한국어병학회지 제37권 제1호 (2024)
J. Fish Pathol., 37(1) : 037~048

www.ksfp.org
pISSN 1226-0819, eISSN 2233-5412
http://dx.doi.org/10.7847/jfp.2024.37.1.037

In vitro antimicrobial properties of *Bacillus subtilis* KCTC 1326 for fish bacterial disease management

Ji-Yoon Park[*], In-Joo Shin[*], So-Ri Han[**], Sung-Hyun Kim[**], Youhee Kim[***] and Se Ryun Kwon[*†]

[*]*Department of Aquatic Life Medical Sciences, Summon University, Asan 31460, Korea*
[**]*Fishcare laboratory, Jeju 63626, Korea*
[***]*Department of Smart Aquaculture, Gangwon State University, Gangneung 25425, Korea*

This study evaluated *in vitro* antimicrobial properties of *Bacillus subtilis* KCTC 1326 as an environmentally friendly alternative to antibiotics. *B. subtilis* KCTC 1326 was characterized on biochemical properties and antibiotics susceptibility. It exhibited antimicrobial effects against all 12 species of fish bacteria used in this experiment. Among them, the largest antibacterial zone was observed for *Streptococcus parauberis* (34 mm), while the smallest antibacterial zone was observed for *Citrobacter freundii* (8 mm). Additionally, in the co-culture inhibitory assay of *B. subtilis* and *Edwardsiella piscicida*, the growth of *E. piscicida* was suppressed with increasing concentrations of *B. subtilis* KCTC 1326, with complete inhibition observed at 10^7 and 10^8 CFU/mL of *B. subtilis* KCTC 1326 after 24 hours of incubation. Moreover, at 48 hours of incubation, the growth of *E. piscicida* was inhibited across all concentration ranges of *B. subtilis* KCTC 1326. Therefore, this study indicated the utilizing of *B. subtilis* KCTC 1326 as an antimicrobial for controlling fish bacterial diseases.

Key words: Antimicrobial activity, *Bacillus subtilis* KCTC 1326, Fish bacteria

Introduction

Bacterial diseases cause significant economic losses to the aquaculture industry worldwide aquaculture (Pereira *et al.*, 2022; Nair *et al.*, 2021). These bacterial diseases can be controlled using appropriate antibiotics and chemotherapy drugs. However, this can have a negative impact on the environment due to residual residues, and overuse of antibiotics can lead to the development of antibiotic-resistant bacterial strains or antibiotic-resistant genes (El-Saadony *et al.*, 2021; Nair *et al.*, 2021; Abarike *et al.*, 2018). To overcome these problems, new environmentally friendly alternatives are needed.

Bacillus sp. are widely used in aquaculture as promising alternatives to overcome these issues as antibiotic substitutes. They offer advantages of safety and non-pathogenicity, along with the ability to tolerate a broad range of physiological conditions and produce a wide range of enzymes, antibiotics, metabolites, and other physiologically active compounds (Nayak, 2021). The most commonly used *Bacillus* sp. include *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus*, and *Bacillus amyloliquefaciens* (Caulier *et al.*, 2019; Kuebutornye *et al.*, 2019).

Bacillus subtilis is a gram-positive, rod-shaped aerobic bacterium (Lee *et al.*, 2017). They can also form spores, are more heat-stable than non-spore-forming bacteria and can survive and reach the intes-

[†]Corresponding author: Se Ryun Kwon
Tel: +82-41-530-2289; Fax: +82-41-530-2917
E-mail: srkwon@sunmoon.ac.kr

tines even with high bile acid and the low pH of the gastric barrier (Guo *et al.*, 2016). Additionally, *B. subtilis* has been used as a substitute for antibiotics by adding it to feed for a long time and has been reported to improve growth, digestive enzyme activity, and disease resistance (Abarike *et al.*, 2018; Hao *et al.*, 2017; Zuenko *et al.*, 2017).

In this study, the biochemical and antibiotic susceptibility characteristics of *B. subtilis* KCTC 1326 were investigated. In addition, an antimicrobial activity was evaluated on fish-derived bacterial species to determine whether it could be used as an eco-friendly alternative to fish bacterial diseases.

Materials and Methods

Bacteria strains and culture conditions

The fish disease bacteria and culture temperatures used in the experiment are shown in Table 1. *Bacillus subtilis* KCTC 1326 (ATCC 33234; J. Spizizen strain 168) was supplied from Korean collection for type cultures (KCTC, Korea). The fish pathogens *Aeromonas hydrophila*, *A. salmonicida*, *Lactococcus garvieae*, and *Vibrio harveyi* were supplied by professor Do-Hyung Kim at Pukyong national university. Additionally, the other bacterial strains were isolated directly in our laboratory (Kim *et al.*, 2023a; Kim *et al.*, 2023b).

Characterization of *Bacillus subtilis* KCTC 1326

1) API 50 CHB biochemical tests

The sugar utilization ability of the bacteria was tested using the API 50 CHB tests. Following the manufacturer's protocol, *B. subtilis* KCTC 1326 was cultured overnight on Nutrient agar (NA; Sigma-Aldrich, USA) at 37°C. For the inoculum, several colonies of *B. subtilis* KCTC 1326 were taken in API 50 CHB medium (bioMérieux, France), and the turbidity was adjusted with 2 McFarland. The inoculum was distributed to 50 test strips and mineral oil was added. The results were read after incubation at 37°C for 48 hours. The resulting readout was tabulated +/- according to the color change.

2) Protease activity

Protease activity was performed to see enzymatic activity. Protease activity was assessed by dropping 20 μL of *B. subtilis* KCTC 1326 cultured in de Man, Rogosa and Sharpe (MRS) broth (BD Difco ™, USA) onto MRS agar supplemented with 1% skim milk and then culturing at 37°C for 24 hours to confirm the presence of a clear zone.

