Antimicrobial Activity of Combination Bacteriocin and Asam Sunti Extract (*Averrhoa bilimbi* L. fermented) Against Multidrug Resistant *Escherichia coli* in Lettuces (*Lactuca sativa*)

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Abstract

The ready-to-eat vegetables are often associated with the presence of multidrug-resistant (MDR) bacteria. This study aimed to evaluate the potency of bacteriocin, Asam Sunti extract, and their combination against MDR *E. coli* in lettuce. Their antimicrobial activity was assessed using the disk diffusion method and bacterial enumeration after direct application in pre-inoculated lettuce with MDR *E. coli*. The bacteriocin was produced by *Lactobacillus plantarum* BP102 at optimum production time or during the stationary phase at 18 h. These bacteriocins were able to inhibit five MDR *E. coli* isolates, while Asam Sunti extract and the combination of bacteriocin and Asam Sunti extract were only able to inhibit three MDR *E. coli* (LL1.2, LL3.11, and LL3.12) and (LL1.2, LL1.3, and LL3.11), respectively. In direct application to pre-inoculated fresh lettuce, higher inhibition of MDR *E. coli* was observed after applying the combination of bacteriocin and Asam Sunti extract with a ratio of 1:1 and 1:2, compared to bacteriocin alone. However, the inhibitory activity of this combination treatment was not significantly different ($p>0.05$) with the Asam Sunti extract alone. The highest rate of decrease in total bacteria in lettuces was 97% occurred in isolate LL1.2 with bacteriocin treatment alone, and isolate LL3.11 with combination treatment of bacteriocin and Asam Sunti extract (1:2). While on MCA media, the best reduction rate of 94% occurred in isolate LL1.2 with treatment using bacteriocin only, Asam Sunti extract only, and their combination (1:2). The inhibition of MDR *E. coli* in fresh lettuces by bacteriocin, Asam Sunti extract, and their combination was strain-dependent which was indicated by various inhibition results in all treatments.

Key words: Asam Sunti extract, Bacteriocin, multidrug resistant.

INTRODUCTION

The emergence of multidrug-resistant bacteria (MDR), where microorganisms are resistant to more than one antibiotic, has become a problem that needs attention. The MDR bacteria are usually found in health-related facilities. Surprisingly, the emergence of MDR bacteria is also reported in foods [1,2,3]. The MDR bacteria found in fresh or raw foods such as vegetables might be due to the natural contamination from irrigation water, organic fertilizers, and soil during cultivation [4].

Fresh vegetables such as lettuce are commonly consumed by Indonesian people as *lalapan* and used as raw materials in the salad. Fresh vegetables that are washed only with water are not properly able to eliminate pathogenic bacteria that contaminate the foods, such as *Escherichia coli* [5]. Ready-to-eat vegetables (RTE) such as lettuce, basil, long beans, and cabbage sold in the Malang market are reported to contain MDR bacteria. Lettuce contains MDR *E. coli* bacteria that are resistant to kanamycin, tetracycline, and streptomycin [6]. In 2018, in the United States and Canada, health problems associated with the consumption of fresh romaine lettuces as reported by The Centers for Disease Control (CDC) that out of 58 people who were sick, five were hospitalized ,and one person died. The fresh lettuces were contaminated with *E. coli* O157:H7. The *E. coli* O157:H7 strain was found in the sediment of the agricultural water reservoir on the farm [7]. MDR bacteria such as gram-negative bacteria that produce ESBL (Extended Spectrum Beta-Lactamase) enzymes, such as *E. coli* and *Klebsiella pneumoniae,* can destroy many clinically important antibiotics. Bacteria expressing ESBL are difficult to control using more than two or three antibiotics [8].

