Antimicrobial Activity of Lactic Acid Bacteria Strains Isolated from Dadih against *Escherichia coli*

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Abstract. Food is a material that is very susceptible to contamination by pathogenic microorganisms that affect the quality, shelf life and safety of food. One way to prevent contamination by pathogenic microorganisms is to add preservatives. Bacteriocin produced by lactic acid bacteria (LAB) is one of the natural food preservatives that are safe for human consumption. The aims of this study were to screen the ability of LAB isolated from dadih to inhibit the growth of *E. coli* and to evaluate antimicrobial components that play a role in inhibiting the growth of *E. coli*. The antimicrobial sensibility of LAB to pH, heat and proteolytic enzymes was carried out using the referenced method. The results showed that supernatant from 12 LAB strains of*dadih* were able to inhibit the growth of *E. coli* with various inhibition zones. However, out of the 12 LAB, only 9 strains were found to have an inhibition zone of more than 4 mm. Then the 9 strains were tested for antimicrobial compounds, and it was found that 3 strains had antimicrobial activity derived from organic acids especially lactic acid, and 6 strains namely R-43, R-32, R-19, R-55, R-45 and R-41 had an antimicrobial effect in the form of bacteriocins based on the sensitivity test for pH, heat and enzyme treatments. Crude bacteriocin derived from 6 LAB strains inhibited the growth of *E. coli*, and the highest antimicrobial activity was found on *Streptococcus lactis* sub sp. diacetylactis R-43 with average inhibition zone of 8.9 mm. Based on this study, it can be concluded that the bacteriocin produced by *S. lactis* subsp. diacetylactis R-43 can be used as one of the natural preservatives for prevention from food-borne pathogen, *E. coli*.

1. Introduction

*Escherichia coli* is a gram-negative bacterium, facultative anaerobic, rod-shaped coliform bacterium of the genus *Escherichia* that is commonly found in the lower intestinal of warm-blooded organisms or endotherms [1,2]. Most *E. coli* strains are harmless, but some serotypes can cause serious food poisoning in their hosts, and are occasionally responsible for product recalls due to food contamination[3,4]. *E. coli* is expelled into the environment within fecal matter. The bacterium grows massively in fresh fecal matter under aerobic conditions for 3 days, but its numbers decline slowly afterwards [5]. *E.coli* and other facultative anaerobes constitute about 0.1% of gut microbiota [6] and fecal–oral transmission is the major route through which pathogenic strains of the bacterium cause disease. Cells are able to survive outside the body for a limited amount of time, which makes them potential indicator organisms to test environmental samples for fecal contamination [7,8]. Foodborne pathogens have become important social topics and have received much attention from consumers and food safety regulatory agencies around the world because of the frequent outbreaks of microbial infections.
It is estimated that 175,905 Shiga-toxigenic Escherichia coli (STEC) foodborne disease cases occur in the United States each year, with non-O157 STEC being reportedly the causative agents in 64.1% of cases [9]. The US Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) declared raw non-intact beef, as well as intact beef intended to be processed into non-intact beef, adulterated if found positive for E. coli belonging to the groups O26, O45, O103, O111, O121, O145, and/or O157 [10].

Lactic acid bacteria (LAB) are the most important microorganisms associated with fermentation. These nonsporulating gram-positive bacteria are widely distributed in nature. Their main product is lactic acid, which is produced as they ferment carbohydrates. LAB utilize and convert carbohydrates, primarily glucose, in the raw materials to produce various metabolites, which give the food its unique flavor and nutritional value and are not present before fermentation [11]. Many LABs have probiotic properties and antimicrobial effects [12,13] suggesting that LAB can be used in food preservation. Hosono et al. [14] has isolated 4 genera of BAL from dadih, fermented food from buffalo milk that is similar to yogurt, namely Lactobacillus sp, Streptococcus sp, Leuconostoc sp and Lactococcus sp. These dadih’s LAB have never been investigated for its antimicrobial activity even though these strains had antimutagenic ability against various mutagen compounds [15]. The present study purposes were to evaluate the ability of BAL isolated from dadih to inhibit the growth of E. coli and to determine the type of antimicrobial compounds produced by dadih’s LAB.

2. Materials and Method

2.1 Dadih’s LAB and Pathogenic Bacteria
In this study 12 strains of LAB used were isolated from dadih, West Sumatera, Indonesia [14]. Whereas the pathogenic gram-positive bacteria used was Escherichia coli FNCC-19.

2.2 Activation of LAB Culture and Pathogenic Bacteria
Active culture was made by taking 0.1 ml of dadih’s LAB stock culture and put in a test tube containing 5 ml of MRS Broth, then shake until blended and incubated at 37°C for 18 hours. The pathogenic bacteria was activated by inoculating 0.1 ml of the test bacterial stock culture into 5 ml of the Nutrient Broth, then shaken evenly and incubated at 37°C for 18 hours.