3) Gelatinase activity

Gelatinase activity was determined using gelatinase from the API 20E kit (bioMérieux, France). The experiment was performed according to the manufactur-

Table 1. Bacterial isolates and culture condition used in this study

Bacteria species	Strain	Medium	Temperature	Origin
Aeromonas hydrophila	AC133	TSA	27°C	Crucian carp
Aeromonas salmonicida	18KR23	TSA	20°C	Rainbow trout
Aeromonas sobria	OMK91	BHIA	27°C	Rainbow trout
Aeromonas veronii	OMI61	BHIA	27°C	Rainbow trout
Edwardsiella piscicida	BYPO22	BHIA (1% NaCl)	27°C	Olive flounder
Lactococcus garvieae	KEO3-MRL9	BHIA (1% NaCl)	27°C	Olive flounder
Streptococcus parauberis	JJPO21	BHIA (1% NaCl)	27°C	Olive flounder
Vibrio harveyi	DHPO20	TSA (1% NaCl)	27°C	Olive flounder
Yersinia ruckeri	OMI25	BHIA	22°C	Rainbow trout
Citrobacter freundii	AJ28	BHIA	27°C	Japanese eel
Enterobacter cloacae	AJ191	BHIA	27°C	Japanese eel
Plesiomonas shigelloides	AJ24	BHIA	27°C	Japanese eel

er's instructions. Briefly, a single colony of *B. subtilis* KCTC 1326 was collected, suspended in 5 mL of suspension medium and distributed onto strips. Then, after overnight incubation at 37°C, the gelatinase reaction was confirmed.

Antimicrobial effects of *B. subtilis* KCTC 1326 against fish bacteria

B. subtilis KCTC 1326 was inoculated in MRS broth with 2% of the preculture and then placed in a shaking incubator at 37°C for 24 hours at 180 rpm. An empty paper disc was prepared by applying 10 μL of *B. subtilis* KCTC 1326 at an OD of 1.5 (625 nm). The prepared discs were then used for the disk diffusion method with modifications based on the Clinical and Laboratory Standards Institute (CLSI) guidelines. In brief, diluted bacterial were spread onto sterilized cotton swabs and smeared onto Mueller-Hinton agar. Subsequently, *B. subtilis* discs were placed onto the agar inoculated with the pathogenic bacteria. Cultures were then incubated at the respective optimal temperatures for each bacterial strain.

1) Antibiotic susceptibility test

The antibiotic susceptibility test was conducted with modifications based on the CLSI and the Becton, Dickinson and Company (BD) antibiotics manual. For fish disease bacteria, 2% of the preculture was cultured as described in Table 1 and used with turbidity adjusted to OD 0.01 (625 nm). After smearing the bacterial solution on Mueller-Hinton agar using a sterile cotton swab and then 9 types of antibiotics discs (ampicillin, amoxicillin/clavulanic acid, erythromycin, kanamycin, tetracycline, sulfamethoxazole/trimethoprim, nalidixic acid, ciprofloxacin, and norfloxacin; BD Difco ™, USA) were used to compare the antimicrobial activity of *B. subtilis* KCTC 1326 with those of conventional antibiotics.

2) Co-culture inhibitory assay

Co-culture inhibitory assay was *B. subtilis* KCTC 1326 precultured in MRS broth and *Edwardsiella piscicida* precultured in brain heart infusion (BHI; BD Difco ™, USA) broth containing 1% NaCl were used. Control group added BHI broth containing 1% NaCl and incubated for 96 hours at 27°C at 180 rpm. Every day, 100 μL of the culture medium was taken, serially diluted 10-fold until 10^{-10}, and then 10 μL was added dropwise to Salmonella-Shigella (SS) agar (BD Difco ™ USA). The plates were incubated at 27°C for 24 hours and the colonies obtained on the plates were counted and expressed as log10 CFU/mL.

Minimal inhibitory concentration (MIC)

To evaluate the minimal inhibitory concentration (MIC), *B. subtilis* KCTC 1326 was incubated overnight at 37°C on an MRS agar. The bacterial solution was adjusted to 0.5 McFarland by picking 2-3 colonies in DW. After mixing 50 μL of bacterial solution and 11 mL of Mueller-Hinton broth for each panel (KRAQ3, KRAQ4), 100 μL per well was dispensed using a multi-pipette. After attached sealing paper to the well plate, the MIC result was read by overnight at 37°C.

Results

Characterization of *B. subtilis* KCTC 1326

B. subtilis KCTC 1326 presented positive results for glycerol, L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, inositol, D-mannitol, D-sorbitol, Methyl-α D-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, esculin, salicin, D-cellobiose, D-maltose, D-lactose (bovine origin), D-melibiose, D-saccharose (sucrose), D-trehalose, inulin, D-raffinose, amidon (starch), glycogen, gentiobiose, protease, and gelatinase. But *B. subtilis* KCTC 1326 isolate cannot utilize erythritol, D-arabinose, L-xylose, D-adonitol, Methyl-β D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, Methyl-α D-mannopyranside, D-melezitose, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol,

L-arabitol, potassium gluconate, potassium 2-ketogluconate, potassium 5-ketogluconate (Table 2).

Comparison of inhibitory effects of *B. subtilis* KCTC 1326 against fish bacteria

Bacillus subtilis (BS) exhibited the highest pathogen inhibition against *Aeromonas*, with a diameter of 25 mm observed on *A. veronii*. Conversely, the lowest inhibitory effect was observed on *A. salmonicida*, with a diameter of 11.5 mm, indicating the lowest antibacterial activity among the 9 types of antibiotics compared (Fig. 1). Bacteria isolated from marine fish, BS demonstrated the highest antibacterial activity against *S. parauberis*, with a diameter of 34 mm. This activity surpassed that of ampicillin, kanamycin, tetracycline, nalidixic acid, and norfloxacin (Fig. 2). Lastly, a group of intestinal bacteria isolated from freshwater fish, the BS in *E. cloacae* was 31 mm, showing higher antibacterial activity than the antibiotics ampicillin, amoxicillin/clavulanic acid, erythromycin, tetracycline, and sulfamethoxazole/trimethoprim. On the other hand, *C. freundii* had the lowest antibacterial activity among the isolated enteric bacteria at 8 mm, and among the nine antibiotics, all except ciprofloxacin, nalidixic acid, and norfloxacin showed antibiotic resistance (Fig. 3, Table 3).

