Antioxidant and Antibacterial Activities of *Lactobacillus*-fermented *Artemisia annua* L. as a Potential Fish Feed Additive

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Fermented medical herbs using *Lactobacilli* have attracted significant attention due to their enhanced biological activities. A traditional medicinal plant, *Artemisia annua* L., was fermented using a probiotic strain, *L. plantarum* SK3494. The strain was isolated from *Artemisia princeps* var. *orientalis* and molecularly identified through sequence similarities and phylogenetic tree analysis. The antioxidant activity of *L. plantarum*-fermented *A. annua* L. (LFA) was determined using the DPPH free radical scavenging assay. Cellular antioxidant activity of LFA was examined using the superoxide radical reduction assay in MAT-C cells. Total polyphenol contents (TPC) and flavonoid contents (TFC) of LFA were determined. The antibacterial activity of LFA against fish pathogens was also determined in this study. The viable cell number (9.38 log10 CFU/ml) and pH (4.1) results showed good adaptive ability of the selected strain during fermentation. LFA was found to have enhanced antioxidant activity compared to non-fermented *A. annua* L. (NFA) based on the DPPH assay. Cellular antioxidant activity was present in both LFA and NFA. After 24 hr and 48 hr of fermentation, the LFA also showed antibacterial activities against fish pathogens *Photobacterium damselae* subsp. *damselae* and *Vibrio ichthyoenteri*. These results suggest that *L. plantarum*-fermented *A. annua* L. may have potential as a feed additive in aquaculture.

Key words : Antioxidant activity, antibacterial activity, *Artemisia annua* L., *Lactobacillus plantarum*, fermentation

Introduction

Farming of aquatic organisms is important to satisfy the increasing consumption of seafood globally. Meanwhile, safe and environmental sustainability, infection disease outbreak, and attenuated immunity are challenges that the aquaculture industry is facing [19]. Researchers have attempted to solve these problems by using functional feed additives. Probiotics [37] and phytochemicals [8] are frequently used as feed additives in aquaculture. *Lactobacilli* are normally considered as probiotics. They exhibit antioxidant potential by reducing oxidative damage, scavenging oxidant free radicals, and modifying anti-oxidative enzymes [38]. The antibacterial ac-

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tivity of *Lactobacillus* strains against fish pathogens such as: *Vibrio ichthyoenteri*, *Edwardsiella tarda,* and *Streptococcus iniae* has been reported previously [26]. Plant-derived phytochemicals such as flavonoids, polyphenol, pigments, steroids, and essentials oils are also widely used in aquaculture due to their antibacterial, antioxidant, and anti-stress activities [7]. *Artemisia annua* L. is a quite common herbal medicine in Asia [42]. It contains enriching artemisinin that is well known for its effect on malaria, inflammation and parasitic infection [30, 52]. *A. annua* L. is also rich in antioxidant compounds such as flavonoids and phenolics [24, 54].

A previous study has shown that lactic fermented herbal teas have higher contents in phenolic compounds, flavonoid compounds, and higher antioxidant [23]. Another study has also reported that the antioxidant and antibacterial activities of medicinal plant (*Bletilla striata*) fermented with fungi are improved compared to unfermented control [12]. *Lactobacillus*-fermented *Artemisia princeps* has been applied as a functional ingredient to improve the growth performance, meat lipid stability, and intestinal health of chickens [27].

A synergistic biological effect can be achieved by ferment-

ing natural plant sources with a suitable probiotic strain. Up to date, few studies have reported the functional properties of fermented medicinal plants as a potential feed additive in aquaculture. Therefore, the objective of this study was to determine the *in vitro* antioxidant and antibacterial activities of fermented *A. annua* L. using an isolated *Lactobacillus* strain.