Bacteriocins are a potential candidate to replace antibiotics as antimicrobial agents against MDR bacteria [9]. However, bacteriocins are less effective in controlling the growth of Gram-negative bacteria such as MDR *E. coli.* The outer membrane of these bacteria acts as a barrier to cell permeability towards antimicrobial substances reaching the cytoplasmic membrane. In addition, bacteriocin activity is also influenced by the presence of the proteolytic enzyme produced by those bacteria [10]. Therefore,

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bacteriocin alone does not guarantee its efficacy in inhibiting Gram-negative bacteria such as MDR *E. coli*. The combination of bacteriocins with other antimicrobial compounds and physical treatments that can work synergistically, providing better activity [11]. The combination of bacteriocin (nisin) and essential oil of *Ocimum basilicum, Salvia officinalis,* and *Trachyspermum ammi* showed a synergistic interaction in inhibiting the bacteria *E. coli* O:157 [12].

Asam Sunti is a fermented starfruit (*Averrhoa bilimbi* L.) used as a spice in food by the people in Aceh Province. Asam Sunti contains organic acids such as oxalic acid, malic acid, lactic acid, citric acid and ascorbic acid. In addition, it also contains phytochemical compounds with antimicrobial activity such as alkaloids, tannins, flavonoids, phenols, and saponin can be used as antibacterial agents [22]. The combination of bacteriocin with natural compounds such as Asam Sunti extract is expected to increase its effectiveness in inhibiting MDR bacteria. Thus, the purpose of this study was to evaluate the potential of the combination of bacteriocin and Asam Sunti extract in inhibiting MDR *E. coli* bacteria in lettuce.

MATERIAL AND METHOD

Bacterial Cultures

Lactobacillus plantarum BP102 was a bacteriocin producer isolated from garlic bulbs (*Allium sativum*) [13]. Indicator pathogen bacteria used were MDR *E. coli* strain LL1.2, LL1.3, LL3.11, LL3.12, and LL3.13, which were isolated from RTE vegetables from Malang markets [6].

The Optimum Production of Bacteriocins

The liquid culture of *L. plantarum* BP102 (10 mL) was inoculated in 90 mL of MRS broth and incubated at 37°C for 36 h. The bacterial culture was taken as much as 5 mL every hour for six hours and then four hours up to 38 hours, then the optical density (OD) was determined using a spectrophotometer with a wavelength of 600 nm. The bacterial cultures were centrifuged at 10.000 rpm for 20 minutes at 4°C. The supernatant was taken, and the pH was adjusted to pH 6.5. The neutralized supernatant was filtered using a sterile membrane filter (0.22 µm) to obtain a cell-free supernatant (CFS). The inhibitory test was carried out using agar disk-diffusion to determine the bacteriocin activity (Equation 1). After that, the CFS was freeze-dried to be used for further tests [14].

$$\text{Bacteriocin activity } (mm^2.mL^{-1}) = \frac{Lz - Ls}{V} \quad \text{.....(1)}$$

Description:
Lz = Diameter of clear zone (mm^2)
Ls = Diameter of blank disk (mm^2)
V = Volume of sample (mL)

Extraction of Asam Sunti

The extraction of Asam Sunti was carried out by the maceration method using ethanol as a solvent [34]. Then, the Asam Sunti extract in the form of paste with a pH of 1.3 was adjusted to pH 2.5, which was then freeze-dried. The salinity of Asam Sunti extracts was also measured using a refractometer.

Phytochemical Analysis of Asam Sunti Extract

The analysis of Asam Sunti extract was carried out by qualitative test according to the Harborne (1987) method. It detected qualitatively the presence of metabolite compounds, such as tannins, phenols, saponins, steroids, alkaloids, and flavonoids.

The Combination of Bacteriocin and Asam Sunti Extract *in Vitro*

One loopful of MDR *E. coli* isolates as indicator bacteria was taken and inoculated in 25 mL Nutrient broth incubated at 37°C for 24 h. The indicator bacteria (0.1 mL) with a cell density of 10^6 cells.mL^{-1} were inoculated into Nutrient agar using the spread plate technique. In this experiment, three treatments were used, namely 1% bacteriocin, 1% Asam Sunti extract, and a combination of bacteriocin and asam sunti extract ($^v/_v$) with a ratio (%) of 1:1, 1:2, and 2:1. Method for preparing a combination immersion solution of bacteriocin and Asam Sunti extract (1%) for immersion were prepared by dissolving 1 g of bacteriocin or Asam Sunti extract in 100 mL of sterile distilled water. The 50 µL of each sample was used for antimicrobial activity test using the agar disk diffusion method [15]. The antimicrobial activity was calculated using Equation 1.