Table 2. Biochemical characters of *B. subtilis* KCTC 1326

Active ingredients	Result	Percentage positive results (Logan and Berkeley, 1984)	Active ingredients	Result	Percentage positive results (Logan and Berkeley, 1984)
Glycerol	+	97	Salicin	+	100
Erythritol	-	0	D-cellobiose	+	100
D-arabinose	-	0	D-maltose	+	100
L-arabinose	+	98	D-lactose (Bovine origin)	+	48
D-ribose	+	99	D-melibiose	+	80
D-xylose	+	89	D-saccharose (Sucrose)	+	100
L-xylose	-	0	D-trehalose	+	100
D-adonitol	-	0	Inulin	+	83
Methyl-β D-xylopyranoside	-	0	D-melezitose	-	0
D-galactose	+	30	D-raffinose	+	90
D-glucose	+	100	Amidon (Starch)	+	98
D-fructose	+	100	Glycogen	+	98
D-mannose	+	94	Xylitol	-	0
L-sorbose	-	2	Gentiobiose	+	96
L-rhamnose	-	2	D-turanose	-	97
Dulcitol	-	0	D-lyxose	-	0
Inositol	+	95	D-tagatose	-	0
D-mannitol	+	96	D-fucose	-	0
D-sorbitol	+	88	L-fucose	-	0
Methyl-α D-mannopyranoside	-	0	D-arabitol	-	0
Methyl-α D-Glucopyranoside	+	99	L-arabitol	-	0
N-Acetylglucosamine	+	22	Potassium gluconate	-	4
Amygdalin	+	99	Potassium 2-ketogluconate	-	0
Arbutin	+	100	Potassium 5-ketogluconate	-	2
Esculin	+	100	Gelatinase	+	100

+, positive reaction; -, negative reaction.

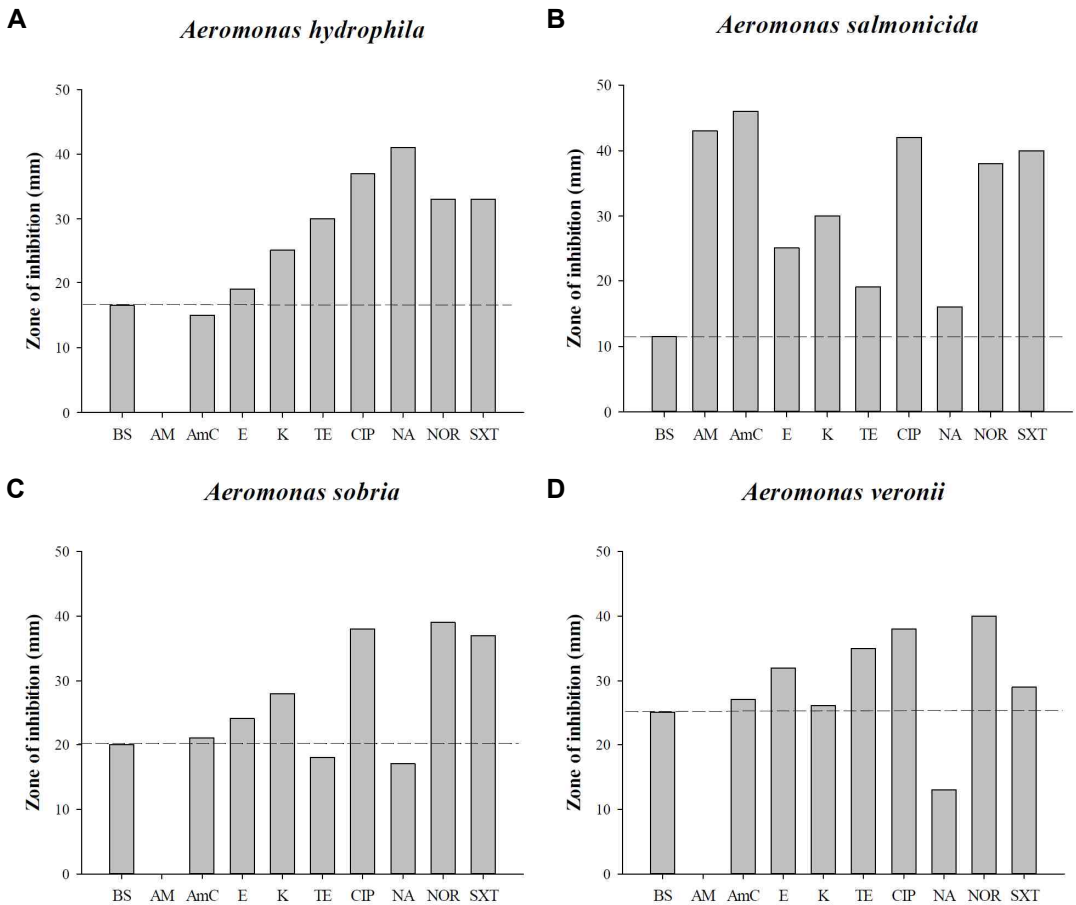

Fig. 1. Antibacterial activity of *B. subtilis* KCTC1326 against *Aeromonas* species and comparison with antibiotics BS, *Bacillus subtilis*; AM, ampicillin (10 μg); AmC, amoxicillin/clavulanic acid (20/10 μg); E, erythromycin (15 μg); K, kanamycin (30 μg); TE, tetracycline (30 μg); CIP, ciprofloxacin (5 μg); NA, nalidixic acid (30 μg); NOR, norfloxacin (10 μg); SXT, sulfamethoxazole/trimethoprim (23.75/1.25 μg).

