



Characterization and safety evaluation of partially purified bacteriocin produced by *Escherichia coli* E isolated from fermented pineapple *Ananas comosus* (L.) Merr

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Abstract

Antibacterial activity of cell-free supernatant from *Escherichia coli* E against selected pathogenic bacteria in food and aquaculture was the highest against *Edwardsiella tarda* 3, a significant aquaculture pathogen. Biochemical properties of the bacteriocins were studied and bacteriocin was found to be sensitive to proteinase K, demonstrating its proteinaceous nature. In addition, pH and temperature affected bacteriocin activity and stability. The bacteriocins were partially purified by ammonium sulfate precipitation. The antibacterial activity was only detected in 20% ammonium sulfate fraction and direct detection of its activity was performed by overlaying on the indicator strains. The inhibition zone associated with the antibacterial activity was detected in the sample overlaid by *E. tarda* 3 and *Staphylococcus aureus* DMST8840 with the relative molecular mass of about 27 kDa and 10 kDa, respectively. Bacteriocin showed no cytotoxic effect on NIH-3T3 cell line; however, two virulence genes, *aer* and *sfa*, were detected in the genome of *E. coli* E by PCR. The characteristics of bacteriocins produced by *E. coli* E exhibited the antibacterial activity against both Gram-positive and Gram-negative pathogenic bacteria and the safe use determined by cytotoxicity test which may have interesting biotechnological applications.

Keywords Bacteriocin · Cytotoxicity assay · *Escherichia coli* E · Safety evaluation · Virulence genes

Introduction

Despite the growth of antibiotic-resistant bacteria, the development of new and effective antibiotic is steeply declining [1]. The discovery and development of novel antibacterial agents have therefore been implemented by many research groups to

develop alternatives to antibiotic treatment [2–4]. New methods are currently under consideration including the development of bacteriocins, which are antibacterial peptides [2, 5, 6]. Bacteriocins are ribosomally synthesized by bacteria that inhibit or kill closely related species [7, 8]. Bacteriocins differ from classical antibiotics in term of synthesis and spectrum of killing activity, and some can be considered pathogen-specific designer drugs [5, 6]. The high level of diversity of bacteriocins produced by Gram-positive and Gram-negative bacteria has attracted great interest because of their potential applications in food preservation, human health, livestock rearing, agriculture, and aquaculture [6, 9–11].

Aquaculture is the fastest-growing food production industry. However, intensive culture has led to outbreaks of various bacterial diseases [12]. Bacterial species belonging to at least 13 genera have been reported to be pathogenic to aquatic animals including Gram-negative bacteria such as *Aeromonas*, *Edwardsiella*, *Flavobacterium*, *Francisella*, *Photobacterium*, *Piscirickettsia*, *Pseudomonas*, *Tenacibaculum*, *Vibrio*, and *Yersinia* and Gram-positive bacteria such as *Lactococcus*, *Renibacterium*, and *Streptococcus*

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[12, 13]. *Edwardsiella tarda* is an intracellular pathogen accounting for enormous economic losses in cultured seawater and freshwater fish [14, 15]. To control bacterial diseases, vaccines and antibiotics have been used [6, 12]. However, these methods can result in high cost, high stress to the animals, the long half-life of drugs, and selection for antibiotic-resistant bacteria [6, 9, 15]. Therefore, the use of bacteriocin-producing probiotic bacteria is an alternative approach to disease prevention in aquaculture [6, 9, 13]. In addition to pathogenic bacteria in aquaculture, food contamination by pathogenic bacteria has been a serious health problem. *Salmonella enterica*, *Escherichia coli* O157:H7, *Shigella* spp., and *Staphylococcus aureus* are among the major causes of outbreaks of foodborne diseases [16, 17]. Many studies carried out in recent years clearly demonstrate that the application of bacteriocins in food preservation can decrease the risks for transmission of foodborne pathogens through the food chain, extend shelf life, and reduce the application of chemical preservation [18–20].

Escherichia coli is known to produce two types of bacteriocins: colicins and microcins [3, 6, 21]. Bacteriocins produced by nonpathogenic *E. coli* strains are a potential candidate for inhibition of pathogens such as *Salmonella* [22]. The study showed that the majority of bacteriocin-producing *E. coli* strains produce more than one bacteriocin [21]. Colicins are diverse and they are the most extensively studied bacteriocins in *E. coli* [6]. The colicins of *E. coli* have served as a model system for investigating the mechanisms of bacteriocin structure and function, genetic organization, ecology, and evolution [6, 8, 23]. They can kill by either membrane permeabilization, nuclease activity, or inhibition of peptidoglycan and lipopolysaccharide biosynthesis [7, 24, 25]. However, colicin is not active against the producing bacteria due to the presence of a specific inhibitor called the immunity protein [7]. Microcins are bacteriocins produced by enterobacteria with molecular masses below 10 kDa [4, 26, 27]. Microcins exhibit a diversity of structures and mechanisms of action such as the inhibition of vital enzymatic functions and damage to the inner membrane [26].

