Veterinary World, EISSN: 2231-0916
Available at www.veterinaryworld.org/Vol.7/November-2014/21.pdf

RESEARCH ARTICLE
Open Access

The effectiveness of novel bacteriocin derived from *Escherichia coli* colonized in the fermented pineapple *Ananas comosus* (L.) Merr. against pathogenic bacteria isolated from aquaculture sites

S. W. Lee[1], W. Wendy[2], L. Montira[3] and A. U. Siti Hazirah[1]

1. Faculty of Agro Based Industry, Universiti Malaysia Kelantan Campus Jeli, 17600, Jeli, Kelantan, Malaysia; 2. School of Fisheries and Aquaculture Sciences, Universiti Malaysia Terengganu, Kuala Terengganu, 21030, Terengganu, Malaysia; 3. Department of Science, Biology Division, Faculty of Science and Technology, Prince of Songkla University, Pattani Campus, Pattani, 94000, Thailand.

Corresponding author: S. W. Lee, e-mail: leeseongwei@yahoo.com, WW: wendy@umt.edu.my, LM: montiral@yahoo.com, AUSH: hazirah@yahoo.com

Received: 07-07-2014, **Revised:** 21-09-2014, **Accepted:** 24-09-2014, **Published online:** 30-11-2014

doi: 10.14202/vetworld.2014.1014-1018. **How to cite this article:** Lee SW, Wendy W, Montira L, Siti Hazirah AU (2014) The effectiveness of novel bacteriocin derived from *Escherichia coli* colonized in the fermented pineapple *Ananas comosus* (L.) Merr. against pathogenic bacteria isolated from aquaculture sites, *Veterinary World 7*(11): 1014-1018.

Abstract

Aim: The aim was to evaluate antimicrobial property of bacteriocin isolated from *Escherichia coli* against pathogenic bacteria from aquaculture sites.

Materials and Methods: *E. coli* was isolated from fermented pineapple *Ananas comosus* using eosin methylene blue agar. The antimicrobial activity of the isolated *E. coli* was screened using hole-plate diffusion method. The bacterial strain that showed the widest inhibition zone was selected and grown in tryptic soy broth, followed by partial purification of bacteriocin by using ammonium sulphate. Bacteriocin derived from the *E. coli* was subjected to the antimicrobial test against 55 bacteria strains namely *Aeromonas hydrophila* (n=10), *Citrobacter freundii* (n=5), *Edwardsiella tarda* (n=10), *Flavobacterium* spp. (n=10), *Pseudomonas* spp. (n=10), *Vibrio parahaemolyticus* (n=5) and *Vibrio alginolyticus* (n=5) by using twofold broth microdilution method to determine minimum inhibitory concentration (MIC) values of the bacteriocin against the tested bacteria.

Results: The results of the present study showed that the MIC values of the partially purified bacteriocin against present pathogenic bacteria isolates ranged from 7.81 to 31.25 ppm whereas the MIC values of kanamycin (positive control) ranged from 15.63 to 125 ppm.

Conclusion: The results of the present study showed the bacteriocin derived from *E. coli* can control all the present bacterial isolates indicating the huge potential of the bacteriocin as a new antimicrobial agent for aquaculture uses.

Keywords: *Escherichia coli*, partially purified bacteriocin, pathogenic bacteria.

Introduction

Traditionally, bacteriocin was widely used for food preservative and human health. The well-known nisin was the first bacteriocin that applied as a food preservative to prevent the growth of *Clostridium botulinum* spores in cheese [1]. Gradually, this bacteriocin was accepted by 45 countries as bio-preservative in food and till present it was widely used as commercial bacteriocin as a food preservative [2]. Apart of bacteriocin used as food preservative, it was also reported used as a broad spectrum antibiotic in order to kill multi-resistant human bacterial pathogens instead of using conventional antibiotics [3] that were gradually not more effective in controlling human pathogenic bacteria due to the increasing of antibiotic resistance case among human pathogenic bacteria [4]. Similar case was also reported among pathogenic bacteria from aquaculture sites.