1) Co-culture inhibitory assay

Co-culture was performed to observe the antagonistic effect of *B. subtilis* KCTC 1326 against the pathogen *E. piscicida*. When compared to the group without *B. subtilis* KCTC 1326 inoculation, the growth of *E. piscicida* was inhibited in the *B. subtilis* KCTC 1326 (10^5-10^6 CFU/mL), with respective counts of 4.44 x 10^3 CFU/mL and 5.64 x 10^3 CFU/mL after 24 hours of co-culture. Furthermore, 100% inhibition of *E. piscicida* growth was observed in the *B. subtilis* KCTC 1326 (10^7-10^8 CFU/mL) at higher concentrations. From 48 hours of cultivation onwards, the growth of *E. piscicida* was completely inhibited in all concentration intervals where *B. subtilis* KCTC 1326 was co-cultured (Fig. 4).

MIC value of *B. subtilis* KCTC 1326

MIC values of *B. subtilis* KCTC 1326 was shown in Table 4. Ceftiofur from the cephalosporin family showed an MIC value of 0.5 μg/mL, amoxicillin from the β-Lactam family showed MIC values of 0.12 μg/mL, and amoxicillin/clavulanic acid showed MIC values of 0.12/0.06 μg/mL. For the lincomycin class, clindamycin exhibited a MIC value of 0.5 μg/mL,

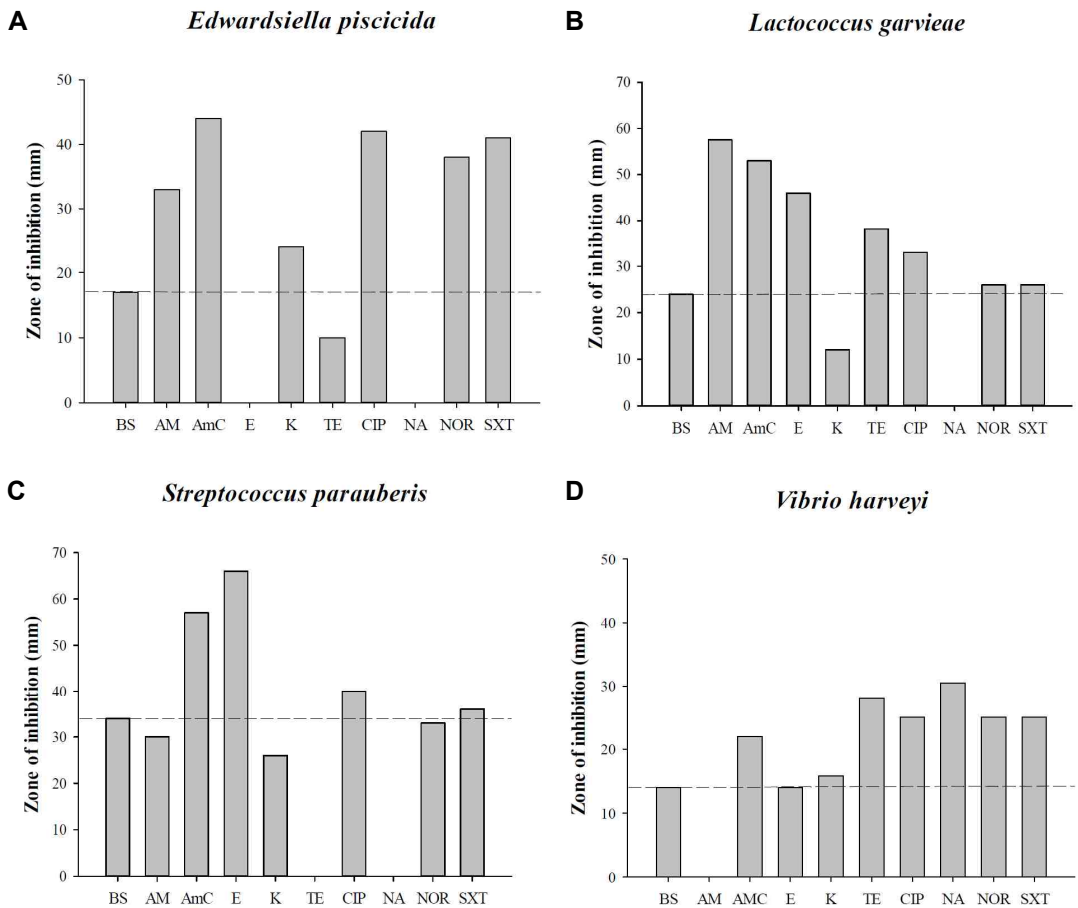

Fig. 2. Antibacterial activity of *B. subtilis* KCTC1326 against marine fish bacteria and comparison with antibiotics BS, *Bacillus subtilis*; AM, ampicillin (10 μg); AmC, amoxicillin/clavulanic acid (20/10 μg); E, erythromycin (15 μg); K, kanamycin (30 μg); TE, tetracycline (30 μg); CIP, ciprofloxacin (5 μg); NA, nalidixic acid (30 μg); NOR, norfloxacin (10 μg); SXT, sulfamethoxazole/trimethoprim (23.75/1.25 μg).

while for the macrolide class, erythromycin had a MIC value of 0.12 μg/mL, and for the phenicol class, florfenicol had a MIC value of 1 μg/mL. Additionally, for the tetracycline class, doxycycline, oxytetracycline, and tetracycline showed MIC values of 1, 64, and 8 μg/mL, respectively. Moreover, for the quinolone class, ciprofloxacin and enrofloxacin had MIC values of 0.06 μg/mL, flumequine had a MIC value of 0.5 μg/mL, and nalidixic acid had a MIC value of 8 μg/mL. Furthermore, no antibiotic resistance was observed for the aminoglycoside and sulfonamide classes.

Discussion

Bacillus sp. are known to produce a variety of antimicrobial compounds, including peptides and lipopeptides, which act as antibiotics (Blibech *et al.*, 2019). Among them, *B. subtilis* has been used as a substitute for antibiotics by adding it to feed for a long time and has been confirmed to inhibit the growth of *E. piscicida* in zebrafish (Ren *et al.*, 2019). It was confirmed that when fed to flounder, it induces a non-specific immune response and strengthens resistance to *S. parauberis* (Kim and Heo, 2019).