Application of Bacteriocin and Asam Sunti Extract on Lettuces

Fresh lettuces (2 g) were artificially contaminated by immersing in 10 mL NB medium containing 10^6 cells.mL^{-1} of MDR *E. coli* at room temperature for 5 mins. The strain of MDR *E. coli* used was the sensitive strain according to the result of *in vitro* tests. Then, the lettuce samples were then placed on sterile filter paper. Each contaminated lettuce was then immersed for 5 mins in 5 mL of 1% bacteriocin, 1% Asam Sunti extract, and a combination of both. While fresh

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lettuces (2 g) immersed in 10 mL of sterile distilled water were used as the control. Lettuce sample was rinsed with 5 mL of sterile salt water (0.85% NaCl) before it was diluted 10-fold and inoculated into Nutrient agar (to detect total of bacteria) and MacConkey agar (selective medium used for the detection of *E. coli* that can be seen from the characteristic morphology) using the pour plate method with three replications. Petri dishes were incubated at 37°C for 24 h, and colony counts were conducted using total plate count [14]. The percentage of reduction of the total number of bacteria growing on the media was calculated according to Equation 2.

$$\textbf{Cell number reduction (\%)} = \frac{No - Nt}{No} \textbf{ X 100}\% \quad \text{.....(2)}$$

Description:
N_o = Number of bacterial colonies in the control.
N_t = Number of bacterial colonies in the treatment.

Data Analysis

The data obtained from the *in vitro* test and application of fresh lettuce were analyzed using a one-way analysis of variance (ANOVA) with $p<0.05$. The results that are significantly different were further tested using Tukey's test. Data analysis was performed using SPSS 21.0 for Windows.

RESULT AND DISCUSSION

Bacteriocin Activity and its Growth Curve

The growth curve of *L. plantarum* BP102 (Fig. 1) shows the adaptation/lag phase that occurred at the first hour of incubation. The exponential phase occurred at 2 h to 10 h of incubation time and was followed by the stationary phase at 10 h to 38 h. The bacteriocin activity of *L. plantarum* BP102 was detected during the exponential and stationary phase, but it reached the optimum activity at 18 h during the stationary phase indicated by the highest inhibition activity against *E. coli* LL1.2 and LL1.3. Similar results were reported that the optimum production of bacteriocin of *L. plantarum* was produced after 14 h of incubation [16]. Other studies have shown that the optimum production of bacteriocins was at 19 to 30 h, depending on the high biomass growth medium [17].

The incubation time (Fig. 1) showed the different inhibitory activities. However, the inhibitory activity shown was not much different. The highest inhibitory activity against the two test bacteria was found at 18 h. In the test, the highest inhibition of bacteriocin against *E. coli* LL1.2 was 1.22 $mm^2.mL^{-1}$ and for *E. coli* LL1.3 was 1.16 $mm^2.mL^{-1}$. The more incubation time, the more bacteriocin activity increased and reached its optimum in the stationary phase.

A study reported that *L. plantarum* ATCC 8014 can produce metabolite compounds that inhibit Gram-positive (*Staphylococcus aureus* and *Listeria monocytogenes*) and Gram-negative (*E. coli* and *Salmonella typhimurium*) bacteria. Tests were carried out at 15, 18, 21, and 24 h, all of which indicated an inhibition zone. The highest zone of inhibition was found at 24 h in three bacteria (*S. typhimurium, S. aureus,* and *L. monocytogenes*), but in *E. coli*, the highest inhibition zone was at 18 h [18].

Another study reported that the optimum bacteriocin production in de Man Rogosa and Sharpe Broth (MRSB) at 12 h during the final exponential growth phase and maximum at 32 h during the stationary phase. [19]. Another study reported the optimum production of bacteriocins by LAB at an incubation time of 24 h and a maximum of 48 h. Therefore, the bacteriocin activity depends on the type of bacteria and media conditions [20].

