the original *E. cava* and 0.1 and 0.5% of EPE on week 16 (Fig. 2-5). In particular, fishfed a diet supplemented with 1.0% of the original *E. cava* showed the markedly highest lysozyme activity, compared to the others.

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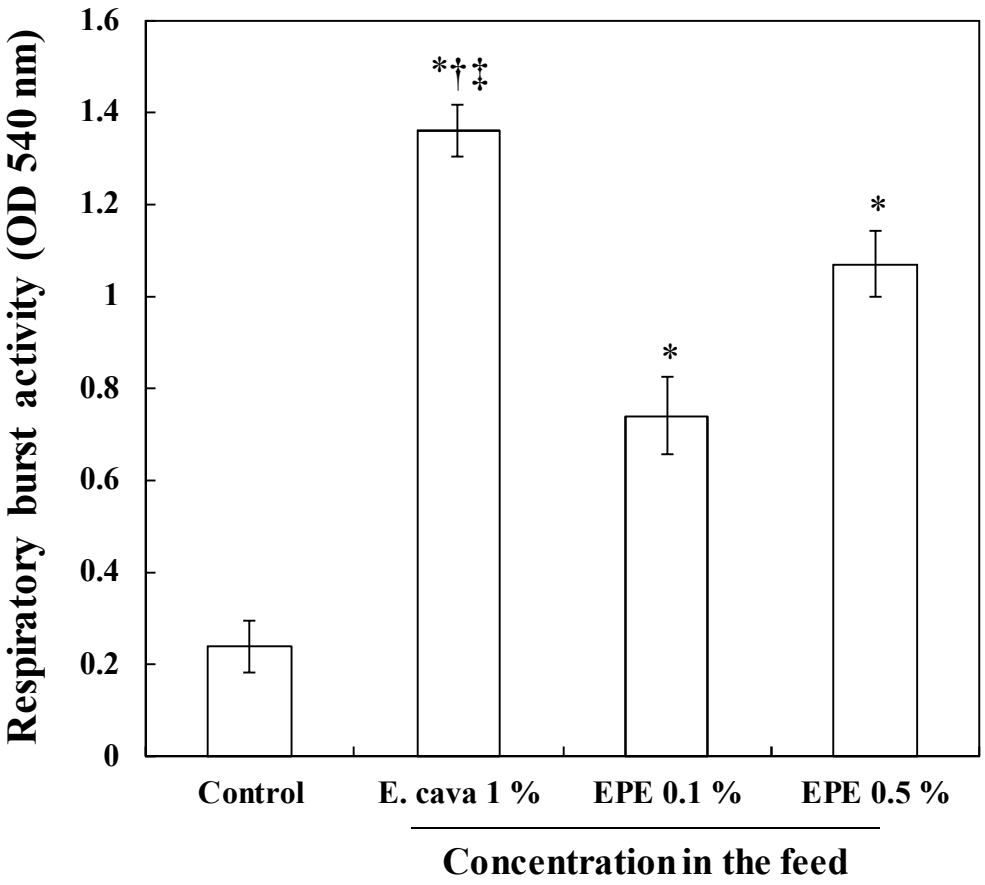

Figure 2-4.