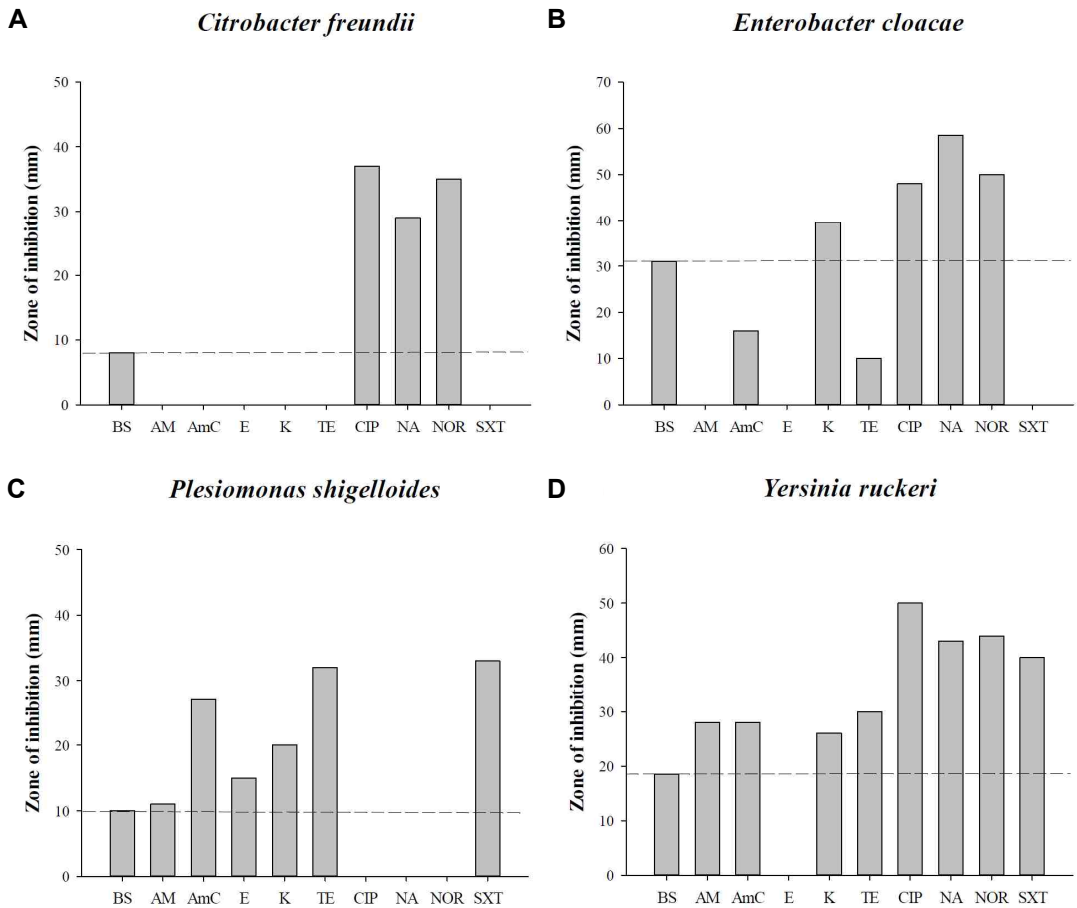

Fig. 3. Antibacterial activity of *B. subtilis* KCTC1326 against gut bacteria isolated from freshwater and comparison with antibiotics BS, *Bacillus subtilis*; AM, ampicillin (10 μg); AmC, amoxicillin/clavulanic acid (20/10 μg); E, erythromycin (15 μg); K, kanamycin (30 μg); TE, tetracycline (30 μg); CIP, ciprofloxacin (5 μg); NA, nalidixic acid (30 μg); NOR, norfloxacin (10 μg); SXT, sulfamethoxazole/trimethoprim (23.75/1.25 μg).

Additionally, supplementation of *B. subtilis* in aquaculture improved water quality, growth performance, and digestive enzyme activity in *Litopenaeus vannamei*, while also enhancing resistance against *V. harveyi* infection (Zokaeifar *et al.*, 2014). Additionally, *B. subtilis* has the advantage of being spore-forming and heat-stable. Therefore, it is not inactivated by the heat generated during product manufacturing and can be stored in a dry form (De Souza *et al.*, 2012).

This study conducted to evaluate the biochemistry characteristics of *B. subtilis* KCTC 1326. Logan and Berkeley (1984) performed API 50CHB test on 131 *B. subtilis* isolates and presented the percentage positive results for each tests. While they presented 97% of positive results of D-turanose, this result showed negative. The percentage positive results of L-sorbose, L-rhamnose, potassium gluconate, and potassium 5-ketogluconate ranged 2~4%, but in this study, they were found to be negative. Other reactions were consistent with 131 *B. subtilis* isolates. Furthermore, the results of this study showed that the protease activity was consistent with the findings of the previous paper (Hashmi *et al.*, 2022).

In this study, 9 types of antibiotics were used.

Table 3. Antimicrobial activity of *B. subtilis* KCTC 1326 and comparison with antibiotics

	BS	Antibiotics (mm)								
		AM	AmC	E	K	TE	CIP	NA	NOR	SXT
Aeromonas hydrophila	16.5	0	15	19	25	30	37	41	33	33
Aeromonas salmonicida	11.5	43	46	25	30	19	42	16	38	40
Aeromonas sobria	20	0	21	24	28	18	38	17	39	37
Aeromonas veronii	25	0	27	32	26	35	38	13	40	29
Edwardsiella piscicida	17	33	44	0	24	10	42	0	38	41
Lactococcus garvieae	24	57.5	53	46	12	38	33	0	26	26
Streptococcus parauberis	34	30	57	66	26	0	40	0	33	36
Vibrio harveyi	14	0	22	14	15.8	28	25	30.5	25	25
Citrobacter freundii	8	0	0	0	0	0	37	29	35	0
Enterobacter cloacae	31	0	16	0	39.5	10	48	58.5	50	0
Plesiomonas shigelloides	10	11	27	15	20	32	0	0	0	33
Yersinia ruckeri	18.5	28	28	0	26	30	50	43	44	40

AM, ampicillin; AmC, amoxicillin/clavulanic acid; E, erythromycin; K, kanamycin; TE, tetracycline; CIP, ciprofloxacin; NA, nalidixic acid; NOR, norfloxacin; SXT, sulfamethoxazole/trimethoprim.