A few studies have reported the cytotoxicity and virulence factors of bacteriocins [5, 28, 29]. Virulence factors have played an important role in the pathogenicity of *E. coli* strains associated with urinary tract infection by overcoming host defense mechanisms and causing disease [30]. Several genes encoding virulence factors such as hemolysin (*hly*), cytotoxic necrotizing factor I (*cnf I*), S-family adhesions (*sfa*), pyelonephritis-associated pili (*pap*), and aerobactin (*aer*) have been detected by PCR and shown to have higher prevalence of the presence of virulence genes in uropathogenic *E. coli* strains [31, 32]. Therefore, for a safe application of using bacteriocin as an antibacterial agent in aquaculture and food industry, several genetic determinants of virulence factors should be of concern. Thus, the aim of this study was to

investigate a new antibacterial agent from bacteriocin-producing bacteria isolated from fermented fruit. *E. coli* E was previously isolated from fermented pineapple, *Ananas comosus* and the antibacterial activity against pathogenic bacteria in aquaculture was determined [33]. Due to its potential application as an antibacterial agent, the safety of bacteriocins was evaluated. In addition, virulence factors which play an important role in the pathogenicity of *E. coli* E were also investigated.

Materials and methods

Bacterial strains and culture conditions

The *E. coli* E strain was isolated as described previously [33]. Selected pathogenic bacteria in food and aquaculture including *Bacillus cereus*, *Escherichia coli*, *Streptococcus* sp., *Salmonella* sp., *Staphylococcus aureus* DMST8840, *Shigella* sp., *Pseudomonas aeruginosa* 5, *Vibrio cholera* 7, *Vibrio parahaemolyticus* 5, *Vibrio alginolyticus* 3, *Aeromonas hydrophila* 9, and *Edwardsiella tarda* 3 were laboratory strains/isolate which obtained from the culture collection of the Microbiology Laboratory, Faculty of Science and Technology, Prince of Songkla University and Faculty of Agro Based Industry, Universiti Malaysia Kelantan Campus Jeli. Bacteriocin producer *E. coli* E were cultured in a Luria-Bertani (LB) medium with shaking at 37 °C and maintained in 20% (v/v) glycerol. All pathogenic bacteria were cultured in nutrient broth (NB) in a 37 °C shaker. All *Vibrio* strains were grown on NB supplemented with 3% NaCl.

Bacteriocin detection and production

The pre-culture of *E. coli* E strain was inoculated into 250 ml of sterilized LB. Cell culture was then adjusted to the same initial optical density ($OD_{600nm} = 0.05$) and incubated in a 37 °C shaker at 180 rpm for 6 h. The obtained culture was centrifuged in order to get the cell-free supernatant (CFS).

The activity of bacteriocin expressed in arbitrary units ($AU\ ml^{-1}$) was determined through a twofold broth dilution method against *E. tarda* 3. One AU is defined as the highest serial twofold dilution which prevents the visible turbidity of an indicator strain in a well per 1 ml after 24 h incubation [34].

Antibacterial activity of *E. coli* E against all pathogenic microorganisms was determined by a well diffusion method [35]. The pathogenic bacteria were inoculated into 25 ml sterilized NA medium (with final concentration $10^6\ CFU\ ml^{-1}$). Wells with the diameter of 5 mm were then filled up with 90 μ l of CFS. The bacteriocin activity was determined by measuring the diameter of the inhibition zone around the wells after incubation for 12–36 h at room temperature (28–30 °C) depending on the growth of the pathogenic strains. At least two

independent experiments were done for each tested pathogenic bacterium.

Sensitivity to heat, pH, and enzymes

The thermal sensitivity of bacteriocin was tested by incubation of 1 ml CFS at 45 °C, 70 °C, and 90 °C for 15, 30, 60, and 120 min and 121 °C for 20 min then stored immediately on ice followed by a subsequent antibacterial assay. A positive control (no heat treatment at 30 °C) and a negative control (culture medium) were included.

To determine the effect of pH on antibacterial activity, CFS was adjusted to different pH values [2–12] using 1 M HCl and 1 M NaOH and then incubated at room temperature for 30 and 120 min. The agar diffusion method was performed to measure the residual activity after neutralizing treated samples to pH 7.0 [36].

The sensitivity to enzymatic proteolysis was evaluated by treatment of CFS with the final concentration of 1 mg ml⁻¹ of the following proteolytic enzymes: proteinase K (Vivantis, Malaysia) in Tris buffer pH 8.0, trypsin (Sigma, USA) in sodium phosphate buffer (pH 7.0), catalase (Sigma, USA) in phosphate buffer pH 7.0 (potassium phosphate buffer). After treatment, the enzymes were heat inactivated at 100 °C for 3 min. CFS without enzymes and culture medium were used as a positive control and negative control, respectively. The residual activity was then determined by using the well diffusion method against the most sensitive pathogenic bacteria in food and aquaculture including *Shigella* sp. and *E. tarda* 3.