2.3 Antimicrobial Activity of Dadih’s LAB in in vitro Test
The antibacterial activity of the supernatant obtained from 12 strains dadih’s LAB was determined using the paper disk diffusion method as described by Ref. [16,17, 24]. The cultures of dadih’s LAB were incubated aerobically at 37°C for 24h. The indicator bacteria, E. coli was grown in Nutrient Broth at 37°C for 24h. Then 100 µl of pathogenic microorganism were placed and spread using glass hockey sticks on the surface of MRS Agar. The sterile paper disc (6mm) dipped into LAB supernatants and into sterile MRS broth as negative control, and then put onto the surface of MRS agar plates previously seeded with the indicator bacteria. The plates were incubated at 37°C for 24 h. After incubation, the diameter of zone of growth inhibition was measured.

2.4 Characterization of Bacteriocin

2.4.1 Effect of pH
In order to determine effect of pH, 0.5 ml of purified bacteriocin was added into 4.5 ml of nutrient broth at different pH values (3 to 11) and incubated for 30 min at 37°C. Each of the crude bacteriocin samples treated at different pH values was assayed against indicator bacteria by agar diffusion method [16,17].

2.4.2 Effect of Temperature
Crude bacteriocin (0.5 ml) was added into 4.5 ml of nutrient broth in the test tube. Each test tube was then overlaid with paraffin oil to prevent evaporation and then heated at different temperatures (30, 40, 50, 60, 70, 80, 90, 100 and 121°C) for 10 min. The preparations containing nutrient broth (4.5 ml) and crude bacteriocin (0.5 ml) in test tubes were plugged with non-absorbent cotton and covered with aluminium foil and kept in an autoclave at 121°C or 15 lbs pressure for 10 min to check its activity at
very high autoclaving temperature. The crude bacteriocin activity of above different heat-treated was measured by agar diffusion method method [16,17].

2.4.3 Effect of Amylase and Proteolytic Enzyme
Effect of amylase and proteolytic enzyme on the activity of crude bacteriocin was studied as described by Zhou et al. [18]. Crude bacteriocin was treated with amylase (5 mg/ml), trypsin (5 mg/ml), and proteinase (5 mg/ml), respectively, and phosphate buffer (0.5M, pH 7.0) was used as control. The effect of the enzymes on crude bacteriocin activity was studied by agar diffusion method [16,17] using above preparations against *E. coli* as the indicator bacteria.

2.4.4 Production of Crude Bacteriocin
The dadih’s LAB were propagated in MRS broth (1000 ml) seeded with 10% inoculum of overnight culture and incubated at 37°C for 24 h. After incubation, the whole broth was centrifuged at 10,000×g for 15 min and the cell-free supernatant was used as crude bacteriocin [19].

3. Results and Discussion
In this study the antimicrobial ability of 12 BAL strains isolated from dadih against *Escherichia coli* is presented in Table 1.

| Dadih’s LAB | Inhibition zone (mm) |
|-------------|---------------------|
| *Leuconostoc paramesenteroides* R-8 | 3.83 |
| *Streptococcus cremoris* R-14 | 3.60 |
| *Streptococcus faecalis* subsp. *liquefaciens* R-19 | 4.20 |
| *Streptococcus lactis* subsp. *diacetylactis* R-22 | 6.33 |
| *Leuconostoc paramesenteroides* R-31 | 3.07 |
| *Streptococcus faecalis* subsp. *liquefaciens* R-32 | 3.10 |
| *Streptococcus lactis* subsp. *diacetylactis* R-41 | 1.93 |
| *Streptococcus lactis* subsp. *diacetylactis* R-43 | 2.83 |
| *Leuconostoc paramesenteroides* R-45 | 5.53 |
| *Leuconostoc paramesenteroides* R-49 | 2.63 |
| *Streptococcus faecalis* subsp. *liquefaciens* R-55 | 3.63 |
| *Streptococcus faecalis* subsp. *liquefaciens* R-56 | 4.63 |