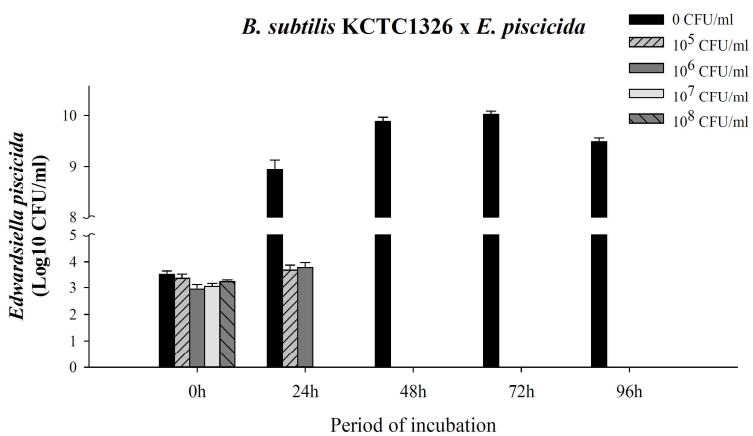

Fig. 4. Inhibitory activity of *B. subtilis* KCTC 1326 against *E. piscicida* in co-culture. The data represent the average values of triplicate samples and are presented as mean ± standard deviation (SD).

Among them, ciprofloxacin and norfloxacin are fluoroquinolones. Although the use of fluoroquinolones is currently prohibited in aquaculture, they were utilized in this experiment for scientific research purposes. When comparing the antibacterial activity against bacteria isolated from freshwater fish, intestinal bacteria, and bacteria isolated from marine fish, *B. subtilis* KCTC 1326 exhibited antibacterial effects regardless of salinity conditions in both freshwater and marine environments. All *Aeromonas* genus used in the experiments, except for *A. salmonicida*, exhibited resistance to the antibiotic ampicillin. *B. subtilis* KCTC 1326 showed as much antibacterial activity as other antibiotics against *A. sobria* and *A. veronii*. In the case of marine-derived bacteria, overall, *B. subtilis* KCTC 1326 exhibited high antimicrobial activity, with *S. parauberis* showing the highest antimicrobial effect at 34 mm. *C. freundii*, *E. cloacae*, *P. shigelloides*, and *Y. ruckeri* belonging to the *Enterobacterales* order exhibited higher antibiotic resistance compared to bacteria isolated from *Aeromonas* genus and marine fish. Specifically, in this study, *C. freundii* showed resistance to 6 types of 9 types of antibiotics tested (ampicillin, amoxicillin/clavulanic

Table 4. Antibiotics susceptibility analysis of *Bacillus subtilis* KCTC 1326 using minimum inhibitory concentration (MIC) test

Antibiotics Group	Antibiotics	Range	MIC (μg/mL)
Cephalosporin	Ceftiofur	0.03~32	0.5
	Cephalexin	0.25~64	-
β-Lactam	Ampicillin	0.25~128	-
	Amoxicillin	0.06~16	0.12
	Amoxicillin/Clavulanic acid	0.06/0.03~8/4	0.12/0.06
Lincomycin	Clindamycin	0.015~16	0.5
Macrolide	Erythromycin	0.03~64	0.12
Phenicol	Florfenicol	0.06~64	1
Aminoglycoside	Gentamycin	0.12~32	-
	Neomycin	0.5~64	-
Tetracycline	Doxycycline	0.12~128	1
	Oxytetracycline	0.12~256	64
	Tetracycline	0.06~64	8
Sulfonamide	Trimethoprim/Sulfadiazine	0.12/2.38~16/304	-
Quinolone	Ciprofloxacin	0.015~64	0.06
	Enrofloxacin	0.03~32	0.06
	Flumequine	0.12~128	0.5
	Nalidixic acid	0.5~64	8
	Oxolinic acid	0.5~32	-

acid, erythromycin, kanamycin, tetracycline, sulfamethoxazole/trimethoprim), which aligns with previous findings showing resistance to β-lactam antibiotics such as ampicillin, clindamycin, lincomycin, and penicillin (Zhao *et al.*, 2022). Additionally, *E. cloacae* and *P. shigelloides* also demonstrated resistance to various antibiotics in line with our study, consistent with the results of other researchers (Mabrok *et al.*, 2024; Adesiyan *et al.*, 2019).

For our co-culture inhibition study, *B. subtilis* first needed to demonstrate antimicrobial effects against the fish pathogenic bacteria. Additionally, it was necessary to distinguish between the colonies of *B. subtilis* and the fish pathogenic bacteria for CFU measurement. In this regard, *E. piscicida* was selected as the optimal bacterium for co-culture inhibition experiments because *B. subtilis* KCTC 1326 does not grow on the selective SS agar, whereas *E. piscicida* produces H_2S and forms black colonies on SS agar. In this study, *B. subtilis* KCTC 1326 and *E. piscicida* were co-cultured to observe inhibition of *E. piscicida* growth. Co-culture experiments showed that increasing concentrations of *B. subtilis* KCTC 1326 inhibited the growth of *E. piscicida in vitro*. This could be due to the inhibition caused by antibacterial substances produced by *B. subtilis*, such as lantibiotics, rhizocticin, surfactin, and mycosubtilin (Abriouel *et al.*, 2011).

According to our research findings, *B. subtilis* exhibited discursive antimicrobial activity against all 12 species of fish disease bacteria, indicating promising potential for its application as a probiotic in aquaculture. When fed a diet supplemented with *Bacillus* spp., Nile tilapia showed increased immunity and intestinal microvilli (Costa *et al.*, 2024). Additionally, for future use as probiotics in fish, they must not only be safe for fish but also should not harbor acquired and transferable antibiotic resistance (Guo *et al.*, 2016). Therefore, our study also measured MIC to assess the potential as probiotics in the future.