The increasing of antibiotic resistance case among pathogenic bacteria in the aquaculture led to the most of the commercial antibiotics was no more effective in controlling bacterial diseases in aquaculture [5-7]. This happens due to the misuse or overuse of the commercial antibiotic among the fish farmers [7,8]. Subsequently, fish farmers were left with no option. They have to seek the alternative resources as antimicrobial agents to overcome bacterial diseases outbreak [9,10].

Thus, in the present study, we attempted to reveal the potential of the bacteriocin isolated from *Escherichia coli* as a new antimicrobial agent for aquaculture uses.

Materials and Methods

Fermented pineapple *Ananas comosus*

Pineapple purchased from local wet market were washed, peeled and grated. The prepared pineapple was then fermented with 1% molasses at room temperature for 1 month. *E. coli* were isolated from this sample.

Bacteria isolates

Fifty-five bacteria strains namely *Aeromonas hydrophila* (n=10), *Citrobacter frecundii* (n=5),

Edwardsiella tarda (n=10), *Flavobacterium* spp. (n=10), *Pseudomonas* spp. (n=10), *Vibrio parahaemolyticus* (n=5) and *Vibrio alginolyticus* (n=5). *A. hydrophila*, *C. freundii*, *E. tarda* and *Flavobacterium* spp. were obtained from diseased red hybrid tilapia, *Oreochromis* spp. whereas *V. parahaemolyticus* and *V. alginolyticus* were isolated from Asian seabass, *Lates calcarifer*.

Isolation and identification of bacteriocin producing *E. coli*

E. coli was isolated by pour plate method by using eosin methylin blue (EMB) agar (Merck, Germany) as described in the study of Lee *et al*. [5]. After 24 h incubation, the bacterial colonies that growth on EMB agar with metallic green sheen in color were picked up and stored in tryptic soy agar deep tube (Merck, Germany) for further study. The suspected bacterial isolates were identified by using conventional biochemical tests and confirmed by bacteria identification kit (BBL, USA) as described in the study of Lee *et al*. [11]. The antimicrobial activity of the isolated *E. coli* was screened by using well diffusion method as described by Lee *et al*. [8] and Lee and Wendy [12]. Mueller Hinton agar (Merck, Germany) was used in the antimicrobial test. All the pathogenic bacteria were culture in the tryptic soy broth (TSB) for 24 h at room temperature and adjusted to 10^9 CFU/mL by using physiological saline after 24 h. The concentration was cross check with a Biophotometer (Eppendorf, Germany). The pathogenic bacteria were then spread on the Mueller Hinton agar. A well with a depth of 3 mm was made to fill up with 100 µL of cell-free supernatant fluid of the isolated *E. coli* after growing them in the TSB broth for 24 h and diluted appropriately. The inoculated media were incubated for 24 h at room temperature. The antimicrobial activity was determined by the measurement of the diameter of the inhibition zone around the wells [10]. The bacterial isolate showing the widest zone was used for further study.

Partial purification of bacteriocin

Partial purification of bacteriocin was done as described Joshi *et al*. [13]. *E. coli* that showed the widest inhibition zone was cultured in TSB for 24 h at room temperature. After incubation, the broth was centrifuged at 14,000 rpm for 10 min to separate out the bacterial cells. The supernatant was used as a crude bacteriocin. Different concentrations (10-70% of the total volume of the crude supernatant) of ammonium sulfate (Merck, Germany) were added to the supernatant and kept undisturbed at 4°C overnight after vortex. Precipitate formed after overnight incubation was collected by centrifugation at 14,000 rpm for 10 min and dissolved in 20 mmol sodium phosphate buffer at pH=6.0. Each fraction was screened for their antimicrobial activity and recorded in comparison with the crude bacteriocin. Fraction recorded with the widest inhibition zone was used for minimum inhibitory concentration (MIC) test.