Materials and Methods

Isolation and identification of *Artemisia*-inhabiting bacteria

Artemisia princeps var. *orientalis* was collected from the field. After drying at room temperature, *A. princeps* var. *orientalis* was ground using a blender (DA5000, Gyeonggi-do, Korea). For the isolation of *Artemisia*-inhabiting bacteria, *A. princeps* var. *orientalis* powder (10 g) was added into 100 ml of MRS broth (Man, Rogosa and Sharpe, Difco, NJ, USA) or *Bacillus* minimal medium (BMM) composed of Na_2HPO_4 (33.51 mM), KH_2PO_4 (22.04 mM), NaCl (8.56 mM), NH_4Cl (18.69 mM), $MgSO_4$ (1 mM), and $CaCl_2$ (0.1 mM) followed by incubation at 37℃ for three days with shaking (100 rpm). A single colony was picked and identified by 16S rRNA gene sequencing using universal 27F primer (5′-AGA GTT TGA TCC TGG CTC AG-3′). BLAST (Basic Local Alignment Search Tool, NCBI) was used for similarity analysis. Mega software (version 7) was used to construct phylogenetic tree. The stability of the group was determined by running a bootstrap analysis (1,000 replicates).

Fermentation of *Artemisia annua* L.

A. annua L. powder was purchased from a local market of Chengsong, Kyeongbuk, Korea. The plant was dried at 50℃ for 3 days and smashed into fine powder (150-mesh). *L. plantarum* SK3494 was pre-cultured in MRS broth at 37℃ for 16 hr ($2.5×10^9$ CFU/ml). Then 1% of the pre-cultured SK3494 was inoculated into the sterilized medium containing of *A. annua* L. powder (5.0 g) and MRS powder (1.1 g) in 100 ml distilled water (D.W.). Fermentation was conducted at 37℃ for 48 hr. Cultured medium (10 ml) was sampled every 12 hr. The pH value was immediately measured using a pH meter (pH/ISE Meter 735P, Istek Co., Ltd, Seoul, Korea). Viable cell numbers were determined by drop plate method [17]. Supernatants were prepared by centrifugation (Mega 17R, Hanil science industrial, Incheon, South Korea) at 12,000 rpm, for 10 minutes at 4℃. They were subsequently

used to determine antioxidant and antibacterial activities.

DPPH free radical scavenging activity

The antioxidant activity was determined using the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity according to the method of Saeed [46] with some modifications. Briefly, 50 µl of the prepared supernatants (0, 12 hr, 24 hr, 36 hr, 48 hr) were diluted with 450 µl of D.W. and reacted with 2 ml of DPPH solution. The solution was incubated in the dark for 15 minutes at room temperature with shaking (100 rpm). Then 2.5 ml of D.W. was added to the solution and the optical density was measured at 517 nm using a spectrophotometer (UV-1601, SHIMADZU Co., Ltd, Kyoto, Japan). D.W was used as control and compared with the supernatant. The scavenging activity was calculated using the following equation:

DPPH radical scavenging activity (%)=
    (1-Absorbance$_{sample}$/ Absorbance$_{control}$) ×100%

Superoxide radical reduction in MAC-T cells

The supernatants of non-fermented *A. annua* L. (NFA) and 24 hr-fermented *A. annua* L. (LFA) and pure cell (LO) of SK3494 (10^7 CFU/ml) were prepared by centrifugation (12,000 rpm, 4℃, 10 minutes). The superoxide radical reduction activities of these prepared samples were determined in primary bovine mammary alveolar cells (MAC-T) by stable transfection with SV-40 large T-antigen [21] using the method described previously [16]. MAC-T cells were grown to confluency on cover glass in 6-well plate. Following treatments, dihydroethidium (DHE) was added to cells to reach a final concentration of 1 µM followed by incubation in a 5% CO_2 incubator for 30 minutes. After the 30 minutes of incubation, cells were washed three times with phosphate buffered saline (1X PBS, containing NaCl 8 g/l, KCl 0.2 g/l, Na_2HPO_4 1.42 g/l, KH_2PO_4 0.24 g/l), fixed with 4% formaldehyde, and washed with PBS again three times. Slides were mounted with ProLong Gold Antifade reagent containing 4′, 6-diamidino-2-phenylindole (DAPI) (Invitrogen, Carlsbad, USA) to visualize the nuclei. Slides were observed under a fluorescence microscope (Olympus IX71). Images were captured digitally using an Olympus DP71 camera and DP controller software (Olympus Optical Co. Ltd, Tokyo, Japan). Prepared samples were used to treat with cells and cells were then stained with DHE. The intensity of red fluorescence was assessed using a fluorescence microscope. The red areas in cells represented oxidized DHE, indicating the

production of superoxide. The images shown in this study were representatives of three independent experiments.