Table 1 shows that all supernatants from the dadih’s LAB were able to inhibit all pathogenic and decaying bacteria with inhibitory zones varying from 1.93 - 6.33 mm. LAB which shows high antimicrobial activity against all pathogenic and spoilage bacteria is *Streptococcus lactis* subsp. *diacetylactis* R-22, *Leuconostoc paramesenteroides* R-45, *Streptococcus faecalis* subsp. *liquefaciens* R-19, and the low antimicrobial activity on *Streptococcus lactis* subsp. *diacetylactis* R-41 and *Leuconostoc paramesenteroides* R-49. Ref. [20, 21, 22] reported the ability of some LAB isolates to inhibit various types of pathogenic bacteria. Therefore, the last three LABs were not continued for further testing. To find out the antimicrobial compounds of 9 dadih’s LAB, it was continued to evaluate supernatant sensitivity to various pH and heat treatments, and the results were shown in Tables 2 and 3.
Table 2. Sensitivity of antimicrobial activity of supernatant from dadih’s LAB against *Escherichia coli* at various pH

| Dadih’s LAB               | Control | pH 3 | pH 5 | pH 7 | pH 9 | pH 11 |
|---------------------------|---------|------|------|------|------|-------|
| *S. faecalis* subsp. *liquefaciens* R-19 | 12.30   | 9.20 | 7.20 | 8.10 | 4.30 | 8.20  |
| *S. lactis* subsp. *diacetylactis* R-22  | 7.70    | 2.60 | 3.00 | 5.70 | 0.00 | 0.00  |
| *S. faecalis* subsp. *liquefaciens* R-32  | 8.60    | 10.50| 15.40| 14.70| 14.40| 15.40 |
| *S. lactis* subsp. *diacetylactis* R-41  | 4.10    | 5.70 | 5.00 | 4.20 | 6.60 | 5.20  |
| *S. lactis* subsp. *diacetylactis* R-43  | 11.50   | 10.40| 6.70 | 8.70 | 6.30 | 9.80  |
| *L. paramesenteroides* R-45            | 5.50    | 7.70 | 6.10 | 6.50 | 8.80 | 7.90  |
| *L. paramesenteroides* R-49            | 1.60    | 9.80 | 11.30| 0.00 | 0.00 | 0.00  |
| *S. faecalis* subsp. *liquefaciens* R-55 | 3.20    | 5.70 | 4.40 | 8.30 | 12.50| 11.50 |
| *S. faecalis* subsp. *liquefaciens* R-56 | 2.70    | 3.30 | 2.90 | 0.00 | 0.00 | 0.00  |

Table 3. Sensitivity of antimicrobial activity of supernatant from dadih’s LAB against *Escherichia coli* at various heating temperatures

| Dadih’s LAB               | Inhibition zone (mm) | Temperature (°C) |
|---------------------------|----------------------|------------------|
|                           | Control | 30  | 50  | 70  | 90  | 100 | 121 |
| *S. faecalis* subsp. *liquefaciens* R-19  | 5.90    | 4.80 | 7.80 | 8.10 | 7.10 | 7.80 | 7.40 |
| *S. faecalis* subsp. *liquefaciens* R-32  | 8.30    | 9.10 | 9.70 | 9.30 | 7.00 | 8.70 | 6.70 |
| *S. lactis* subsp. *diacetylactis* R-43  | 5.90    | 4.40 | 5.20 | 7.20 | 7.50 | 5.60 | 6.20 |
| *L. paramesenteroides* R-45             | 4.90    | 5.70 | 5.00 | 6.10 | 4.30 | 4.50 | 5.20 |
| *L. paramesenteroides* R-49             | 8.80    | 6.40 | 8.10 | 5.10 | 4.90 | 7.10 | 7.30 |
| *S. faecalis* subsp. *liquefaciens* R-55 | 5.20    | 8.10 | 5.50 | 6.70 | 6.20 | 6.80 | 6.10 |

Data in Tables 2 and 3 show that the antimicrobial activity of *Streptococcus lactis* subsp. *diacetylactis* R-22, *Lecuonostoc paramesenteroides* R-49 and *Streptococcus faecalis* subsp. *liquefaciens* R-56 against *E. coli* were lost after the supernatants were adjusted to pH from 7 to 11. This is because the antimicrobial activity of these 3 LABs was derived from organic acids, especially lactic acid which is produced from degradation of simple sugar during their growth. The same finding was reported by Ref. [23]. Growth of *E. coli* in beef was also inhibited by lactic acid and citric acid during cold storage of beef [24]. The other 6 LAB strains have antimicrobial properties that are most likely derived from bacteriocin which are characterized by no loss of inhibitory zones after the pH is adjusted to pH 11 (Figure 1).