MIC values determination

Crude bacteriocin partially purified with 20% of ammonium sulfate showed the widest inhibition zone against the present pathogenic bacteria. The values of MIC of the crude bacteriocin fraction against pathogenic bacterial isolates were determined through a two-fold broth microdilution method [9,10]. The final bacterial cells concentration was 10^5 CFU/mL [14]. The bacterial isolates were cultured in TSB for 24 h at room temperature, and the concentration of these cultures was adjusted to 10^9 CFU/mL by using physiological saline. The concentration was cross-check with a Biophotometer (Eppendorf, Germany). The bacterial suspensions were then inoculated into a microtiter plate that contained a serial dilution of crude bacteriocin fraction and kanamycin as a positive control. The microplate was then incubated at room temperature for 24 h. The MIC values were defined as the lowest concentration of the crude bacteriocin fraction in the wells of the microtiter plate that showed no visible turbidity after 24 h incubation.

Results

In the present study, *E. coli* producing bacteriocin was successfully isolated from fermented pineapple, *A. comosus*. The bacteriocin derived by partial purification using 20% of ammonium sulfate showed the widest inhibition zone against all the present pathogenic bacteria namely *A. hydrophila* (n=10), *C. freundii* (n=5), *E. tarda* (n=10), *Flavobacterium* spp. (n=10), *Pseudomonas* spp. (n=10), *V. parahaemolyticus* (n=5) and *V. alginolyticus* (n=5) (Figures-1-3).

The widest inhibition zone of *E. coli* strain against all the present pathogenic bacteria was selected to determine the MIC values of its bacteriocin The MIC values of partially purified bacteriocin and kanamycin recorded in the present study ranged from 7.81 to 31.25 ppm and 15.63 to 125 ppm, respectively (Table-1), against all the present

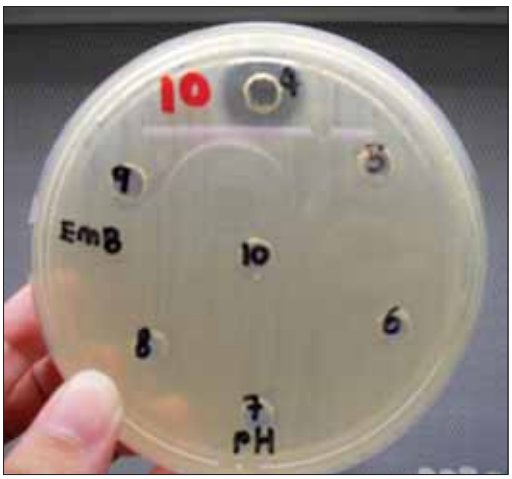

Figure-1: Clear inhibition zone of bacteriocin against *Escherichia coli*.

Table-1: MIC values of partially purified bacteriocin with 20% of ammonium sulfate and kanamycin against pathogenic bacteria from aquaculture sites.