Total polyphenol contents

Total polyphenol contents were determined using published method [55]. Briefly, 0.1 ml of prepared supernatants (0, 12 hr, 24 hr, 36 hr, 48 hr) were added into the test tube containing 1 N Folin and Ciocalteau's phenol reagent (0.2 ml), 1 N sodium carbonate solution (0.2 ml) and D.W was used to fill the volume up to 10 ml. The absorbance was measured at wavelength of 700 nm after 30 minutes of reaction in the dark. Gallic acid was used as a standard phenolic compound. Total polyphenol contents were calculated as mg equivalent gallic acid/ml and expressed as GE mg/ml.

Total flavonoids contents

Total flavonoids contents were estimated using the method described by Moreno [39] with slight modifications. Briefly, 0.1 ml of the prepared supernatants (0, 12 hr, 24 hr, 36 hr, 48 hr) were diluted with 0.9 ml of 80% ethanol. Then 0.5 ml of the solution was taken to a test tube containing 0.1 ml of 10% aluminum nitrate, 0.1 ml of 1 M aqueous potassium acetate and 4.3 ml of 80% ethanol. The absorbance of the mixture was measured at wavelength of 415 nm after 40 minutes of incubation at room temperature. Total flavonoids contents were calculated using quercetin as a standard flavonoid compound.

Antibacterial activity against fish pathogens

Antibacterial activities of NFA and LFA cultures against two fish pathogens *Photobacterium damselae* subsp. *damselae* ATCC 33539 and *Vibrio ichthyoenteri* ATCC 700023 were determined using agar well diffusion method [5]. Fish pathogens were pre-cultured in marine broth (MB) at 37℃ for 16 hr. The culture solutions were covered onto the surface of marine agar using a sterile cotton swab. The 0 hr, 24 hr and 48 hr cultured supernatants (100 µl) were used to fill with the holes (6 mm/diameter) made by sterile Pasteur tubes in the agar plates. Antibacterial activity was evaluated by the diameter size of clear zone after 24 hr of incubation at 37℃.

Statistical analysis

IBM SPSS statistics 24 for windows (IBM, New York, US) was used for all data analysis. Data are presented as mean ± standard deviation. Significant differences were determined using Duncan's multiple range test at $p<0.05$ level.

Results and Discussion

Identification of *Artemisia*-inhabiting bacteria

A total of 13 strains were isolated and identified based on 16S RNA sequencing (Table 1). SK3493 and SK3494 showed high sequence similarities with lactic acid bacteria (LAB). SK3497 and SK3499 showed high sequence similarities with *Bacillus* species. Both LAB and *Bacillus* species are well known probiotics with diverse beneficial effects for their hosts [35, 10]. *Lactobacillus plantarum*, a normal LAB species, is generally considered as safe and used in different animals [14, 33, 47, 49]. In addition, *L. plantarum* is also very frequently used in the fermentation of plant sources [43].

Table 1. Identification of bacteria isolated from *Artemisia princeps* var. *orientalis*