Because the supernatants of the 6 dadih’s LAB had antimicrobial activity that is resistant to various heating temperatures and pH, then it was continued to isolate the crude bacteriocin from these LAB. Antimicrobial ability of crude bacteriocin against *E. coli* was presented in Table 4. The data in Table 4 shows that crude bacteriocin from 6 dadih’s LAB were able to inhibit the growth of *E. coli* with different inhibition zone. The highest antimicrobial activity was produced by *Streptococcus lactis* subsp. *diacetylactis* R-43, and lowest activity in *Streptococcus faecalis* subsp. *liquefaciens* R-55.
Figure 1. Inhibition zone of supernatant from strains R-41 and R-19 against Escherichia coli at various pH

Table 4. Antimicrobial activity of crude bacteriocin from dadih’s LAB against Escherichia coli

| Dadih’s LAB                                             | Inhibition zone (mm) |
|---------------------------------------------------------|----------------------|
| *Streptococcus faecalis* subsp. *liquefaciens* R-19     | 6.30                 |
| *Streptococcus faecalis* subsp. *liquefaciens* R-32     | 4.57                 |
| *Streptococcus lactis* subsp. *diacetylactis* R-41      | 5.32                 |
| *Streptococcus lactis* subsp. *diacetylactis* R-43      | 8.90                 |
| *Leuconostoc paramesenteroides* R-45                    | 3.90                 |
| *Streptococcus faecalis* subsp. *liquefaciens* R-55     | 3.57                 |
Furthermore, a crude bacteriocin was evaluated for its sensitivity at various heating temperatures on the growth of *E. coli* and the results are presented in Table 5.

**Table 5.** Antimicrobial activity of crude bacteriocin from dadih’s LAB against *Escherichia coli* at various heating temperatures

| Dadih’s LAB | Inhibition zone (mm) | Temperature (°C) |
|-------------|----------------------|-----------------|
|             | Control 30 50 70 90 100 121 |
| *S. faecalis* subsp. *liquefaciens* R-19 | 4.56 5.55 4.34 5.87 5.66 7.09 3.20 |
| *S. faecalis* subsp. *liquefaciens* R-32 | 4.79 5.43 4.53 5.88 3.43 6.20 0.67 |
| *S. lactis* subsp. *diacetylactis* R-43 | 5.76 6.55 4.43 5.77 5.66 9.63 1.30 |
| *L. parmesenteroides* R-45 | 4.56 4.66 4.42 5.32 5.53 4.66 0.83 |
| *L. parmesenteroides* R-49 | 5.87 7.76 5.56 6.66 5.97 5.33 3.10 |
| *S. faecalis* subsp. *liquefaciens* R-55 | 6.78 7.77 6.00 6.00 6.33 5.86 0.50 |

The data in Table 5 shows that the antimicrobial activity of crude bacteriocin was relatively resistant to heating temperatures up to 121°C. These results confirm the resistance of bacteriocin present in supernatants (Table 3) that were heated in the same temperatures. To ensure that the antimicrobial component is bacteriocin, sensitivity was tested at various enzymes, and the results are presented in Table 6.

**Table 6.** Antimicrobial activity of crude bacteriocin from dadih’s LAB against *Escherichia coli* treated with various enzymes

| Dadih’s LAB | Control | Inhibition zone (mm) |
|-------------|---------|----------------------|
|             | Amilase Trypsin Proteinase K |
| *S. faecalis* subsp. *liquefaciens* R-19 | 8.87 5.90 0.0 0.0 |
| *S. faecalis* subsp. *liquefaciens* R-32 | 5.32 8.43 0.0 0.0 |
| *S. lactis* subsp. *diacetylactis* R-43 | 5.50 9.66 0.0 0.0 |
| *L. parmesenteroides* R-45 | 7.78 4.56 0.0 0.0 |
| *L. parmesenteroides* R-49 | 5.77 7.43 0.0 0.0 |
| *S. faecalis* subsp. *liquefaciens* R-55 | 5.30 8.43 0.0 0.0 |

The data in Table 6 shows that the antimicrobial activity of crude bacteriocin was not lost when treated with amylase, but the activity was lost when treated with proteolytic enzymes (trypsin and proteinase K). This means that the antimicrobial component of the 6 LABs was actually a bacteriocin which is protein or peptide compounds. Bacteriocin is protein compound that is biologically active in inhibiting bacterial growth from a group of related bacteria [25]. Most bacteriocins of LAB are peptide compounds with small molecular weights (<10 kDa), heat-resistant, cationic and amphiphilic [26, 27]. Various types of LAB have been reported to produce bacteriocin to inhibit the growth of pathogenic bacteria [19], [16], [18], [28, 29].

**Conclusion**

Among 12 LAB, only 9 strains were found to have an inhibition zone of more than 4 mm. The antimicrobial compound of 3 strains derived from organic acids mainly lactic acid, and other 6 strains namely R-43, R-32, R-19, R-55, R-45 and R-41 derived from bacteriocin based on sensitivity to pH, enzymes and heat treatments. Crude bacteriocin derived from 6 LAB strains inhibited the growth of *Escherichia coli*, and the highest antimicrobial activity was obtained in *Streptococcus lactis* subsp. *diacetylactis* R-43 followed by *Streptococcus faecalis* subsp. *liquefaciens* R-19 and *Leuconostoc parmesenteroides* R-41.
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