Acid and bile tolerance

Acid and bile tolerance was determined using the modified methods according to previously described [37]. For acid tolerance test, 1 ml of the culture was centrifuged at 1677g when the OD_{600nm} reached 1.0. The cell pellets were then washed with 1× PBS (phosphate buffered saline) twice and resuspended in 1 ml of the same buffer. One hundred microliters of cell suspensions was mixed into 900 µl of 1× PBS with the pH values of 1, 2, 3, and 4 (adjusted using 1 mol l⁻¹ HCl). The mixtures were incubated at 37 °C for 0, 1, 2, and 3 h. The serial dilutions were made with 0.9% NaCl and plated onto NA media by the spread plate method. The plates were incubated at 37 °C for 24 h. The acid tolerance of *E. coli* E was expressed by the number of survival cells.

For bile tolerance test, the culture was collected when the growth of *E. coli* E reached OD_{600nm} 1.0. One hundred microliters of the culture was spread onto NA agar with bile salt concentrations of 0.1%, 0.2%, 0.3%, and 0.6%. The growth of *E. coli* E was detected using the standard agar plate method after incubation at 37 °C for 24 h.

Partial purification

CFS obtained from 1 L of culture was precipitated with ammonium sulfate at different concentrations (20%, 30%, and 50%) at 4 °C for 24 h using the modified method as previously described [33]. The pellet was collected by centrifugation at 20,130g for 20 min and dissolved with 25 mM Tris-HCl (pH 8) and then dialyzed against the same buffer for 12 h at 4 °C. The partially purified products were concentrated using freeze dryer and lyophilized products were stored in -20 °C. Each resuspended fraction was filtered with filter membrane (0.45 µm) and screened for its antibacterial activity using the well diffusion method against two indicator strains including *Shigella* sp. and *E. tarda* 3. The fraction which exhibited inhibition activity was collected for molecular weight determination.

Direct activity and molecular weight determination

The partially purified bacteriocin was detected through 18% separating gel and 5% stacking gel with 30% acrylamide and 0.8% bis-acrylamide under denaturing and non-denaturing conditions [36]. Duplicate bacteriocin samples and a protein ladder (10–250 kDa, New England BioLabs) were loaded onto a polyacrylamide gel. After electrophoresis, the gel was cut into two parts: the first part containing one of the bacteriocin samples and standard protein was stained with Coomassie brilliant blue G-250 (Sigma-Aldrich, Steinheim, Germany) to determine molecular weight. The second part containing other bacteriocin samples was assayed for direct detection of activity. This part was washed repeatedly for 24 h with distilled water and placed on a pre-poured TSA plate which was then overlaid with 5 ml melted TSA containing indicator strains including *Shigella* sp., *E. tarda* 3, and *S. aureus* DMST8840 [28]. The plate was then incubated at room temperature for 24 h and observed for the clear zone around bands. Protein concentration was determined by the Bradford protein assay (Bio-Rad) using BSA as the standard.

Cell culture

Mouse embryonic fibroblast NIH-3T3 cells (ATCC®CRL1658™) were obtained from the Faculty of Medicine, Khon Kaen University. The cells were cultured in Dulbecco's minimal Eagle medium (DMEM, Invitrogen, CA, USA) supplemented with 10% fetal bovine serum, 100 U ml⁻¹ penicillin, and 100 U ml⁻¹ streptomycin (Invitrogen). Cells were cultured in a humidified 5% CO₂ incubator at 37 °C. Cells were subcultured with 0.25% trypsin-EDTA when reaching 90% confluence. Cells with more than 90% viability were used in all experiments.

Cytotoxicity assay

To determine the effect of CFS on NIH-3T3 viability, a cytotoxicity assay was performed using thiazolyl blue tetrazolium bromide (MTT, Sigma) according to previously described [38]. Cells were seeded in 96-well plate at 5×10^5 cells per well in the complete media and incubated for 24 h. The media were replaced by CFS in various dilutions (1/8, 1/16, 1/32, 1/64, and 1/128) and incubated for 72 h. DMEM containing 6.25 mg ml^{-1} nisin (Sigma) was used as a positive control. All conditions were added with fresh media to $150 \mu\text{l}$ per well prior to add with 2 mg ml^{-1} of MTT at $50 \mu\text{l}$ per well and incubated for 4 h. The supernatants were removed and formazan crystals were dissolved by DMSO. The amount of formazan crystal was measured at 570 nm by a microplate reader (Tecan, Switzerland) and converted to a percentage of viable cells compared to control. All samples were used in three independent experiments.

DNA extraction and PCR amplification of virulence genes

Genomic DNA from *E. coli* E was isolated with DNAzol® reagent (Invitrogen, USA) as recommended by the manufacturer. The extracted DNA was analyzed with 1% (w/v) agarose gel electrophoresis. Detection of *hly*, *aer*, *cnf I*, *sfa*, and *pap* genes was performed by amplifying the genes by PCR. The primers sequences were previously reported in the literatures [31, 32] (Table 1). Primers for *hly*, *aer*, *cnf I*, *sfa*, and *pap* amplified DNA fragments of 1177, 602, 498, 410, and 328 bp, respectively. Expected sizes of the amplicons were ascertained by electrophoresis in 1% agarose gel with an appropriate molecular size marker (GeneRuler™ 1 kb, Fermentas). PCR products were visualized under UV after ethidium bromide staining.