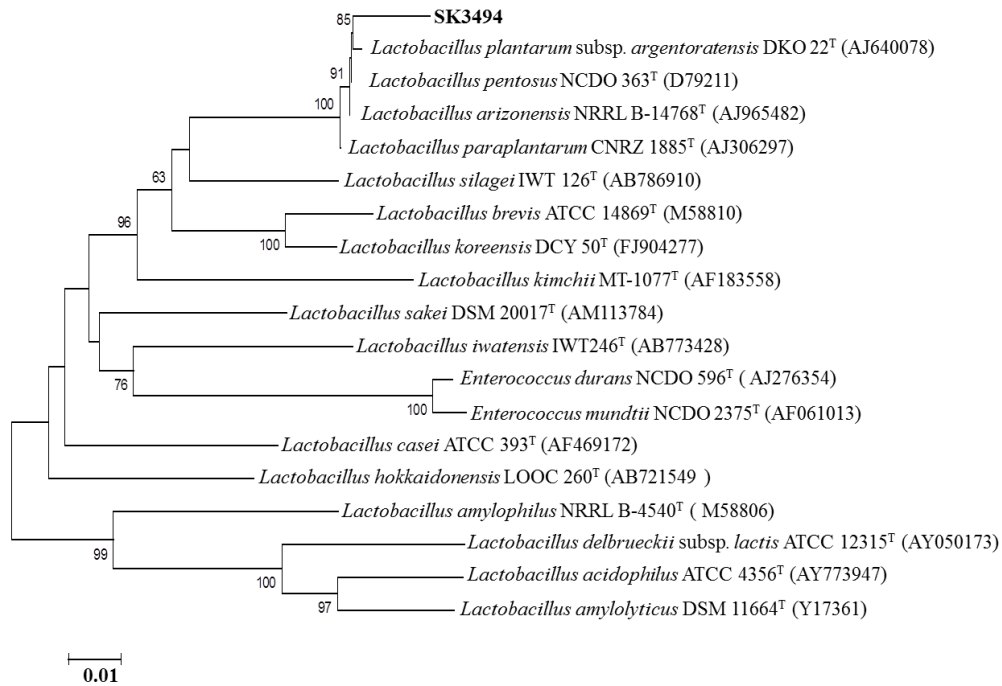| Strain	Isolated media	Homology	Query coverage	Max identity	Characteristics	References
SK3493	MRS	*Weissella* sp. Rrt5	99	99	Probiotic	36
SK3494	MRS	*Lactobacillus plantarum*	98	99	Probiotic	42-45
SK3495	BMM	*Rummeliibacillus stabekisii*	98	99	Unknown	11
SK3496	BMM	*Enterococcus mundtii*	99	99	Probiotic; Pathogen	3, 18
SK3497	BMM	*Bacillus amyloliquefaciens*	100	99	Probiotic	2, 47
SK3498	BMM	*Pantoea agglomerans*	100	100	Opportunistic pathogen	13
SK3499	MRS	*Bacillus megaterium*	100	100	Probiotic	1
SK3500	MRS	*Enterococcus hirae*	100	100	Pathogen	4
SK3501	BMM	*Bacillus thuringiensis*	100	100	Biopesticide	44
SK3502	BMM	*Bacillus oleronius*	100	99	Pathogen	50
SK3503	BMM	*Escherichia hermannii*	100	100	Pathogen	40
SK3504	BMM	*Acinetobacter radioresistens*	100	100	Opportunistic pathogen	39
SK3505	BMM	*Citrobacter* sp.	100	99	Opportunistic pathogen	30

Fig. 1. Phylogenetic neighbor-joining tree of SK3494 based on 16S rRNA sequences showing its relationship with related taxa.

Strain SK3494 showed high sequence similarities with *L. plantarum.* It shared the highest sequence similarities with *Lactobacillus plantarum* subsup. *argentoratensis* DKO 363^T (AJ640078, type strain) based on phylogenetic neighbor-joining tree analysis (Fig. 1). Herein it was designated as *L. plantarum* SK3494 and used in the fermentation of *Artemisia annua* L. The other strains identified as *Artemisia*-inhabiting bacteria were potential pathogens, biopesticides, or unknown bacteria.

Growth ability and pH change

Viable cells and pH of *L. plantarum* SK3494 were directly measured during the 48 hr of fermentation. As shown in Fig. 2, a stationary phase was achieved after 12 hr of incubation. The highest cell number reached 9.38 log10 CFU/ml after 24 hr of incubation. It was then dramatically decreased after 48 hr of incubation. The pH value was decreased after 12 hr of incubation and kept steady at around 4.1. Wang [53] has reported that viable cells of *L. plantarum*-fermented noni juice have reached almost 1.0×10^9 CFU/ml after 48 hr of fermentation and the pH is decreased to 3.7. Kim [29] has also reported that viable cells of grape pomace fermented by *L. plantarum* are about 1.0×10^7 CFU/ml with a pH of 3.49 after 24 hr of fermentation. Diverse organic acids are produced during the lactic acid fermentation. They can cause the rapid decrease of pH along with increasing

cell numbers [15]. In this study, *L. plantarum* SK3494 demonstrated good adaptability by using *A. annua* L. as a nutritional source, suggesting that the isolate might be a suitable strain for the fermentation of *A. annua* L.