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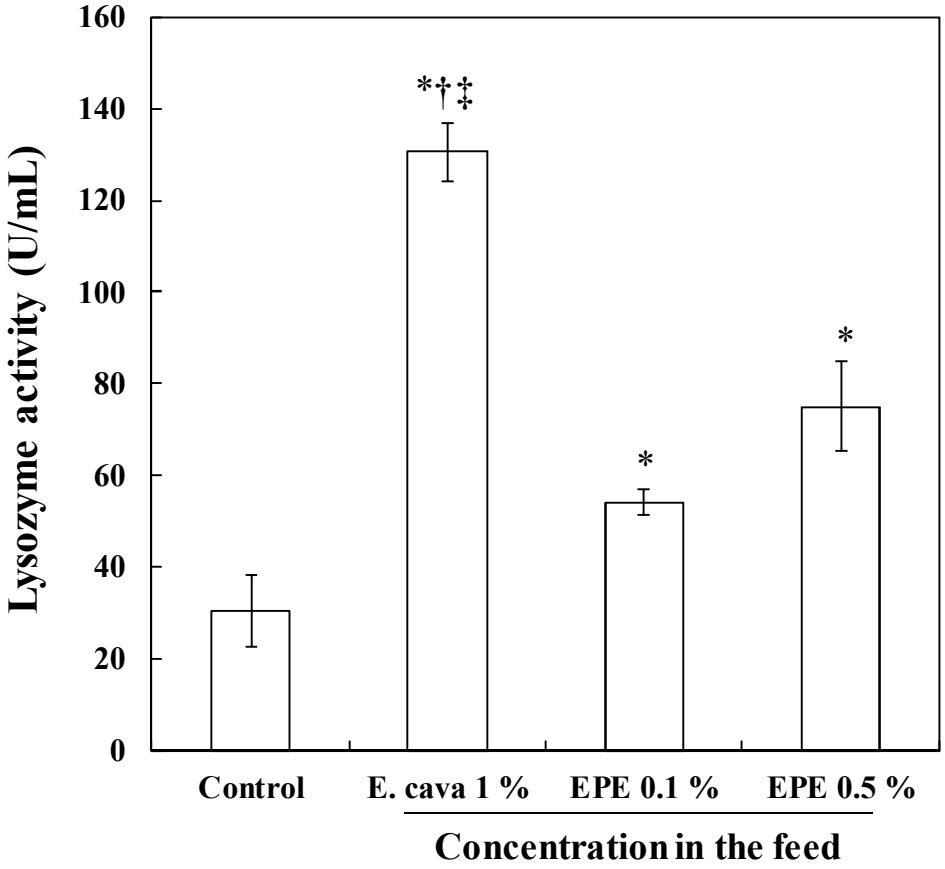

Figure 2-5.

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3.6. The supplements of the 1% original *E. cava* showed the highest myeloperoxidase activityin oliveflounder

At the conclusion of the rearing experiment, fish from the experimentalgroups were fed a diet supplemented with 1.0% of the original *E. cava* and 0.1 and 0.5% of EPE. At 16 wks, the myeloperoxidase activity was measured. The myeloperoxidase activity was significantly enhanced in fish fedwith 1.0% of the original *E. cava* and 0.1 of EPE on week 16, whereas did not show significant change in the fish fed with 0.5% EPE (Fig. 2-5). Particularly, fishfed a diet supplemented with 1.0% of the original *E. cava* showed the markedly highest myeloperoxidase activity, compared to the others.

3.7. The supplements of the 1% original *E. cava* and 0.1% and/or 0.5% EPE markedly decreased the cumulative mortality of oliveflounder

In the *in vivo* trial, mortality from *Edwardsiella tarda, Streptococcus iniae* and*Vibrio harveyi* in fish fed diets of 1.0% of the original *E. cava* and 0.1 and 0.5% of EPEwas accessed. First of all, to identify whether the pathogen bacteria (*E. tarda, S. iniae* and*V. harveyi*) were injected to fish, PCR assay was performed. Agarose gel electrophoresis showed the mRNA expression levels of specific regions of *V. harveyi,E. tarda* and *S. iniae*(Fig. 2-7).