Statistical analyses

All results were presented as mean \pm SD. Multiple comparisons among treatments were made using ANOVA with Tukey's multiple comparison test.

Results

Bacteriocin production and inhibitory activity

The inhibitory spectrum of the bacteriocins by *E. coli* strain E against different pathogenic bacteria in food and aquaculture was determined by the well diffusion method. The results indicated that the CFS obtained from *E. coli* E was able to inhibit various selected pathogens as shown in Table 2. *S. aureus* DMST8840 and *Shigella* sp. were the most sensitive

to CFS among food pathogens. The highest antibacterial activity was found against *E. tarda* 3, the most significant pathogen in aquaculture, with an inhibition zone of $25.6 \pm 0.58 \text{ mm}$. These indicator stains were then selected for further experiments. Unlike some bacteriocins from *E. coli* H22 exhibiting inhibitory activity against Gram-negative indicator strains tested of the family *Enterobacteriaceae* [25], *E. coli* E exhibited a relatively broad spectrum of antibiotic activity. Culture supernatants from *E. coli* E were sampled during growth cycle under shaking and non-shaking conditions at $37 \text{ }^\circ\text{C}$ and tested for bacteriocin activity. The highest bacteriocin activity (1422 AU ml^{-1}) was reached during the early stationary phase after 5–6 h of incubation (Fig. 1). Under static conditions, the highest activity was found after a long period of cultivation (25–30 h) (data not shown). The bacteriocin production strongly decreased during stationary phase in both conditions, probably as a consequence of bacteriocin proteolysis.

Biochemical properties of bacteriocin

Crude bacterial cultures were collected and treated with various enzymes such as proteinase K, trypsin, and catalase. An enzyme sensitivity assay showed that the antibacterial activity exhibited by *E. coli* E was completely inactivated by the treatment of proteinase K. However, trypsin and catalase displayed no effect on the activity of CFS from *E. coli* E. Characterization of the proteinaceous inhibitor confirmed that the antibacterial substances produced by *E. coli* E were bacteriocins. The effect of pH and temperatures on antibacterial activity against the growth of *E. tarda* 3 by bacteriocins is shown in Fig. 2. There was no significant difference in bacteriocin activity between control (pH 7) and sample adjusted to pH 8 after the first 15 min (Fig. 2a). In addition, the results indicated that bacteriocins were the most stable at pH range 6.0–8.0 and could retain more than 80% of original activity after 120 min (Fig. 2b). However, bacteriocin activity was totally lost at extreme pH (pH 2.0 and 12.0) within the first 15 min of treatment. To evaluate the thermal stability of bacteriocin, CFS was incubated at $30 \text{ }^\circ\text{C}$ (control), $45 \text{ }^\circ\text{C}$, $70 \text{ }^\circ\text{C}$, $90 \text{ }^\circ\text{C}$, and $121 \text{ }^\circ\text{C}$ for various periods. Results indicated that the inhibitory activity significantly decreased with increasing temperature (Fig. 2c). Bacteriocins could maintain their activity after a short treatment period at $45 \text{ }^\circ\text{C}$ and $70 \text{ }^\circ\text{C}$. Its antibacterial activity remained for 120 min at $30 \text{ }^\circ\text{C}$ (Fig. 2d). However, it was found to be unstable with the loss of the antibacterial activity after autoclave treatment.

Bile salt and acid tolerance

E. coli E was further examined for the probiotic properties including acidic tolerance and bile salt tolerance. Bile salt resistance tests showed that *E. coli* E was able to survive at

Table 1 Primer pairs used for the detection of virulence genes in *E. coli* E

Gene and DNA region amplified	Primer	Primer sequence (5'–3')	References
<i>hly</i>	F	AACAAGGATAAGCACTGTTCTGGCT	[31, 32]
	R	ACCATATAAGCGGTCATTCCCCTCA	
<i>cnfI</i>	F	AAGATGGAGTTTCCCTATGCAGGAG	
	R	CATTGAGATCCTGCCCTCATTAT	
<i>sfa</i>	F	CTCCGGAGAAGTGGGTGCATCTTAC	
	R	CATCAAGCTGTTGTTCGTCCGCCG	
<i>pap</i>	F	GACGGCTGTACTGCAGGGTG TGGCG	
	R	ATATCCTTTCTGCAGGGATGCAATA	
<i>aer</i>	F	TACCGATTGTCATATGCAGACCG	[31]
	R	AATATCTTCTCCAGTCCGGAGAAG	

bile salt concentrations of 0.1%, 0.2%, and 0.3% at 37 °C for 24 h. As for acid tolerance tests, *E. coli* E did not tolerate pH 1.0. At pH 2 and 3, cells could survive for 2 h but the viable cell numbers were sharply decreased after 3 h. In this study, pH 4.0 did not affect the cell number after exposure for 3 h.