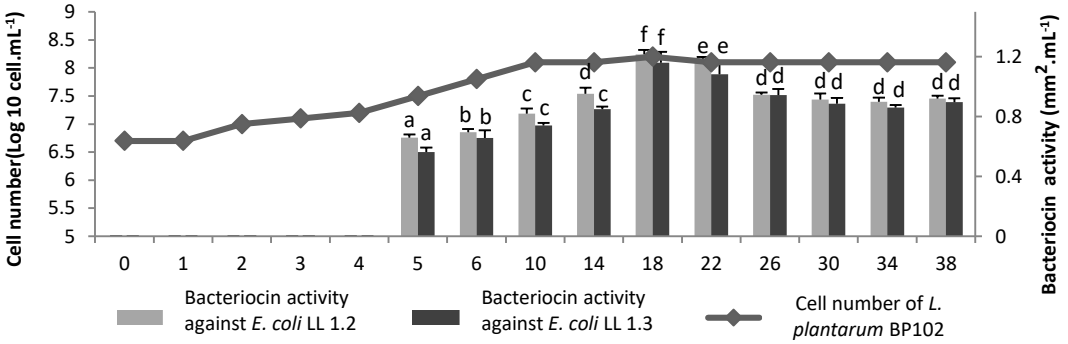

Figure 1. Growth curve and bacteriocin activity of *L. plantarum* BP102 on MDR *E. coli* LL1.2 and LL1.3. Data were expressed as mean ± standard deviation of the three replications. Different notations showed different among treatments ($p<0.05$).

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Phytochemical Analysis of Asam Sunti Extract

Asam Sunti is used by the Indonesian people, especially the people of Aceh Province, as a flavor enhancer. As fruits in general, Asam Sunti contains organic acids. However, the organic acids in Asam Sunti can decrease due to the processing and storage process, except for lactic acid, which tends to increase. The increase of lactic acid indicated the activity of lactic acid bacteria in Asam Sunti [21]. In this study, the phytochemical compounds contained in Asam Sunti extract (Table 1) were saponin and triterpenoid. Only these compounds were successfully identified, possibly because of the salt content in Asam Sunti. The salt content interfered with the phytochemical analysis, so it is necessary to wash it first before the phytochemical content was analyzed. Saponins are compounds that have a strong surface tension that acts as an antimicrobial by disrupting the stability of the bacterial cell membrane, which causes cell lysis. It is because saponins are semipolar compounds that can dissolve in lipids and water so that these compounds will be concentrated in microbial cell membranes [22]. The saponin content in starfruit extract was higher than that of the leaves and petioles. Saponin compounds found in starfruit (*Averrhoa bilimbi* L.) were triterpene saponins [23].

Table 1. The phytochemical compounds in Asam Sunti extract based on qualitative test

No.	Phytochemical compounds	Result
1.	Flavonoids	-
2.	Alkaloids	-
3.	Tannins	-
	Terpenoids	-
4.	Steroids	-
	Triterpenoids	+
5.	Phenol	-
6.	Saponins	+

Description: (+) = Identified), (-) = Not Identified

Combination of Bacteriocin and Asam Sunti Extract *in Vitro*

Based on the qualitative test results of the combination of bacteriocin and Asam Sunti extract on MDR *E. coli* bacteria, the bacteriocin alone showed inhibitory activity against all isolates of MDR *E. coli* (Fig. 2). The highest inhibitory activity was found against strain LL1.2, by 1.14±0.19 mm (p<0.05) and LL3.12 by 1.91±0.04 mm (p<0.05). Meanwhile, the Asam Sunti extract alone showed inhibitory activity against three isolates, namely LL1.2, LL3.11, and LL3.12, with the highest inhibitory activity against strain LL3.11 by 0.83±0.06 mm (p<0.05). The combination of bacteriocin and Asam Sunti extract with a ratio of 1:1 (B1+AS1) showed inhibitory activity against three MDR isolates, namely LL1.2, LL1.3, and LL3.11. Whereas the combination ratio of 1:2 (B1+AS2) and 2:1 (B2+AS1) also showed activity against four strains of MDR *E. coli*, namely LL1.2, LL1.3, LL 3.11, and LL3.12. The MDR *E. coli* can be inhibited by bacteriocins and Asam Sunti extract and the combination of both against two strains, namely LL1.2 and LL3.11. The combination of 1:2 (B1+AS2) and 2:1 (B2+AS1) against strain LL3.11 showed better activity than bacteriocin, Asam Sunti extracts alone, and the combination 1:1 (B1+AS1). The inhibitory activity of the combination of bacteriocin and Asam Sunti extract was not consistent. It can be assumed that the defense pattern of each strain of MDR *E. coli* was different (strain-dependent).