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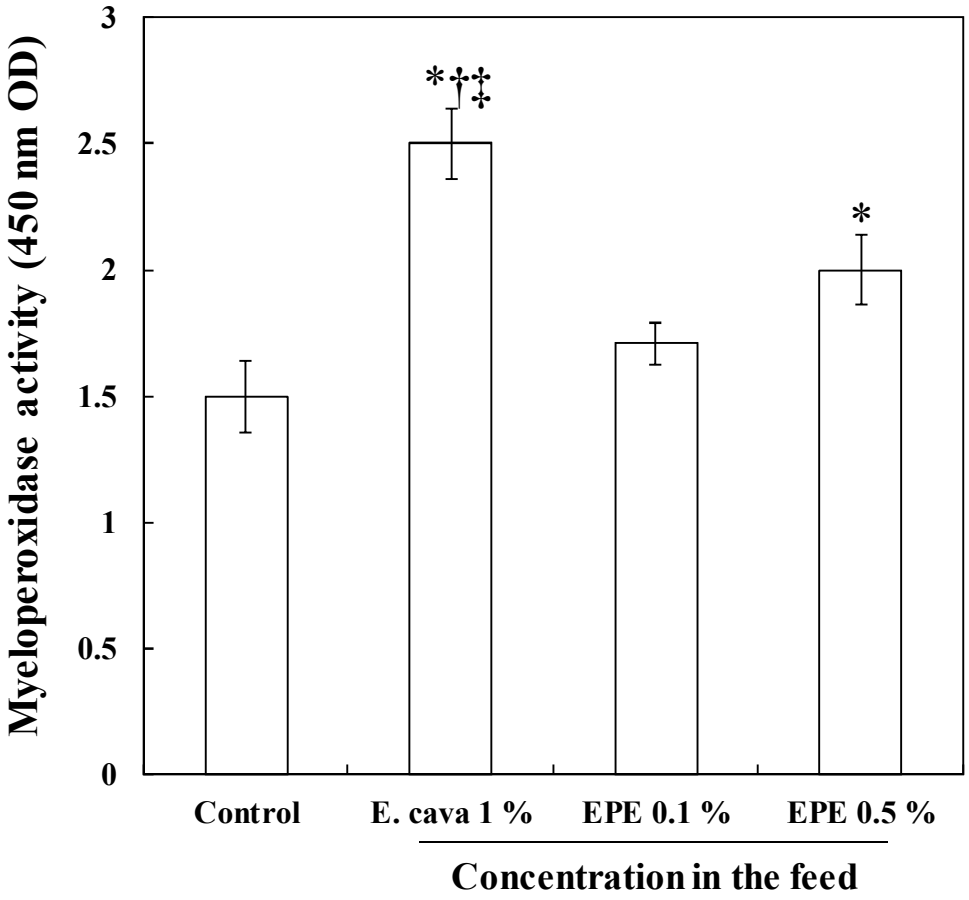

Figure 2-6.

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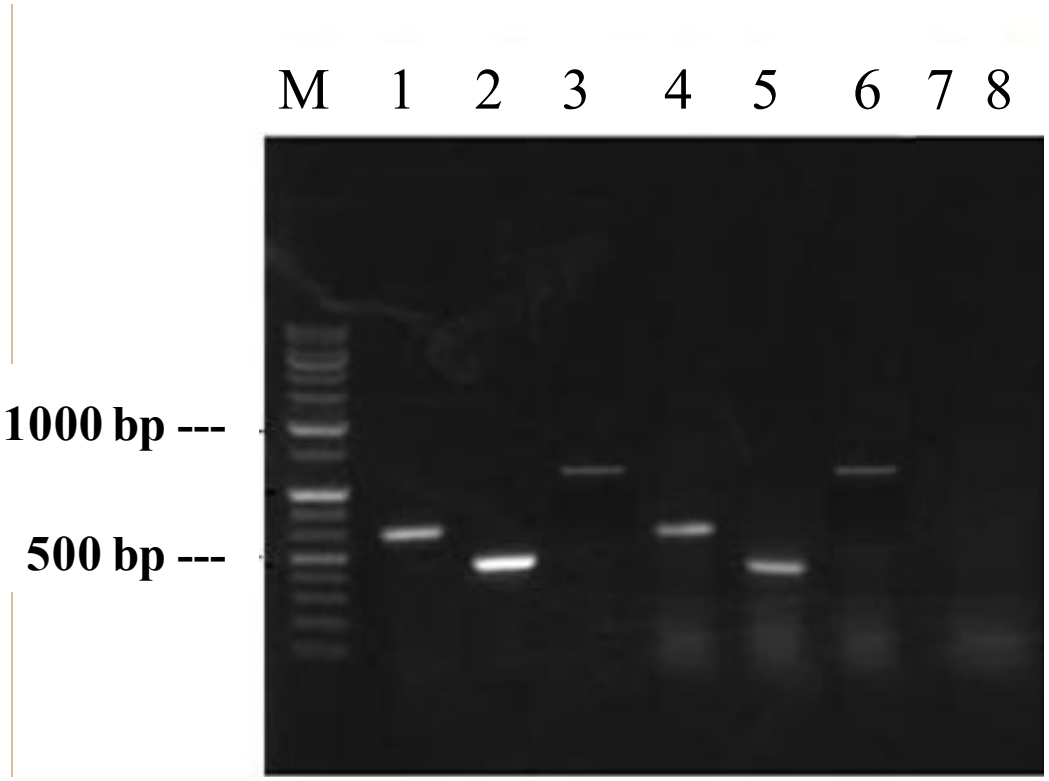

Figure 2-7.