Partial purification and direct activity determination

In this study, bacteriocins produced by *E. coli* E were partially purified by mixing them with ammonium sulfate at concentrations of 20%, 30%, and 50%. The obtained fractions were screened for the antibacterial activity by the well diffusion method against both *Shigella* sp. (Fig. 3a) and *E. tarda* 3 (Fig. 3b). The antagonistic activity was only detected in 20% ammonium sulfate fraction as shown in Fig. 3. In the active fraction, at least three distinct protein bands were detected by SDS-PAGE (Fig. 4a, lane 2). Direct detection of the bacteriocins activity was performed by overlaying them on the indicator strain (Fig. 4b). Only the apparent molecular masses of about 27 kDa and 10 kDa from 20% ammonium sulfate fraction showed inhibitory activity against *E. tarda* 3 and *S. aureus* DMST8840, respectively, whereas other fractions

failed to inhibit the growth of tested bacteria (Fig. 4b, lanes 5a and 6b).

Cytotoxicity and determination of virulence genes in *E. coli* E

The effect of CFS on NIH-3T3 viability was measured using an MTT assay. The result showed that CFS at all conditions and nisin did not significantly alter NIH-3T3 viability compared to that of control (Fig. 5). To confirm the safety of *E. coli* E, PCR was used to detect whether virulence genes were present in the genome. The results revealed that there were two virulence genes including *aer* (602 bp) and *sfa* (410 bp) detected in the genome of *E. coli* E (Fig. 6).

Discussion

We further characterized *E. coli* E which isolated from fermented pineapple from the previous study [33] by using the Clermont *E. coli* phylo-typing method [39]. Base on the quadruplex genotype obtained, an *E. coli* E is assigned to a phylo-group F (+ + - -, indicating *arpA*-, *chuA*+, *yjaA*-, and

Table 2 Inhibitory spectrum of CFS produced by *E. coli* E on several pathogenic bacteria in food and aquaculture

Indicator strains	Diameter of inhibition zone (mm)	Indicator strains	Diameter of inhibition zone (mm)
<i>Bacillus cereus</i>	0 ^a	<i>P. aeruginosa</i> 5	3.5 ^b ± 0.71
<i>Escherichia coli</i>	0 ^a	<i>V. cholerae</i> 7	4.5 ^{bc} ± 0.71
<i>Streptococcus</i> sp.	5.0 ^{bc} ± 1.00	<i>A. hydrophila</i> 9	0 ^a
<i>Salmonella</i> sp.	0 ^a	<i>E. tarda</i> 3	25.6 ^c ± 0.58
<i>S. aureus</i> DMST8840	14.3 ^d ± 1.53	<i>V. alginolyticus</i> 3	5.7 ^{bc} ± 0.58
<i>Shigella</i> sp.	16.0 ^d ± 1.00	<i>V. parahaemolyticus</i> 5	7.0 ^c ± 1.00

Data are mean ± SD from three independent replicates (ANOVA, Tukey's multiple comparison test, $P < 0.01$). Different superscript letters indicate the significant different of mean value by ANOVA with Tukey's multiple comparison test

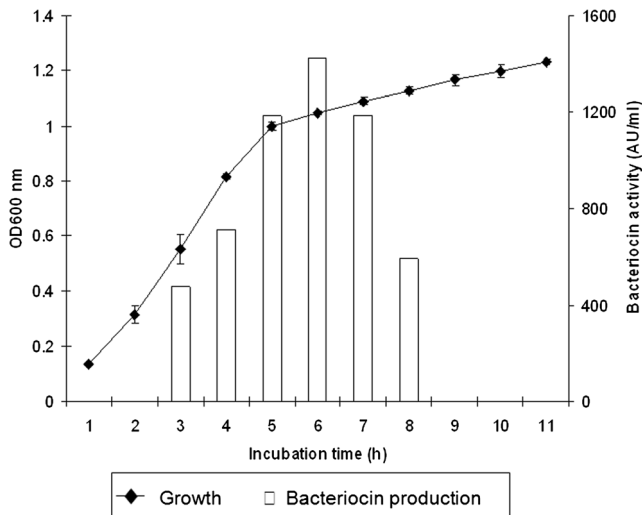
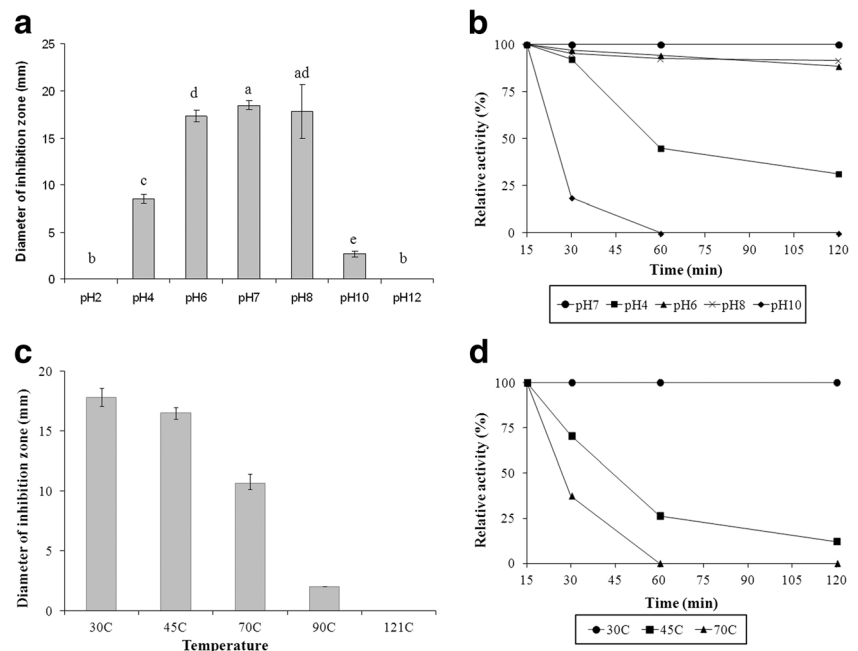


