

## **Inhibitory activity of crude bacteriocin produced by lactic acid bacteria isolated from dadih against *Listeria monocytogenes***

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**Abstract.** Pato U, Yusuf Y, Fitriani S, Jonnadi NN, Wahyuni MS, Feruni JA, Jaswir I. 2020. Inhibitory activity of crude bacteriocin produced by lactic acid bacteria isolated from dadih against *Listeria monocytogenes*. *Biodiversitas* 21: 1295-1302. The use of natural preservatives called bacteriocin derived from lactic acid bacteria (LAB) is one way of preventing food from being contaminated by pathogenic microorganisms such as *Listeria monocytogenes* (LM). The aims of this study were to evaluate the ability of LAB isolated from dadih to inhibit the growth of LM and to obtain the antimicrobial components that play a role in inhibiting the growth of LM. The antimicrobial activity of the supernatant obtained from 12 strains of dadih LAB was determined using the paper disk diffusion method. The results showed that the supernatant from the 12 LAB strains was able to inhibit the growth of LM with various inhibition zones. However, out of the 12 LABs, only 9 strains were found to have an inhibition zone of more than 3.5 mm. The antimicrobial compounds of 9 strains were tested and it was found that the antimicrobial compounds of strains R-8, R-14 and R-49 were derived from lactic acid. In addition, 6 strains namely R-43, R-32, R-19, R-55, R-45 and R-41 were derived from bacteriocin based on their sensitivity to pH, heat and enzyme treatments. Crude bacteriocin derived from 6 LAB strains inhibited the growth of LM, and the highest antimicrobial activity was obtained in *Streptococcus faecalis* subsp. *liquefaciens* R-55 with an average inhibition zone of 13.87 mm. Bacteriocin produced by strain R-55 can be used as natural preservatives for the prevention of food-borne disease caused by LM.

**Keywords:** Antimicrobial activity, lactic acid bacteria, *Listeria monocytogenes*, bacteriocin, preservative compound

### **INTRODUCTION**

*Listeria monocytogenes* (LM) is one of 9 species of *Listeria* that can cause listeriosis in humans. Listeriosis is a potentially fatal infection that is generally transmitted through food, results in a high rate of hospitalization (> 90%) and death (20-30%) in large outbreaks (Dussurget 2008; Hernandez-Milian 2014; McLauchin et al. 2004). There are two types of diseases associated with LM infections, namely non-invasive listeriosis which is a type of mild disease and invasive listeriosis which is a fatal and severe disease (FDA 2012). In most cases, the mild symptoms that are commonly observed include myalgia, headache, diarrhea and a fever (FAO/WHO 2004). The incidence rate of listeriosis is relatively rare, ranging from 1 to 10 cases per million per year. However, listeriosis has an important impact on public health considering that it is responsible for the highest rate of hospitalization and death among food-borne infections (Mead et al. 1999; Bille et al. 2006). The USA Centers for Disease Control and Prevention (CDC 2019) reported that 24 people were infected by LM and among them, 22 people were hospitalized and 2 people died.

LM is often associated with many animals (Leclercq 2015), therefore these pathogens can be found as contaminants in many raw food products derived from animals. They contaminate the resulting processed products during the processing mechanism itself. This is where the

mechanisms involved do not apply the principles of good sanitation and hygiene. The United States Department of Agriculture and the Department of Food Safety and Inspection (USDA-FSIS) found an incidence of contamination of 7.24% in sausages cooked in 1991 and 7.69% in ham and lunch slices in 1996 (Levine et al. 2001), in addition to in sausage fermentation (Thevenot et al. 2005), end products (Gianfranceschi et al. 2006), dairy products especially cheese (Goulet et al. 2001; Makino et al. 2005), meat and fish products and fast food (McLauchin et al. 2004).

Some of the processed food products available in the market use various types of preservatives, generally chemical preservatives that can help the products to last longer without being contaminated by the pathogenic microbes that cause food-borne diseases. Chemical preservatives used commercially includes calcium propionate, sodium nitrate, sodium nitrite and sulfite compounds such as sulfur dioxide, sodium bisulfite, potassium hydrogen sulfite and disodium (US FDA 1993). The massive use of chemical preservatives by the food processing industry is due to the fact that these preservatives are easily obtained and widely traded in chemical stores. However, chemical preservatives are not always safe for human consumption because sometimes they can cause health problems ranging from allergies and asthma through to cancer (Anand and Sati 2013). Therefore it is necessary to look for safer natural food preservatives.