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At the conclusion of the rearing experiment, fish from the experimentalgroups were fed diets supplemented with 1.0% of the original *E. cava* and 0.1 and 0.5% of EPE. The injection of *E. tarda, S. iniae* and*V. harveyi*markedly increased thecumulative mortalities of fish fed a diet without samples (Fig. 2-8, 2-9, and 2-10). When the *E. tarda*was injected to fish, the 1.0% of the original *E. cava* and 0.1 and 0.5% of EPE significantly decreased the mortality increased in a control diet fed and *E. tarda*injected fish (Fig. 2-8). Among them, the 1.0% of the original *E. cava* showed the highest reduction activity on the mortality increased in a control diet fed and *E. tarda* injected fish (Fig. 2-8). In addition, flounder infected with *E. tarda* were found to have heavyviscous liquid in the body, liver congestion, and abnormal kidney and spleengrowth, i.e., similar anatomical symptoms as naturally infected olive flounder. They were inhibited by the supplementations of the 1.0% original *E. cava* and 0.1 and 0.5% of EPE. Also, when the *S. iniae* was injected to fish, the 1.0% of the original *E. cava* and 0.1% of EPE significantly decreased the cumulative mortality increased in a control diet fed and *S. iniae*injected fish, whereas 0.5% of EPE did not affect (Fig. 2-9). Additionally, the supplementations of the 1.0% original *E. cava* and 0.1 % of EPE decreased the symptoms of fish infected with *S. iniae*such as skinulcers confined to scale-covered parts of the body surface, and often diffuseor petechial hemorrhage in internal organs. Moreover, when the *V. harveyi* was injected to fish, the 1.0% of the original *E. cava* and 0.1% of EPE significantly decreased the mortality increased in non-treated and *V. harveyi* injected fish, whereas 0.5% of EPE did not affect (Fig. 2-10). Furthermore, the 1.0% of the original *E. cava* and 0.1% of EPEimproved the symptoms of fish affected by *V. harveyi* with typical signs of generalized septicemia with hemorrhaging on the base of fins,exophthalmia, corneal opacity, and edematous lesions predominantly centeredon the hypodermis.

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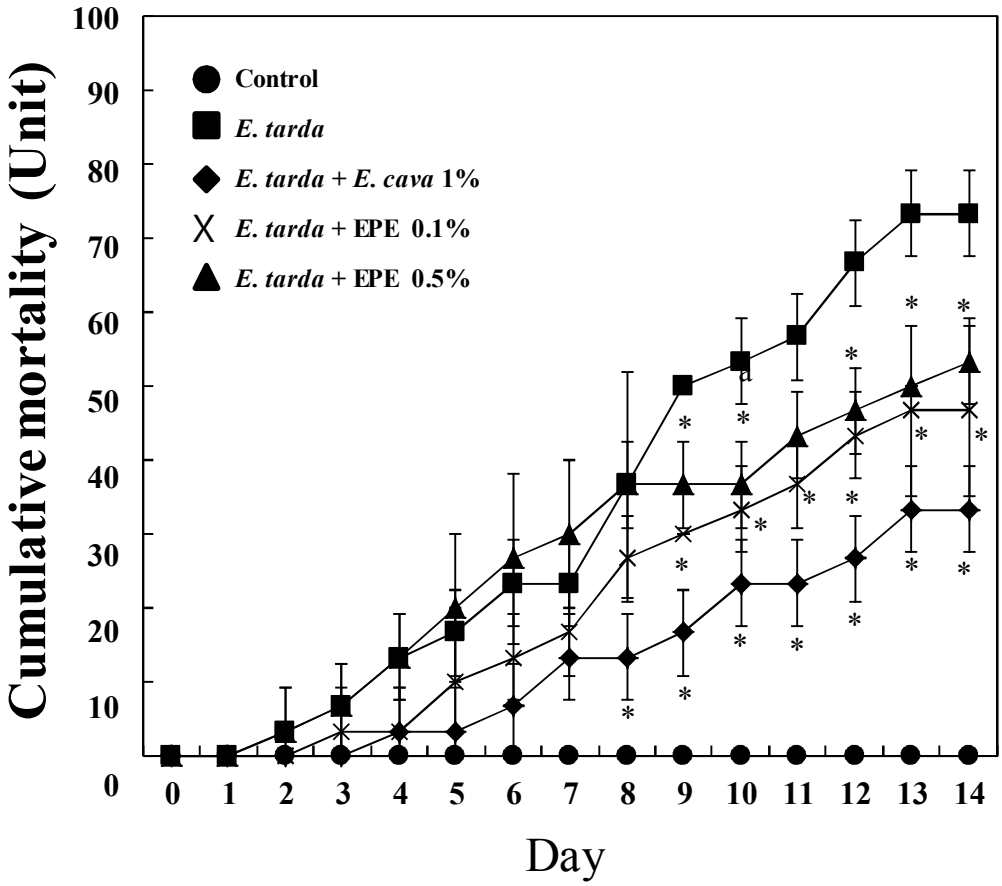

Figure 2-8.