Fig. 1 Growth of *E. coli* E and production of bacteriocin expressed in arbitrary unit (AU ml⁻¹) under shaking condition at 37 °C. The bacterial activity was determined against *E. tarda* 3. All experiments were performed in triplication

TspE4.C2–) (Fig. S1). In addition, bacteriocin of the present study exhibited a bacteriocidal mode of action toward *S. aureus* DMST8840 and *Shigella* sp. which are responsible for foodborne illnesses worldwide. Interestingly, it is highly active against *E. tarda* 3, the most common pathogens of cultured fish, responsible for edwardsiellosis infection of a wide range of freshwater and marine fish leading to major losses in aquaculture worldwide [15, 40]. *E. tarda*, a Gram-negative rod-shaped bacterium, is found to be an intracellular pathogen and is capable of infecting various types of cells [15, 40]. Currently, the common method to treat edwardsiellosis by antibiotics is not effective as treating extracellular bacterial pathogens such as

Vibrio spp. [15]. The previous study showed that *E. coli* strain H22 exhibited the ability to inhibit the growth of pathogenic or non-pathogenic Gram-negative bacteria including *Enterobacter*, *Escherichia*, *Klebsiella*, *Morganella*, *Salmonella*, *Shigella*, and *Yersinia*. The growth inhibition was observed against all strains tested of each genus except for the pathogenic *E. coli* strain p307. However, Gram-positive indicator strains tested such as *B. subtilis* and *S. aureus* were not sensitive [25]. Pyocin SA 189, which is a bacteriocin produced by Gram-negative bacteria *P. aeruginosa* SA 189, showed antibacterial activity against a number of sensitive Gram-positive bacteria including *S. aureus*, *S. pyogenes*, and *Listeria monocytogenes*. However, no inhibitory activity against some species was observed such as *Shigella dysenteriae* and *Salmonella typhi* [41]. Microcin 25, excreted by *E. coli* strain isolated from human feces, showed that the antibiotic was active against *E. coli*, *Salmonella*, and *Shigella* strains of clinical origin by cross-streaking assay [42]. The highest bacteriocin activity was observed to be produced at early stationary phase, which is similar to those in bacteriocin production studies of both Gram-positive and Gram-negative bacteria [28, 43]. The maximum yield of pyocin SA 189 produced by *P. aeruginosa* SA189 was obtained at late log phase and remained constant until the late stationary phase [41]. The amount of bacteriocin released in the culture medium is correlated with the quantity of biomass produced. However, the bacteriocin production strongly decreased when cells entered the stationary phase. The loss of bacteriocin activity may be caused by proteolytic degradation during this growth phase, protein aggregation, and feedback regulation [44]. The production of active bacteriocin is shown to be affected by environmental factors [41, 44, 45]. In our study, *E. coli* E could grow and produce bacteriocins in

Fig. 2 Effect of pH and temperature on the activity of CFS derived from *E. coli* E against the growth of *E. tarda* 3. **a** Effect of different pH on activity for 15 min. **b** pH stability profile during 120 min treatment. **c** Effect of temperature on bacteriocin activity for 15 min. **d** Temperature stability profile during 120 min of treatment. Values shown are the mean ± SD of three independent experiments. (ANOVA, Turkey's multiple comparison test, $P < 0.05$)



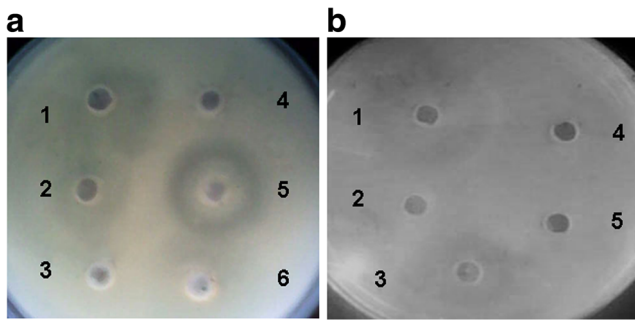


Fig. 3 The antibacterial activity of partial purification fractions against the growth of **a** *Shigella* sp. 1–2: CFS, 3: 50% (NH₄)₂SO₄ saturation, 4: negative control (culture media), 5: 20% (NH₄)₂SO₄ saturation, and 6: 30% (NH₄)₂SO₄ saturation. **b** *E. tarda* 3, 1: 20% (NH₄)₂SO₄ saturation, 2: negative control (culture media), 3: CFS, 4: 30% (NH₄)₂SO₄ saturation, 5: 50% (NH₄)₂SO₄ saturation