The *in vitro* test results on MDR *E. coli* LL3.13 showed that no inhibitory activity by Asam Sunti extract and the combination with bacteriocin, but it could only be inhibited by bacteriocin alone. The ability of the rate of adaptation by MDR *E. coli* LL3.13 may result in decreased sensitivity to the antimicrobial compound used. In Gram-negative bacteria, the inhibitory mechanism is more complex because bacteria have a more complex cell wall structure and thus require a higher concentration. The combination of nisin and *Curcuma zanthorrhiza* essential oil, *Curcuma zedoaria* with a concentration of 4%, showed a bactericidal effect towards *E. coli* FNCC 0091 [24]. Several studies have shown that the antimicrobial activity of nisin (bacteriocin) can often be influenced by several factors, including pH, temperature, composition, structure, and natural microbiota in food [25]. In addition, the decrease of bacteriocin activity may be due to proteases released from cells, protein aggregation, adsorption to the cell surface, or feedback regulation [26].

Bacteriocins showed significant inhibition activity against Gram-negative bacteria such as MDR *E. coli shiga toxin-producing* (STEC), which is the most detected pathogen in the food industry [27]. To be able to control spoilage and contamination caused by pathogenic bacteria, many researchers use bacteriocin as part of the hurdle technology [28].

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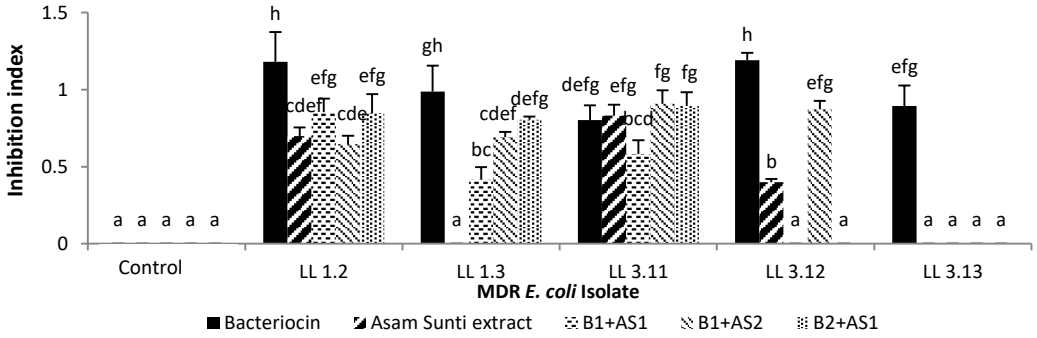

Figure 2. The ability of the combination of bacteriocin and Asam Sunti extract to inhibit MDR *E. coli* bacteria. B1 + AS1: Bacteriocin 1% and Asam Sunti extract 1%, B1 + AS2: Bacteriocin 1% and Asam Sunti extract 2%, and B2 + AS1: Bacteriocin 2% and Asam Sunti extract 1%. Data were expressed as mean ± standard deviation with three replicates. The bars followed by different letters were significantly different (p<0.05).

Meanwhile, the inhibition activity of Asam Sunti extract can occur by the reaction of metabolite compounds such as saponin, which has antimicrobial activity [29]. Natural antimicrobials, including plant extracts, enzymes, bacteriocins, essential oils (EO), bacteriophages, and fermentable substances, have been shown to have the potential to control pathogenic bacteria in food [30].