Antioxidant activity

Fig. 3A shows that both NFA and LFA had a high scavenging effect on DPPH free radical. Increased scavenging
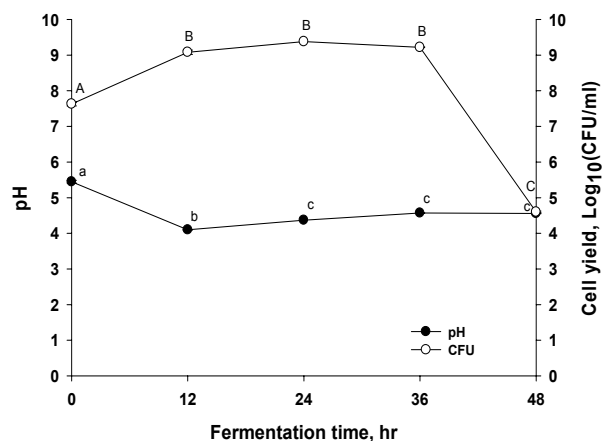

Fig. 2. The growth ability of *L. plantarum* SK3494 and pH change during fermentation of *Artemisia annua* L. Opened and closed circles indicate growth curve and pH change, respectively. Data are presented as mean ± S.D. Small (pH) and capital (CFU) letters are significantly different by Duncan's multiple range test at $p < 0.05$.

activity was observed in LFA samples compared to NFA samples (62~64%). The highest scavenging activity was achieved at 79.86% from the 24 hr fermented-sample. Similar results were obtained for the reduction activities of superoxide radicals in MAC-T cells. MRS medium and *Lactobacillus* pure cell exhibited a certain capability to reduce the level of radicals. Apparently, both NFA and LFA possessed a strong effect on eliminating superoxide radicals. However, there was no difference in superoxide radical reduction activity in MAC-T cells between NFA and LFA treatments (Fig. 3B). It has been reported that *A. annua* L. contains abundant antioxidant compounds with high antioxidant capacity [6]. A high DPPH radical scavenging activity (82.3~91.0%) of artemisinin extracts has been reported in the study of Kim [30]. *Lactobacilli* are generally used as antioxidant sources including their cell pellet [34] and cell-free supernatant (CFS) [44]. In the study of Xing [57], cellular antioxidant activity of *Lactobacilli* CFSs in HepG2 cell line has been found to be associated with the DPPH free radical scavenging results *in vitro*. Hong [20] reported that *Leuconostoc*-fermented Chinese

chives (FC) increased antioxidant activity by DPPH scavenging assay and cellular antioxidant in MAC-T cells compared to pure *Leuconostoc* cell (LO). Fermented *Bletilla striata* (FBS) with *Fusarium* species has been reported to have stronger antioxidant activity compared to unfermented controls [12]. Thus, fermentation is a potential method to improve the antioxidant capacity of natural sources.