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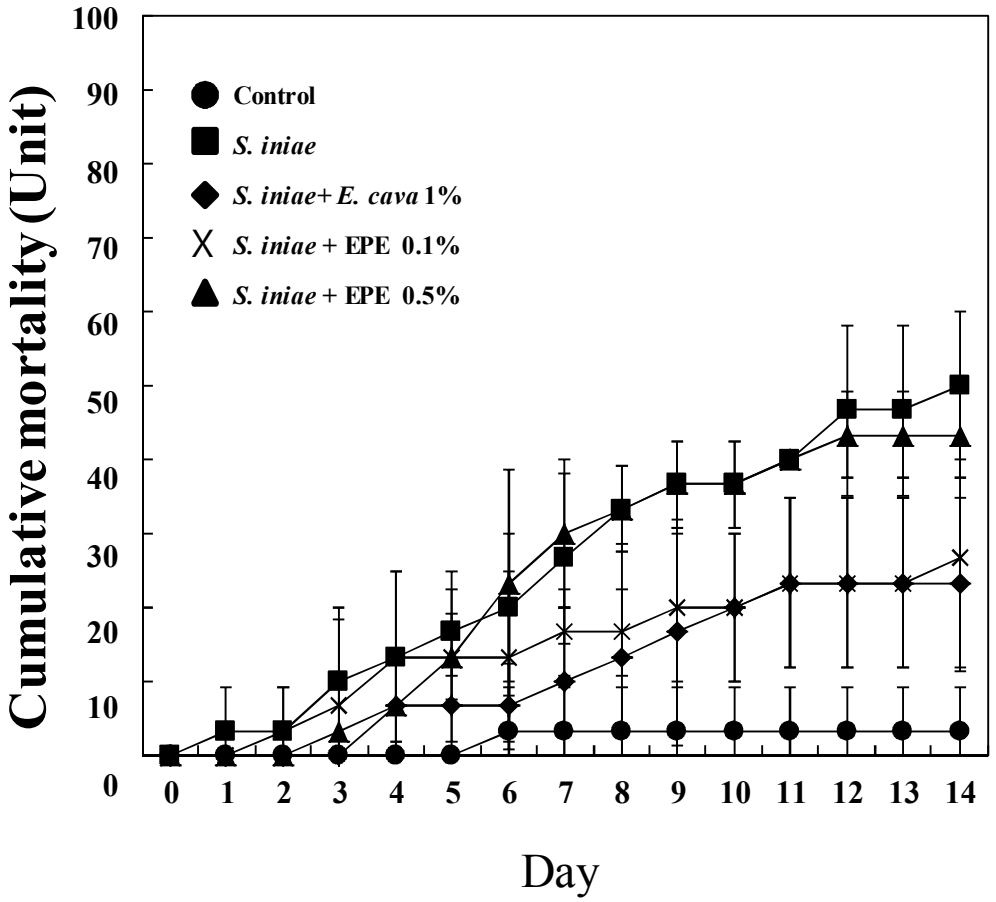

Figure 2-9.