Application of Bacteriocin and Asam Sunti Extract on Lettuces

Based on the results of the *in vitro* test, four isolates of MDR *E. coli* bacteria were selected, which had an inhibition zone in each combination treatment (strain LL1.2, LL1.3, LL3.11 and LL3.12) to be used in the application assay. Based on the application study, strain LL1.2 was inhibited by the bacteriocin alone, with the highest reduction percentage by 97% (Fig. 3). Strain LL1.2 was inhibited by Asam Sunti extract alone, with a reduced level of 62%. By using combination of bacteriocin and Asam Sunti extract, the reduction percentage of the number of bacterial cells was 92% in the 1:1 (B1+AS1) treatment and 95% in the 1:2 (B1+AS2) combination. For the ratio 2:1 (B2+AS1) combination, the decrease was only 58% in nutrient agar (total of heterotrophic bacteria).

The highest reduction in the number of LL1.2 bacteria was found in the bacteriocin treatment and Asam Sunti extract, without treatment around 94%, while the highest reduction combination was the B1+AS2 treatment by 92% and B1+AS1 by 85%. The reduction in the number of bacteria isolates LL1.2 on MCA was found in the B2+AS1 combination by 1%, with the number of bacterial colonies of 1.5×10^4 CFU.mL^{-1}, which was the lowest reduction. In the previous *in vitro* test, the treatment of B2+AS1 against strain LL1.2 showed an inhibition zone. However, when this was treated in lettuces, strain LL1.2 was not inhibited. It might be due to other bacteria originated from lettuce itself (endophytic bacteria).

MDR *E. coli* LL1.3 treated with bacteriocin alone has a reduction percentage of 78%, for Asam Sunti extract alone of 91%, even though the LL1.3 *in vitro* test could not be inhibited by Asam Sunti extract. Whereas the combination of both antimicrobial agents showed the highest reduction in the number of bacteria in the combination of B1+AS2 (93%) and B2+AS1 (97%), while the combination of B1+AS1 the decrease was about 78%, the same as in the washing treatment with bacteriocin alone. The highest decrease in the number of MDR bacteria *E. coli* LL1.3 on MacConkey media was found in asam sunti extract alone by 91%, and the lowest decrease was treated with bacteriocin alone by 87%, the combination B1+AS1 by 89%. The combination of B1+AS2 was 88%, and B2+AS1 was 89%. The reduction in the number of bacterial cells on MacConkey media was not significantly different (p>0.05), especially the decrease in each combination.

Fresh lettuce immersion only in sterile distilled water (control) had a bacterial cell density of 2.5×10^4 CFU.mL^{-1}. MDR *E. coli* LL3.11, which was given bacteriocin alone, decreased the percentage by 10% with the number of cells 2.4×10^4 CFU.mL^{-1} on nutrient agar media. The effectiveness of bacteriocins in food systems is often low due to several factors such as adsorption to food components, enzymatic degradation, poor solubility, or uneven distribution in the food matrix [31].

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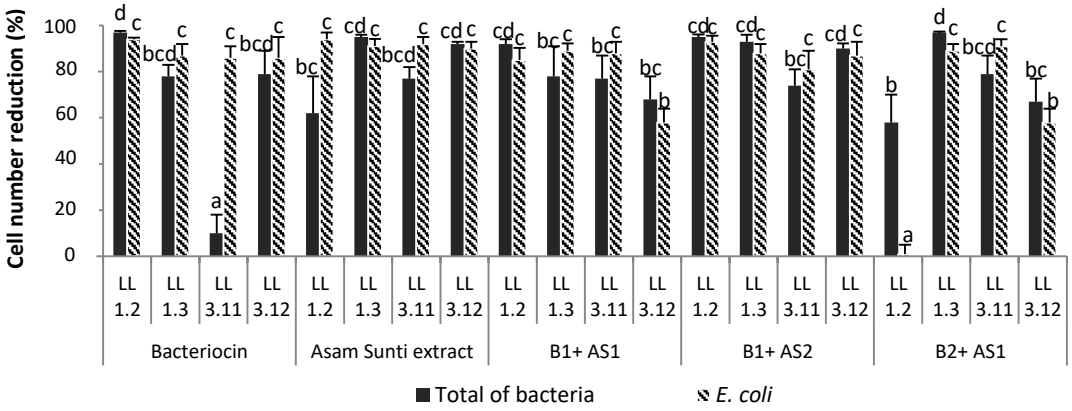

Figure 3. The percentage decrease in the total of bacteria and *E. coli*. Data are expressed as mean ± standard deviation. The different notations on the histogram indicate a significant difference between treatments ($p < 0.05$).