NaCl concentrations up to 4%. The presence of 1.0–4.0% NaCl in culture media did not affect growth compared to control (0% NaCl). The obtained *E. coli* E displayed a strong tolerance to high salt concentrations. Thus, this strain may be applied to high salt environments such as marine or that of food processing. However, the antibacterial activity of bacteriocin was significantly affected by 1.0–4.0% NaCl compared to control (data not shown). NaCl was found to affect cell growth and bacteriocin activity produced by *Lactobacilli* species [45].

Probiotic applications employing bacteria have increased both in research and commercial interest for over 20 years [6, 11, 46]. Bacteriocins have demonstrated their remarkable potential in food preservatives, medical uses, and bacterial disease treatment in aquaculture [6, 9, 13, 18, 24, 43]. The digestive processes in marine animals like fish and shrimp are less variable and generally similar to those found in higher animals [47]. The acid and bile are important antimicrobial components of the digestive system. Being tolerant to acid and bile is a fundamental property for the selection of successful

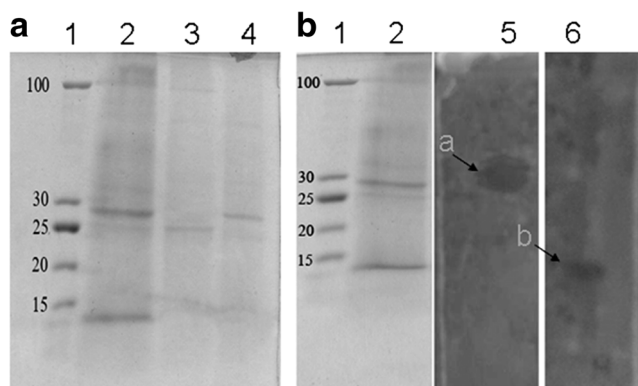


Fig. 4 Partial purification and direct activity detection. **a** SDS-PAGE analysis of ammonium sulfate fractions; lane 1, protein ladder (New England BioLabs); lane 2, 20% (NH₄)₂SO₄ saturation; lane 3, 30% (NH₄)₂SO₄ saturation; and lane 4, 50% (NH₄)₂SO₄ saturation. **b** Gel containing active fraction overlaid with indicator strain *E. tarda* 3 (lane 5) and *S. aureus* DMST8840 (lane 6). Arrows indicate inhibition zones with corresponding active peptides

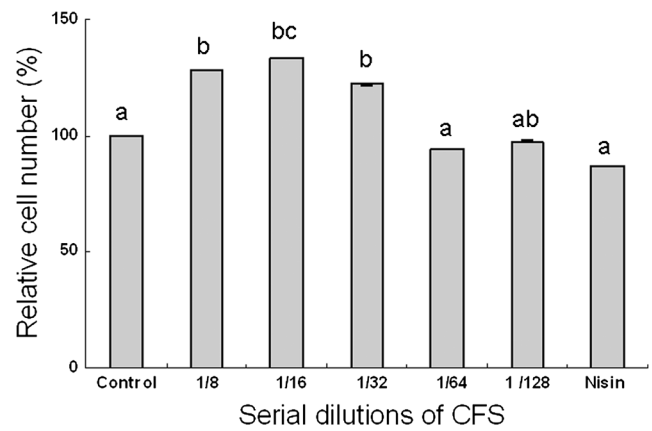


Fig. 5 Cytotoxicity of different concentrations of CFS produced by *E. coli* E against NIH-3T3 determined by MTT assay after 72 h incubation. Values shown are the mean ± SD. (ANOVA, Turkey’s multiple comparison test, *P* < 0.05)

probiotic isolates to assure their viability and functionality [37, 46, 48]. To evaluate the probiotic potential, the acidic and bile salt tolerance of *E. coli* E was determined. In this study, *E. coli* E was able to grow in NA agar supplemented with up to 0.3% bile salt which is considered to be critical and high enough to screen for resistant strains [37, 46]. Two lactic acid bacteria in a commercial probiotic culture were characterized [46]. The results showed that they were able to tolerate bile salt at a concentration of 0.6% and survive at pH 3 but did not at pH 2.0 [46]. Buntin et al. obtained four LAB strains from 160 isolates based on their survival after exposure to pH 2.5 and resistance to bile salt concentration of 0.4%. None of the strains were able to survive when exposed to pH 1 [37]. *B. amyloliquefaciens* LN could survive after incubation at pH 2.0 or 3.0 for 3 h and 0.3% bile salt for 12 h [49]. Depending on the species, feeding rate, diet composition, and time after meals, the fish stomach pH varies between 1