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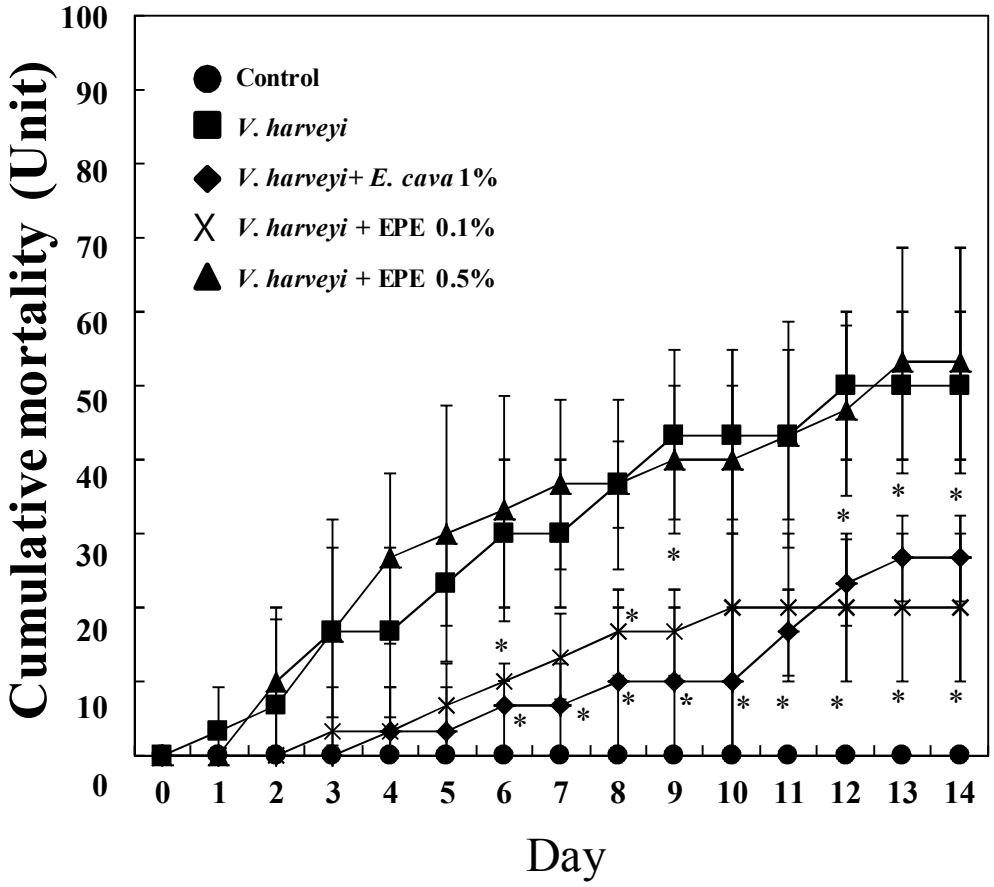

Figure 2-10.

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3. Discussion

The present study first demonstrates that theoriginal *E. cava* induced the growth of olive flounder as increasing the body and decreased mortality of fish. Also, *E. cava* showed the highest lysozyme, myeloperoxidase, and the accumulative mortality of olive flounder infected by pathogen bacteria. These effects were due to the beneficial capacity of *E. cava* as a prebiotic.

A number of approach for inhibiting fish diseases have been attempt with the use of probiotics or beneficial bacteria as dietary supplements which control pathogen bacteria through various mechanism. Indeed, the probiotics as a dietary supplement has been proposed as an alternative mode of improving the growth and health management of cultured organisms (Yano et al., 1992; Amar et al., 2000; Ellis et al., 1990). This has been well documented with several species with probiotics enriched diets such as grouper (Joborn et al., 1997, Miettinen et al., 1996, Panigrahi et al., 2004), tilapia (Nikoskelainen et al., 1997; Ringe et al., Robertson et al., 2000), sea bass (Cuesta et al., 2003, Siwicki et al.1994), and white shrimp (Ellis et al., 2001, Raida et al., 2003, Vendrell et al., 2008) against various pathogens. Our results indicated that the supplement of 1% original *E. cava* with*L. plantarum*effectively improved the growth and decreased the mortality of oliveflounder. Also, the supplement of 0.5% EPE affected to the decrease of the mortality of oliveflounder, but did not affect to the weight. The results indicate that the supplement of 1% original *E. cava*is more effective and valuable in the culture system of oliveflounder. Also, part 1 showed that *E. cava* enhanced the growth of LBA and the secondary metabolites produced from LBA after treatment of *E. cava* inhibited the growth of the pathogen bacteria by playing as role in a prebiotic. In addition, Fuller (1989) reported that the increase in growth rates can be attributed to the improvement in the intestinal microbial flora balance. These results suggest the co-

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application of both *E. cava*and LBA can induce the more beneficial effects on the improvement of the growth and mortality in oliveflounder. At these points, this study is a valuable as a first study.