Total polyphenol content (TPC) and flavonoid content (TFC) were further analyzed to determine the changes of specific antioxidant compounds during the fermentation process. Fig. 3C and 3D showed that TPC and TFC in fermented *A. annua* L. samples were decreased compared to the NFA samples using gallic acid and quercetin as standard compounds. The reduction in TPC and TFC might be due to the utilization of them as substrates for the growth of cells as fermentation characteristics of *A. annua* L. powder. Svensson et al [50] has reported that phenolic acids, phenolic acid esters, and flavonoid glucosides are metabolized during lactic acid fermentation. Different from our results, many studies have reported that phenolics and flavonoids contents
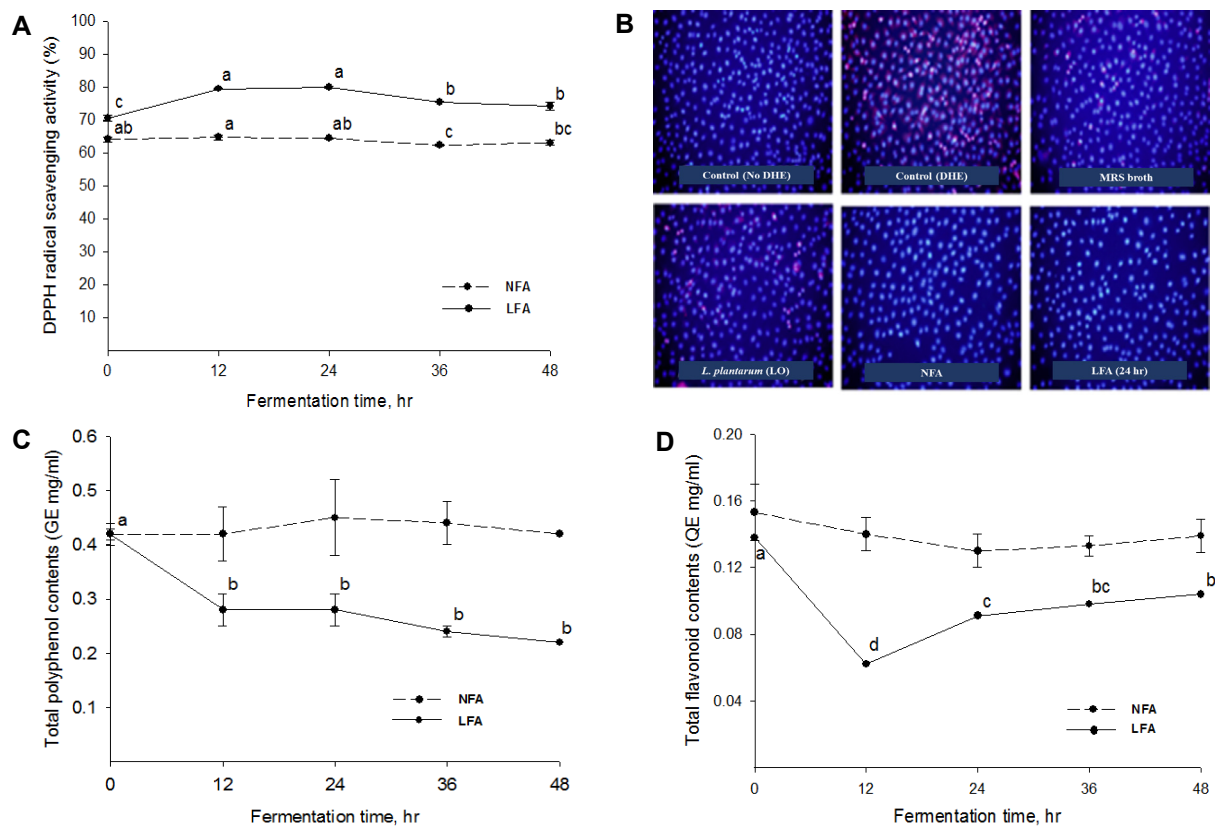

Fig. 3. Antioxidant activity and antioxidants change during fermentation of *Artemisia annua* L. (A) DPPH free radical scavenging activity, (B) Superoxide scavenging activity in bovine cells (MAC-T), (C) Total polyphenol contents, and (D) Total flavonoids contents. Dotted and solid lines indicate NFA and LFA, respectively. Data with different superscripts (a-d) are significantly different by Duncan's multiple range test at $p<0.05$. GE indicates gallic acid equivalent.
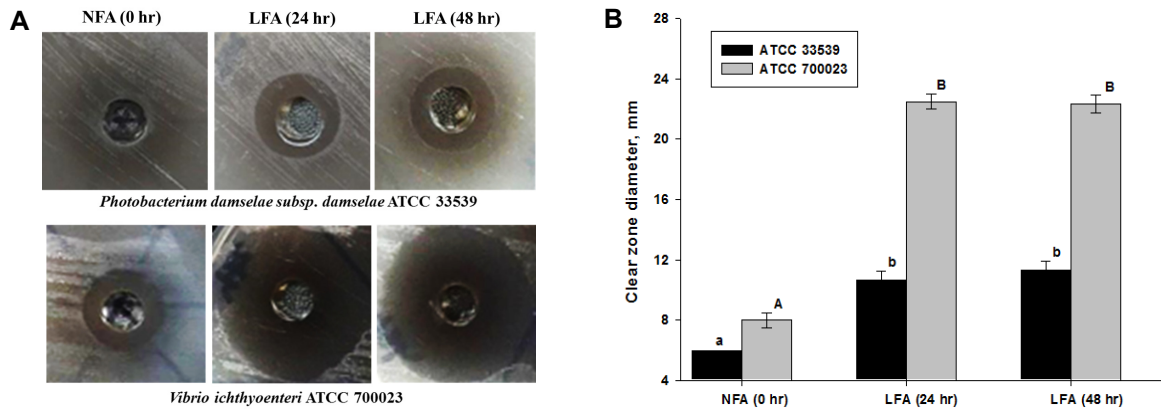
Fig. 4. Antibacterial activity of *L. plantarum*-fermented *Artemisia annua* L. (LFA) against fish pathogens: *Photobacterium damselae* subsp. *damselase* ATCC 33539 and *Vibrio ichthyoenteri* ATCC 700023. (A) Antibacterial images, (B) Quantitative analysis using clear zone diameter (mm). Hole size: 6mm in diameter. Data are presented as mean ± S.D. Small (ATCC 33539) and capital (ATCC 700023) letters are significantly different by Duncan's multiple range test at $p<0.05$.