According to the European food safety authority (EFSA), *B. subtilis* KCTC 1326 has shown antibiotic susceptibility to clindamycin, erythromycin, gentamycin, and tetracycline. However, clear criteria for interpreting MIC values for other antibiotics were not provided. However, in this study, the MIC values of oxytetracycline and nalidixic acid were 64 μg/mL and 8 μg/mL, respectively, which are relatively high compared to other antibiotics, indicating the possibility of antibiotic resistance. This suggests that if *B. subtilis* KCTC 1326 accumulates in the body at high concentrations, it is easily removed by antibiotics.

In conclusion, this study demonstrated the antibacterial effects of *B. subtilis* KCTC 1326 against bacterial diseases occurring in 12 species of fish, indicating its potential as a promising candidate for controlling bacterial diseases in aquaculture. Further exploration into its mechanisms of action, ecological impacts, commercial viability, and long-term effects is warranted to fully assess its utility and safety in aquaculture practices.

Acknowledgement

This work was supported by the National Research Foundation of Korea grant (NRF-2023R1A2C1008182) and the Korea Innovation Foundation grant (2023-DD-UP-0007) funded by the Ministry of Science and ICT (MSIT).

References

Abarike, E.D., Cai, J., Lu, Y., Yu, H., Chen, L., Jian, J., Tang, J., Jun, L. and Kuebutornye, F.K.A.: Effects of a commercial probiotic BS containing *Bacillus subtilis* and *Bacillus licheniformis* on growth, immune response and disease resistance in Nile tilapia, *Oreochromis niloticus*. Fish & shellfish immunology, 82:229-238, 2018. https://doi.org/10.1016/j.fsi.2018.08.037

Abriouel, H., Franz, C.M.A.P., Omar, N.B. and Gálvez, A.: Diversity and applications of *Bacillus* bacteriocins. FEMS microbiology reviews, 35(1):201-232, 2011. https://doi.org/10.1111/j.1574-6976.2010.00244.x

Adesiyan, I.M., Bisi-Johnson, M.A., Ogunfowokan, A.O. and Okoh, A.I.: Incidence and antimicrobial susceptibility fingerprints of *Plesiomonas shigelliodes* isolates in water samples collected from some freshwater resources in Southwest Nigeria. Science of the total environment, 665:632-640, 2019. https://doi.org/10.1016/j.scitotenv.2019.02.062

Anagnostopoulos, C. and Spizizen, J.: Requirements for transformation in *Bacillus subtilis*. Journal of Bacteriology, 81:741-746, 1961. https://doi.org/10.1128/jb.81.5.741-746.1961

Blibech, M., Mouelhi, S., Farhat-Khemakhem, A., Boukhris, I., Ayeb, A.E. and Chouayekh, H.: Selection of *Bacillus subtilis* US191 as a mannanase-producing probiotic candidate. Biotechnology and Applied Biochemistry, 66(5):858-869, 2019. https://doi.org/10.1002/bab.1798

Caulier, S., Nannan, C., Gillis, A., Licciardi, F., Bragard, C. and Mahillon, J.: Overview of the antimicrobial compounds produced by members of the *Bacillus subtilis* group. Frontiers in microbiology, 10:435128, 2019. https://doi.org/10.3389/fmicb.2019.00302

Costa, T.S., Copatti, C.E., da Silva, J.J., Marchão, R.S., da Silva, R.C., da Silva Rocha, A., Pereira, G.A., da Rocha, D.R., de Souza, A.M., da Costa, M.M., Martins, I.P.S., Moreira, J.F. and Melo, J.F.B.: Use of *Bacillus subtilis* multiplicate in the water used for biofloc formation: Growth, hemato-biochemistry, intestinal bacteria colonies, and bacterial resistance evaluations of Nile tilapia. Aquaculture, 590:741039, 2024. https://doi.org/10.1016/j.aquaculture.2024.741039

De Souza, D.M., Suita, S.M., Leite, F.P.L., Romano, L.A., Wasielesky, W. and Ballester, E.L.C.: The use of probiotics during the nursery rearing of the pink shrimp *Farfantepenaeus brasiliensis* (Latreille, 1817) in a zero exchange system. Aquaculture Research, 43(12):1828-1837, 2012. https://doi.org/10.1111/j.1365-2109.2011.02992.x

El-Saadony, M.T., Alagawany, M., Patra, A.K., Kar, I., Tiwari, R., Dawood, M.A.O., Dhama, K. and Abdel-Latif, H.M.R.: The functionality of probiotics in aquaculture: An overview. Fish & shellfish immunology, 117:36-52, 2021. https://doi.org/10.1016/j.fsi.2021.07.007

Guo, X., Chen, D.D., Peng, K.S., Cui, Z.W., Zhang, X.J., Li, S. and Zhang, Y.A.: Identification and characterization of *Bacillus subtilis* from grass carp (*Ctenopharynodon idellus*) for use as probiotic additives in aquatic feed. Fish & shellfish immunology, 52:74-

84, 2016. https://doi.org/10.1016/j.fsi.2016.03.017

Hao, K., Wu, Z.Q., Li, D.L., Yu, X.B., Wang, G.X. and Ling, F.: Effects of dietary administration of *Shewanella xiamenensis* A-1, *Aeromonas veronii* A-7, and *Bacillus subtilis*, single or combined, on the grass carp (*Ctenopharyngodon idella*) intestinal microbiota. Probiotics and antimicrobial proteins, 9:386-396, 2017. https://doi.org/10.1007/s12602-017-9269-7

Hashmi, S., Iqbal, S., Ahmed, I. and Janjua, H.A.: Production, optimization, and partial purification of alkali-thermotolerant proteases from newly isolated *Bacillus subtilis* S1 and *Bacillus amyloliquefaciens* KSM12. Processes, 10(6):1050, 2022. https://doi.org/10.3390/pr10061050