Bacterial strains	MIC values of partially purified bacteriocin with 20% of ammonium sulfate, PPM	MIC values of kanamycin (positive control), PPM
A. hydrophila	7.81	15.63
A. hydrophila	7.81	31.25
A. hydrophila	15.63	31.25
A. hydrophila	31.25	31.25
A. hydrophila	7.81	15.63
A. hydrophila	15.63	31.25
A. hydrophila	7.81	31.25
A. hydrophila	7.81	31.25
A. hydrophila	31.25	31.25
A. hydrophila	7.81	15.63
C. freundii	31.25	62.5
C. freundii	15.63	31.25
C. freundii	7.81	125
C. freundii	15.63	62.5
C. freundii	7.81	15.63
E. tarda	31.25	125
E. tarda	7.81	31.25
E. tarda	31.25	125
E. tarda	15.63	15.63
E. tarda	31.25	31.25
E. tarda	7.81	31.25
E. tarda	15.63	62.5
E. tarda	7.81	15.63
E. tarda	15.63	125
E. tarda	7.81	62.5
Flavobacterium spp.	31.25	15.63
Flavobacterium spp.	7.81	31.25
Flavobacterium spp.	15.63	15.63
Flavobacterium spp.	7.81	62.5
Flavobacterium spp.	7.81	125
Flavobacterium spp.	15.63	15.63
Flavobacterium spp.	31.25	125
Flavobacterium spp.	7.81	62.5
Flavobacterium spp.	7.81	31.25
Flavobacterium spp.	15.63	15.63
Pseudomonas spp.	7.81	125
Pseudomonas spp.	15.63	15.63
Pseudomonas spp.	7.81	125
Pseudomonas spp.	31.25	62.5
Pseudomonas spp.	7.81	15.63
Pseudomonas spp.	7.81	125
Pseudomonas spp.	7.81	125
Pseudomonas spp.	7.81	15.63
Pseudomonas spp.	15.63	125
Pseudomonas spp.	7.81	31.25
V. parahaemolyticus	7.81	15.63
V. parahaemolyticus	7.81	125
V. parahaemolyticus	15.63	31.25
V. parahaemolyticus	7.81	15.63
V. parahaemolyticus	7.81	62.5
V. alginolyticus	15.63	15.63
V. alginolyticus	15.63	125
V. alginolyticus	7.81	15.63
V. alginolyticus	7.81	62.5
V. alginolyticus	15.63	31.25

PPM=Part per million, *V. parahaemolyticus=Vibrio parahaemolyticus, V. alginolyticus=Vibrio alginolyticus, E. tarda=Edwardsiella tarda, C. freundii=Citrobacter freundii, A. hydrophila=Aeromonas hydrophila*, MIC=Minimum inhibitory concentration

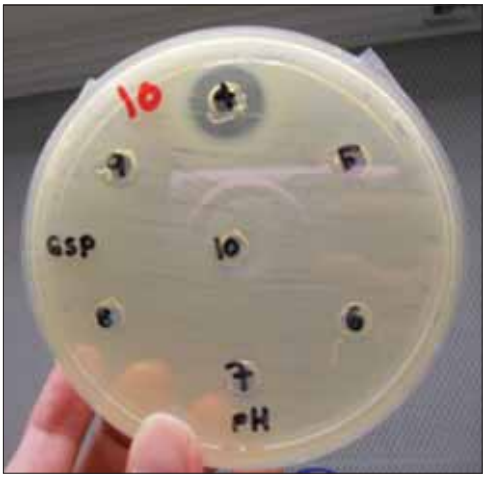

Figure-2: Clear inhibition zone of bacteriocin against *Aeromonas hydrophila.*

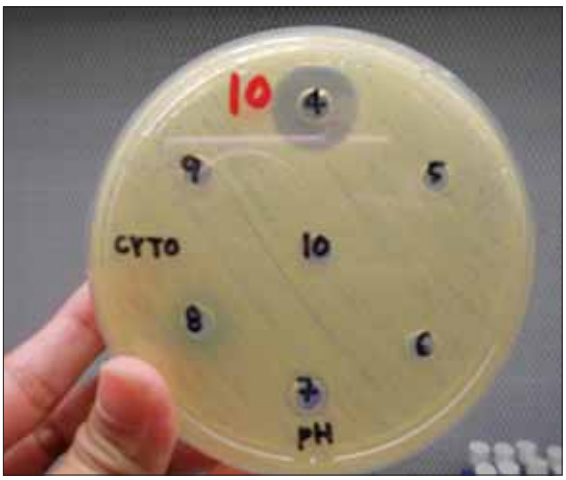

Figure-3: Clear inhibition zone of bacteriocin against *Flavobacterium* sp.

pathogenic bacteria isolates. The MIC values of the partially purified bacteriocin fractions against *A. hydrophila*, *C. freundii*, *E. tarda*, *Flavobacterium* spp. and *Pseudomonas* spp. ranged from 7.81 to 31.25 ppm whereas the MIC values of the bacteriocin against *V. parahaemolyticus* and *V. alginolyticus* ranged from 7.81 to 15.63 ppm.