are increased after *Lactobacillus*-fermentation [9, 55, 56]. To illustrate these unexpected results, Huynh et al [22] have mentioned that a variety of new metabolites are produced during metabolism of phenolic compounds by different bioconversion pathways such as glycosylation, deglycosylation, ring cleavage, methylation and so on others. These newly synthesized compounds may present better antioxidant property. For instance, oleuropein, one major phenolic compound of olive fruits is degraded to hydroxytyrosola, which possesses stronger antioxidant activity during fermentation [32]. This might be related to the degradation and synthesis processes of metabolites during fermentation thereby improving or reducing the antioxidant activity.

Antibacterial activity

NFA showed no inhibitory effect on fish pathogen *Photobacterium damselae* subsp. *damselae* KCTC 2734. It had a slight inhibitory effect against *Vibrio ichthyoenteri* KCCM 40870 (Fig. 4). However, the 24 hr- and 48 hr-LFA samples showed high antibacterial activities against *P. damselae* (12 mm) and *V. ichthyoenteri* (22 mm). *A. annua* L. has been traditionally used as anti-malaria treatment due to its ability to produce artemisinin. It has been reported that water, methanol, ethanol, and acetone extracts of *A. annua* L. can inhibit the growth of pathogenic *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum* subsp. *animalis*, *Fusobacterium nucleatum* subsp. *polymorphum*, and *Prevotella intermedia* [30]. Water extracts of *A. capillaris* can also antagonize the growth of *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhimurium* [28]. It has been reported that fermented medicinal plant (red chicory) with lactic acid bacteria has antimicrobial

activity [25]. Stronger antibacterial activity of fermented *Bletilla striata* with fungus compared to non-fermented control has also been reported [12]. *Lactobacilli* are well known for their antibacterial activity normally associated with organic acid and bacteriocin production [36]. In this study, LFA presented much higher antibacterial effect compared to NFA. This is possibly due to lactic acid production during fermentation.

In the present study, *L. plantarum* SK3494 isolated from *A. princeps* var. *orientalis* was found to be a suitable strain for the fermentation of *A. annua* L. Fermented *A. annua* L. showed enhanced antioxidant activity both by DPPH free radical scavenging activity and cellular antioxidant activity. The mechanism underlying the improved antioxidant activity but decreased level of antioxidant compounds such as polyphenolics and flavonoids during herb fermentation remain unclear. Antioxidant compounds might have been degraded during fermentation and some unknown compounds might have been synthesized during metabolism. In conclusion, Fermented *A. annua* L. showed antioxidant activity and antibacterial ability against fish pathogens, suggesting that it might be used as a potential feed additive in aquaculture.

Acknowledgement

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초록 : 양어 사료첨가제로서의 유산균 발효 개똥쑥의 항산화 및 항균활성

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최근 양어에서는 전염병, 환경오염, 어분 가격 상승 등으로 어려움을 가지고 있다. 본 연구는 쑥에서 분리한 *L. plantarum* SK3494를 이용하여 전통 약초인 개똥쑥을 발효시켜 *in vitro* 생리활성을 측정하고 어류용 사료첨가제로서의 가능성을 연구하였다. 개똥쑥의 발효시 유산균수는 9.38 log10 CFU/ml이며, pH는 4.1로 나타났다. 발효개똥쑥의 항산화활성은 DPPH 측정과 MAC-T 세포 내 과산화물 감소 실험에서 뛰어난 효과를 보였다. 발효개똥쑥은 어류 병원균인 *Photobacterium damselae* subsp. *damselae*와 *Vibrio ichthyoenteri*에 대하여 강한 항균활성을 나타내었다. 따라서 유산균을 이용한 개똥쑥 발효물은 양식용 사료첨가제로써 가능성을 가지고 있는 것으로 판단되었다.