The decrease in MDR *E. coli* LL3.11 on Nutrient agar media by immersion treatment with Asam Sunti extract alone was 77%, while bacteriocin alone was 10% and the combination of 1:1 (B1+AS1), 1:2 (B1+AS2), 2:1 (B2+AS1) was 77%, 74%, and 79%, respectively. It can be concluded that the reduction in the number of heterotrophic bacteria was not significantly different ($p>0.05$) in each combination treatment. MDR *E. coli* LL3.11 on MacConkey agar treated with bacteriocin alone decreased by 86%, Asam Sunti extract alone by 92%, and the combination of 1:1 (B1+AS1), 1:2 (B1+AS2), 2:1 (B2+AS1) was 88%, 81%, and 91%, respectively.

The highest reduction in the total number of *E. coli* LL3.12 on Nutrient agar showed in the treatment of Asam Sunti extract alone (without combination) by 92%, bacteriocin alone (without combination) by 79%, and the highest percentage reduction in the combination of bacteriocin and Asam Sunti extract was found in B1+AS2 by 90%, while the lowest percentage in the combination of 2:1 (B2+AS1) by 67%. In MacConkey media, strain LL3.12 had the highest reduction in the number of cells in the treatment of Asam Sunti extract alone by 90%, and the lowest percentage was found in the combination of B1+AS1 and B2+AS1 by 58%. Therefore, it was assumed that there was an effect of the higher concentration of Asam Sunti extract in inhibiting the growth of *E. coli* LL3.12.

The soaking treatment with Asam Sunti extract has the potential to reduce all MDR *E. coli* in lettuce, while the best combination ratio was 1:1 (B1+AS1) and 1:2 (B1+AS2). The inhibition of MDR bacteria by immersing bacteriocin alone experienced the highest decrease against strain LL1.2 both on NA and MCA media. Whereas in the treatment of Asam Sunti extracts alone, the highest decrease was found towards strains LL1.3 and LL3.12 (NA media) and strain LL1.2 (MCA media), but the reduction value was not significantly different ($p>0.05$). The combination of bacteriocin and Asam Sunti extract in all comparisons was able to inhibit strain LL1.3 with a relatively high percentage value. The reduction in the number of bacterial cells in both media was considered the best if the percentage was above 80%.

The reduced effectiveness of bacteriocins in inhibiting pathogenic bacteria can be attributed to the development of resistance, their interaction, inactivation, or even binding to the bacterial growth medium [32]. The phytochemical compound, especially saponin, was expected to increase the effectiveness of bacteriocin activity in inhibiting MDR bacteria. The combination of enterocin AS-48 and *polyphosphoric acid* (an organic chemical compound) was significantly able to inhibit the growth of the population of *E. coli* O157:H7 in sprouted samples [15]. Application of a combination of bacteriocins and natural compound in foods could potentially be used as part of a hurdle technology that works synergistically and provides a better antibacterial activity [33]. However, future research requires to be elucidated to find the right concentration of combination, and proper extraction of Asam Sunti.

CONCLUSION

In this study, the in vitro test of bacteriocin alone can inhibit all MDR *E. coli* isolates, while the combination of bacteriocin and Asam Sunti extract was only able to inhibit three isolates.

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The combined application of bacteriocin and Asam Sunti extract on fresh lettuce had the best reduction in total bacteria in isolates LL1.2 (bacteriocin only) and LL3.11 (ratio 2:1) by 97%. The best decrease in MDR *E. coli* density was found in isolate LL1.2 by 94% with bacteriocin immersion treatment, Asam Sunti extract, and a combination of 1:2 ratio. From this research, we can know that fresh lettuce contains MDR *E. coli* and can be controlled with bacteriocins and natural compounds. Further research is needed to optimize the combination by considering variations in temperature, pH, and other growth factors, to produce products for washing fresh vegetables.

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