For the control, MIC values of kanamycin against *A. hydrophila* ranged from 15.63 to 31.25 ppm whereas *C. freundii* ranged from 31.25 to 125 ppm. MIC values of kanamycin against *E. tarda*, *Flavobacterium* spp., *Pseudomonas* spp., *V. parahaemolyticus* and *V. alginolyticus* ranged from 15.63 to 125 ppm.

Discussion

To date, there are a lot of studies on the application of bacteriocin in aquaculture. For instance, Byun *et al*. [15] reported the effect of *Lactobacillus* sp. DS-12 in flounder, (*Paralichthys olivaceus*), and Cai *et al*. [16] reported the potential of *Weissella hellenica* DS-12 isolated from flounder intestine as probiotic.

Another studyby Cai *et al*. [17] characterized the lactic bacteria isolated from the intestines of common carp and freshwater prawns. However, none of the studies elaborates the MIC values of the isolated bacteriocin against the pathogenic bacteria. Hence, we claimed that this is the first report on the MIC values of bacteriocin isolated from *E. coli* against pathogenic bacteria isolated from aquaculture sites.

From the literature survey, we found that bacteriocin from *E. coli* namely colicin was most extensively studied [18]. The colicin was first found by Gratia as an antimicrobial protein [19]. In spite of the fact, the partially purified bacteriocin isolated in the present study possesses inhibitory activity against all the present pathogenic bacteria from aquaculture sitesis associated to the colicin. The inhibitory activity showed by the present partially purified bacteriocin from *E. coli* against all the present pathogenic bacteria indicates a huge potential of this bacteriocin to be used as an antimicrobial agent in aquaculture. The partially purified bacteriocin of *E. coli* by using 20% ammonium sulfate possesses inhibitory effects against all the tested pathogenic bacteria may be due to lysozymes, proteases, hydrogen peroxide or organic acid as described by Verschuere *et al*. [20]. In this case, further study should be carried out before we can come to a conclusion.

In comparison to kanamycin, the isolated partially purified bacteriocin was found to be more effective in controlling the present pathogenic bacteria in terms of concentration where the MIC values of partially purified bacteriocin was lower compared to the MIC values of kanamycin against the present pathogenic bacteria. Hence, the potential of this partially purified bacteriocin as a new antimicrobial agent is undoubtedly.

Conclusion

In conclusion, bacteriocin derived from the *E. coli* is proposed to be used as a novel antimicrobial agent against pathogenic aquatic bacteria. However, further studies on the mode of action and the protein profile of the bacteriocin need to be carried out for application in aquaculture.

Authors' Contributions

LSW and ML designed and supervised the study. LSW, WWand SHAU conducted study and analyzed the data. All authors contributed in the draft, revision and approval of the final manuscript.

Acknowledgments

This project was funded by Universiti Malaysia Kelantan Short Term grant R/SGJP/A03.00/00387A/002/2012/000068 and Minister of Education Malaysia under Fundamental Research Grant Scheme (FRGS) vot no: R/FRGS/A0.700/00387A/005/2013/00107.

Competing Interests

The authors declare that they have no competing interests.