Normally, the innate immune system of fish is the first line and primitive of defense against pathogen bacteria in aqua culture system (Harikrishnan et al., 2010). In innate immune system, a variety of immune cells or elements, such as macrophages, monocytes, granulocytes,and humoral elements, such as lysozymes and thecomplement system play as important roles (Secombes and Fletcher, 1992, Magnadóttir, 2006). One proposedmechanism by Cross (2002) is that the regulatory signals generated by probiotics stimulate host immunity to enhance protection against pathogens. Recently, prebiotics also can affect to the innate immunity in *in vitro* and *in vivo* (Sweeney et al., 2011, Ramnani et al. 2012, Nakayama and Oishi, 2013). In addition, modulating immune responses with probiotic bacteria havebeen shown to have several effects in a variety of fish, such asinduction of proinflammatory cytokines, stimulating the activity ofnatural killer cells, increasing mucosal and systemic antibodyproduction, activating phagocytic activity and increasing lysozymeand complement activity (Harikrishnan et al., 2010, Matsuzaki and Chin, 2000, Miettinen et al., 1996, Panigrahi et al, 2004). Generally, the inflammatory response induced by the influx of phagocytesto the infection site is a potential innate defense mechanism of fishagainst pathogens (Ellis, 2001).Phagocytes produce toxic oxygen forms during respiratory burst (Raida et al., 2003), which has been accepted as an accurate parameter to quantifythe intensity of a respiratory burst (Secombes, 1990). In fish, phagocytic cells, such as monocytes/macrophages and/or neutrophils can generate superoxide anion, which is a measure of respiratory burst activity, and affect to kill pathogen (Ellis, 2001, Jørgensen et al., 1993, Sharp and Secombes, 1993). Also, the phagocytic cells can induce the induction of

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serum lysozyme known as a kind of obsonin that leads to bactericidal activity and activates the complement systems and phagocytes (Fletcher and White, 1973, Murray and Fletcher, 1976, Jolles and Jolles, 1984). Myeloperoxidase activity also can be activated by the phagocytic cells (Palic et al., 2005a and 2005b). Compared to the control group, the supplements of the 1% original *E. cava*with*L. plantarum*as a feed for fish markedlyenhanced innate immune system via activating the respiratory burst, the lysozyme, and themyeloperoxidasein oliveflounder. These results were supported by the capacities of LAB on the enhancing the activities of phagocytes, lysozyme, and complement indicated in the previous reports (Panigrahi et al., 2004, Schiffrin et al., 1997) as well as the beneficial capacity of *E. cava* on the growth of LBA indicated in part 1. Possibly, such enhanced factors of the innate immune systemmay have provided protection/defense against infection by the pathogen. Interestingly, the supplements of the 1% original *E. cava* and/or EPEs with*L. plantarum* reduced mortalities following challenge with *E. tarda, S. iniae* and*V. harveyi* for 2 wks. In part 1, this study indicated that a polysaccharide component included in *E. cava* can be a prebiotic as showing the highest growth effect of LAB and the anti-bacteria activity of the secondary metabolite produced from LAB. Previously, dietary supplementation with seaweed extracts has been demonstrated to exert modifying effects on selectgenera of the gut-associated microbiota (Lynch et al., 2010; Reilly et al., 2008). More specifically, consumption of diets supplementedwith fucoidan has demonstrated stimulatory effects on numbers of lactobacilli in the GIT (Lynch et al., 2010, McDonnellet al., 2010). In addition, recent reports showed that novel dietary polysaccharides derived from seaweed species have demonstrated exertive effects on the gut associated microbiota, which includes stimulation of lactic-acid bacteria such as lactobacilli (Lynch et al., 2010; McDonnell et al., 2010) and prohibitive effects on Enterobacteria (Lynch et al., 2010) including Escherichia coli (O'Doherty et al., 2010). With previous finding, these results suggest that the beneficial

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capacities of *E. cava* on the enhancement of innate immunity and the inhibition of bacteria growth, the enhancement of LAB, and the production of secondary metabolites from LAB in gut-related immune system are responsible for the lower mortalities in pathogen bacteria-infected fishes.

According to these results, it could be concluded that the supplement of the original *E. cava*and*L. plantarum*mixed diets can play roles in a prebiotic and be used to enhance the innate immune status and gut-related immune system, thereby improving the diseases resistance in pathogen bacteria-infected oliveflounder. Further studies including field and full commercial cost benefit analysis, are necessary in the future.

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