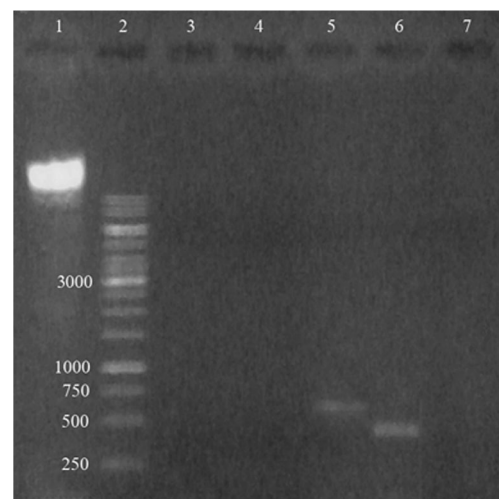


Fig. 6 PCR analysis of virulence genes in *E. coli* E. lane 1, DNA; lane 2, DNA ladder (GeneRuler™ 1kbp); lane 3, *pep*; lane 4, *sfu*; lane 5, *aer*; lane 6, *hly*; and lane 7 *cnf*

and 6 and it is stable until about 6 h after meals then gradually decreases to pH 4 at 24 h [47]. Therefore, the results obtained from this study indicated that *E. coli* E might be able to resist the digestion process in the gastrointestinal tract where it can function effectively.

The production of more than one bacteriocin by *E. coli* has been reported previously [3, 21, 24]. Two types of bacteriocins are commonly produced by *E. coli*, classified by their molecular weight: colicin (25–80 kDa) and microcins (< 10 kDa) [23, 24]. *E. coli* H22 shows antagonistic activity against several enteric bacteria and it produces colicins E1 and IB and microcin C7 [25]. Our data also indicated that *E. coli* E produced more than two types of bacteriocins and these two types were associated with the antibacterial activity (Fig. 4b). Bacteriosin LS2 (4 kDa) isolated from the cell pellet of *Lactobacillus salivarius* BGH01 had antagonistic activity against several indicator strains detected by direct gel detection assay [2]. However, this is the first report to show two specific bacteriocin activities on two different indicator strains. Surprisingly, we could not detect antibacterial activity against *Shigella* sp. by gel direct detection assay. The in vitro inhibition of *Shigella flexneri* 4 by *E. coli* H22 was shown to be mediated by microcin C7 [25]. Bacteriocins produced by *E. coli* E could carry various inhibitory effects or act synergistically against pathogens. One type of bacteriocin classified by molecular weight as colicins (27 kDa) could inhibit *E. tarda* 3 as performed by direct detection of activity. Colicin production could be induced by the SOS response system [7, 24]. Mitomycin C that is able to trigger the SOS response was used in our study. The presence of 1 $\mu\text{g ml}^{-1}$ mitomycin C in culture media could induce about 20% of bacteriocin production compared with control (data not shown). The low molecular weight (10 kDa) could classify it as microcin and it could specifically exhibit antibacterial activity against *S. aureus* DMST8840. The molecular weight of detected microcin was likely to be microcin V (previously known as ColV) (9 kDa) and microcin S (11.67 kDa) produced by *E. coli* and *E. coli* G3/10, respectively [4, 50]. However, further study needs to be carried out to confirm the types of colicin and microcin.

Previous studies showed the co-associations of microcins with other virulence factors such as toxins (*usp*, *hlyA*), siderophores (*iroN*, *iroCD*, *fyuA*, *iucD*), and adhesins (*papC*, *sfa*, *fimH*) [24]. Our study observed two virulence genes, *aer* and *sfa*. Microcins namely H47, M, and ColV were more likely to encode genes associated with siderophores, toxins, and adhesins [24]. However, colicin E1 (57 kDa calculated from the predicted amino acid sequence from the plasmid DNA sequence) was a potentially important virulence factor of certain uropathogenic *E. coli* strains [3, 51].

Very few studies have been reported on the cytotoxicity upon normal eukaryotic cells [5, 52]. Cytotoxicity studies of bacteriocin (enterocin S37) produced by *Enterococcus faecalis* S37 on human colon adenocarcinoma cells were performed and dose-dependent cytotoxicity toward tested cells was observed [5]. The result suggested that enterocin S37 caused some loss of plasma membrane integrity of the tested cells [5]. In our study, an MTT assay was performed to indicate the degree of cytotoxicity of CFS. An increase in relative proliferation in fibroblasts treated with higher CFS dilution (1/8–1/32) was observed, whereas there was no difference between control, dilution of 1/64–1/128 and nisin. Moreover, our data also indicated that CFS dilution did not affect the relative cell number in human head and neck cancer cells, HN18 (data not shown). The results obtained indicate the safe use of bacteriocins including secreted metabolites of *E. coli* E.

Our study demonstrates that bacteriocins produced by *E. coli* E have great potential to be applied as a new antibacterial agent due to its antagonistic activity against both Gram-positive and Gram-negative pathogenic bacteria. Although two virulence genes were found in the *E. coli* E genome, bacteriocins in cell-free supernatants are safe for the normal cell lines. Further experiments should be carried out to elucidate the types of bacteriocins.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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