References

1. Chung, K., Dickson, J. and Crouse, J. (1989) Effects of nisin on growth of bacteria attached to meat. *Appl. Environ. Microbiol.,* 55(6): 1329-1333.
2. Margaret, A.R. and John, E.W. (2002) Bacteriocins: Evolution, ecology and application. *Annu. Rev. Microbiol.,* 56: 117-137.
3. Roy, P.H. (1997) Dissemination of antibiotic resistance. *Med. Sci.* 13: 927-933.
4. Walker, E.S. and Levy, F. (2001) Genetic trends in a population evolving antibiotic resistance. *Evolution,* 55(6): 1110-1122.
5. Lee, S.W., Najiah, M. and Wendy, W. (2010) Bacterial flora from a healthy freshwater Asian sea bass (*Lates calcarifer*) fingerling hatchery with emphasis on their antimicrobial and heavy metal resistance pattern. *Vet. Arh.,* 80(3): 411-420.
6. Lee, S.W., Najiah, M. and Wendy, W. (2010) Bacteria associated with golden pompano (*Trachinotus blochii*) broodstock from commercial hatchery in Malaysia with emphasis on their antibiotic and heavy metal resistances. *Front. Agric. China,* 4(2): 332-336.
7. Lee, S.W. and Wendy, W. (2012) Characterization of *Vibrio alginolyticus* isolated from white leg shrimp (*Litopenaeus vannamei*) with emphasis on its antibiogram and heavy metal resistance pattern. *Vet. Arh.,* 82(2): 221-227.
8. Lee, S.W., Wendy, W. Julius, Y.F.S. and Desy, F.S. (2012) Characterization of anticancer, antimicrobial, antioxidant property and chemical composition of *Phyllanthus urinaria* Linn. leaf extract. *Pharmacol. Online.,* 49(10): 670-674.
9. Lee, S.W. and Najiah, M. (2009) Antimicrobial property of 2-hydroxypropane-1,2,3-tricarboxylic acid isolated from *Citrus microcarpa* extract. *Agric. Sci. China*, 8(6): 740-745.
10. Lee, S.W., Wendy, W., Julius, Y.F.S., Desy, F.S. and Ahmad A.I. (2010) Characterization of antioxidant, antimicrobial, anticancer property and chemical composition of *Murdannia bracteata* leaf extract. *Pharmacol Online*, 3: 930-936.
11. Lee, S.W., Wendy, W. Zalina, C.M., Ruhul Amin, M.D. and Sukree, H. (2013) A study of *Edwardsiella tarda* colonizing live Asian clam, *Corbicula fluminea*, from Pasir Mas, Kelantan, Malaysia with the emphasis on its antibiogram, heavy metal tolerance and genetic diversity. *Vet. Arh.,* 83(3): 323-331.
12. Lee, S.W. and Wendy, W. (2013) Chemical composition and antimicrobial activity of *Cymbopogon nardus* citronella essential oil against systemic bacteria of aquatic animals. *Iran. J. Microbiol.,* 5(2): 147-152.
13. Joshi, V.K., Sharma, S. and Rana, N.S. (2006) Production, purification, stability and efficacy of bacteriocin from isolates of natural lactic acid fermentation of vegetables. *Food Technol. Biotechnol*., 44(3): 435-439.
14. European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID). (2003) Determination of the minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. *Clin. Microbiol. Infect.,* 9(8): 1-7.
15. Byun, J.W., Park, S.C., Ben, Y. and Oh, T.K. (1997) Probiotic effect of *Lactobacillus* sp. DS-12 in flounder (*Paralichthys olivaceus*). *J. Gen. Appl. Microbiol.,* 43(5): 305-308.
16. Cai, Y., Benno, Y. Nakase, T. and Oh, T.K. (1998) Specific probiotic characterization of *Weissella hellenica* DS-12 isolated from flounder intestine. *J. Gen. Appl. Microbiol.,* 44(5): 311-316.

17. Cai, Y., Suyanandana, P., Saman, P. and Benno Y. (1999) Classification and characterization of lactic acid bacteria isolated from the intestines of common carp and freshwater prawns. *J. Gen. Appl. Microbiol*., 45(4): 177-184.
18. Benedetti, H. and Geli, V. (1996) Colicin transport, channel formation and inhibition. *Hand Biol. Phys*., 2: 665-691.
19. Gillor, O., Nigro, L.M. and Margaret, A.R. (2005) Genetically engineered bacteriocins and their potential as the next generation of antimicrobials. *Curr. Pharm. Des*., 11(8): 125-132.
20. Verschuere, L., Rombaut, G., Sorgeloos, P. and Verstraete, W. (2000) Probiotic bacteria as biological control agents in aquaculture. *Microbiol. Mol. Biol. Rev*., 64(4): 655-671.

****