Janda, J.M. and Abbott, S.L.: The changing face of the family *Enterobacteriaceae* (Order: "*Enterobacterales*"): New members, taxonomic issues, geographic expansion, and new diseases and disease syndromes. Clinical microbiology reviews, 34(2):10-1128, 2021. https://doi.org/10.1128/cmr.00174-20

Kim, D.H. and Heo, M.S.: Dietary effect of *Bacillus subtilis* MD-02 on Innate Immune Response and Disease Resistance in Olive Flounder, *Paralichthys olivaceus*. Microbiol. Biotechnol. Lett., 47(1):132–138, 2019. https://doi.org/10.4014/mbl.1807.07003

Kim, H.W., Lee, E.S., Lee, S.J., Han, S.R., Oh, T.J., Kim, M.S., Kim, S.J. and Kwon, S.R.: The antibiotic resistance of *Lactococcus lactis* isolated from the farmed *Anguilla japonica*. J. Fish Pathol., 36(1):107-116, 2023a. https://doi.org/10.7847/jfp.2023.36.1.107

Kim, H.W., Lee, E.S., Lee, S.J., Kim, H.E., Han, S.R., Oh, T.J., Kim, M.S., Kim, S.J. and Kwon, S.R.: Characterization of *Photobacterium* sp. YW2207 isolated from rainbow trout (*Oncorhynchus mykiss*) raised in a fresh water farm in South Korea. J. Fish Pathol., 36(2):251-261, 2023b. https://doi.org/10.7847/jfp.2023.36.2.251

Kuebutornye, F.K., Abarike, E.D. and Lu, Y.: A review on the application of *Bacillus* as probiotics in aquaculture. Fish & shellfish immunology, 87:820-828, 2019. https://doi.org/10.1016/j.fsi.2019.02.010

Lee, S.H., Katya, K., Park, Y.J., Won, S.H., Seong, M.J., Hamidoghli, A. and Bai, S.C.: Comparative evaluation of dietary probiotics *Bacillus subtilis* WB60 and *Lactobacillus plantarum* KCTC3928 on the growth performance, immunological parameters, gut morphology and disease resistance in Japanese eel, *Anguilla japonica*. Fish & shellfish immunology, 61:201-210, 2017. https://doi.org/10.1016/j.fsi.2016.12.035

Logan, N.A. and Berkeley, R.C.W.: Identification of *Bacillus* strains using the API system. Microbiology, 130(7):1871-1882, 1984. https://doi.org/10.1099/00021287-130-7-1871

Mabrok, M., Algammal, A.M., El-Tarabili, R.M., Dessouki, A.A., ElBanna, N.I., Abd-Elnaby, M., El-Lamie M.M.M. and Rodkhum, C.: *Enterobacter cloacae* as a re-emerging pathogen affecting mullets (*Mugil* spp.): Pathogenicity testing, LD_{50}, antibiogram, and encoded antimicrobial resistance genes. Aquaculture, 583:740619, 2024. https://doi.org/10.1016/j.aquaculture.2024.740619

Nair, A.V., Antony, M.L., Praveen, N.K., Sayooj, P., Swaminathan, T.R. and Vijayan, K.K.: Evaluation of *in vitro* and *in vivo* potential of *Bacillus subtilis* MBTDCMFRI Ba37 as a candidate probiont in fish health management. Microbial Pathogenesis, 152:104610, 2021. https://doi.org/10.1016/j.micpath.2020.104610

Nayak, S.K.: Multifaceted applications of probiotic *Bacillus* species in aquaculture with special reference to *Bacillus subtilis*. Reviews in Aquaculture, 13(2):862-906, 2021. https://doi.org/10.1111/raq.12503

Pereira, C., Duarte, J., Costa, P., Braz, M. and Almeida, A.: Bacteriophages in the control of *Aeromonas* sp. in aquaculture systems: an integrative view. Antibiotics, 11(2):163, 2022. https://doi.org/10.3390/antibiotics11020163

Ren, X., Wu, B., Zhao, F., Qi, L., Qiu, X., Li, R., Yang, S., Yi, G., Ding, X., Xia, L. and Sun, Y.: Antagonistic activity and protective effect of a *Bacillus subtilis* isolate against fish pathogen *Edwardsiella piscicida*. Fisheries science, 85:1011-1018, 2019. https://doi.org/10.1007/s12562-019-01346-8

Rohani, M.F., Islam, S.M.M., Hossain, M.K., Ferdous, Z., Siddik, M.A.B., Nuruzzaman, M., Padeniya, U., Brown, C. and Shahjahan, M.: Probiotics, prebiotics and synbiotics improved the functionality of aquafeed: Upgrading growth, reproduction, immunity and disease resistance in fish. Fish & shellfish immunology, 120:569-589, 2022. https://doi.org/10.1016/j.fsi.2021.12.037

Zhao, C., Wen, H., Huang, S., Weng, S. and He, J.: A novel disease (water bubble disease) of the giant freshwater prawn *Macrobrachium rosenbergii* caused by *Citrobacter freundii*: antibiotic treatment and effects on the antioxidant enzyme activity and immune responses. Antioxidants, 11(8):1491, 2022. https://doi.org/10.3390/antiox11081491

Zokaeifar, H., Babaei, N., Saad, C.R., Kamarudin, M.S.,

Sijam, K. and Balcazar, J.L.: Administration of *Bacillus subtilis* strains in the rearing water enhances the water quality, growth performance, immune response, and resistance against *Vibrio harveyi* infection in juvenile white shrimp, *Litopenaeus vannamei*. Fish & shellfish immunology, 36(1):68-74, 2014. https://doi.org/10.1016/j.fsi.2013.10.007

Zuenko, V.A., Laktionov, K.S., Pravdin, I.V., Kravtsova, L.Z. and Ushakova, N.A.: Effect of *Bacillus subtilis* in feed probiotic on the digestion of fish cultured in cages. Journal of ichthyology, 57:152-157, 2017. https://doi.org/10.1134/S0032945217010143

Manuscript Received: May 24, 2024
Revised: Jun 10, 2024
Accepted: Jun 12, 2024