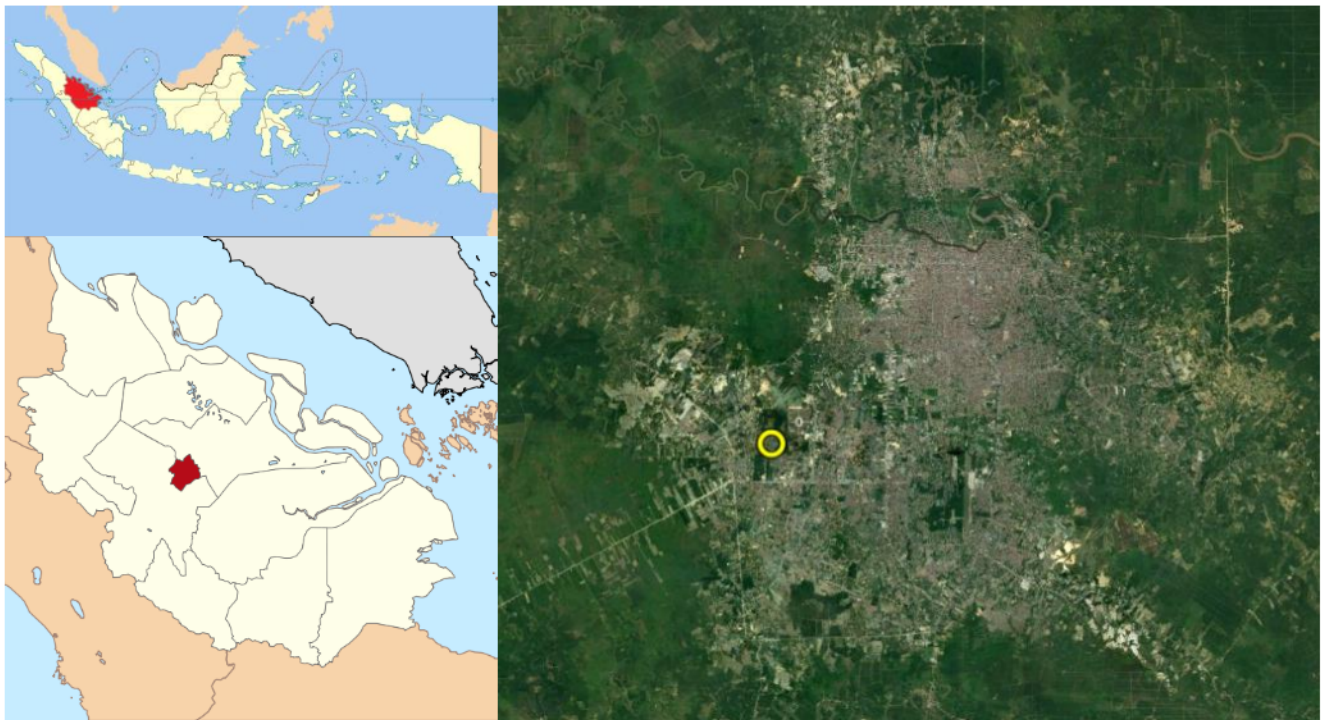
Lactic acid bacteria (LAB) are known for their ability to produce various inhibitors including metabolic end products such as organic acids, hydrogen peroxide and bacteriocin (Rajaram et al. 2010; Sankar et al. 2012; Zhou et al. 2014). The direct application of LAB strains or their antimicrobial products in order to inhibit unwanted bacteria in food is introduced in the concept of biopreservation (Stiles 1996). Many recent studies and reviews have discussed this issue extensively (Cleveland et al. 2001; Deegan et al. 2006; Ross et al. 2002). LAB is the bacteria most often used in fermented foods because it has a long history of safe use. It is a microbe that lives in the digestive tract of both humans and animals (Maragkoudakis et al. 2009). *L. salivarius* isolated from chicken carcasses has the potential to be used as a preservative to increase safety and to extend the shelf life of chicken products (Sakaridis et al. 2014). Bacteriosinogenic LAB has been shown to inhibit the growth of pathogenic and spoilage bacteria in meat (Gálvez et al. 2008; Lücke 2000; Díaz-Ruiz et al. 2012), including in sausage fermentation (Leroy et al. 2006) and some food products (Bredholt et al. 1999; Buchanan and Bagi 1997; Dimitrijevic'et al. 1999; Duffes et al. 1999). Bacteriocin is an alternative bio preservative compound produced by LAB that can be used in food as having bactericidal properties against Gram-positive and Gram-negative bacteria. It is thus very beneficial for the food industry because of its activity of inhibiting the growth of the disease-carrying bacteria that are usually present in food. Bacteriocin has the potential to act as a natural

preservative to replace chemical preservatives in food. Enterococcus, Lactobacillus, Pediococcus, Leuconostoc, and Carnobacterium are genera of LAB that commonly produce bacteriocin (Eijsink et al. 2002). Nisin is produced by *Lactococcus lactis* subsp. *lactis* has been used for commercial food preservation. Hosono et al. (1989) isolated 4 genera of LAB from dadih, a fermented food made from buffalo milk similar to yogurt, namely Lactobacillus sp., Streptococcus sp., Leuconostoc sp. and Lactococcus sp. Dadih's LAB has never been studied for their antimicrobial activity before even though these strains have an anti-mutagenic ability against various mutagen compounds (Hosono et al. 1990). The purposes of the present study were to evaluate the ability of BAL isolated from dadih to inhibit the growth of *Listeria monocytogenes* and to determine the type of antimicrobial compounds involved.

## MATERIALS AND METHODS

### Study area

The study was conducted in the Laboratory of Agricultural Product Analysis, Faculty of Agriculture, Universitas Pekanbaru, Riau, Indonesia (0°25' - 0°45' N and 101°14' - 101°34' E). The map of study area is shown in Figure 1.



**Figure 1.** Study area in Universitas Riau (yellow circle), Pekanbaru, Riau Provinsi, Indonesia (0°25' - 0°45' N and 101°14' - 101°34' E)

### Dadih's LAB and pathogenic bacteria

In this study, 12 strains of LAB namely *Leu. paramesenteroides* R-8, *St. cremoris* R-14, *St. faecalis* subsp. *liquefaciens* R-19, *St. lactis* subsp. *diacetylactis* R-22, *Leu. paramesenteroides* R-31, *St. lactis* subsp. *diacetylactis* R-41, *St. lactis* subsp. *diacetylactis* R-43, *Leu. paramesenteroides* R-45, *Leu. paramesenteroides* R-49, *St. faecalis* subsp. *liquefaciens* R-55 and *St. faecalis* subsp. *Liquefaciens* R-56 isolated from dadih (Hosono et al. 1989) were screened for their antimicrobial activity. The pathogenic gram-positive bacterium used was *Listeria monocytogenes* FNCC-0156.

### Activation of the LAB culture and pathogenic bacteria

The active culture was made by taking 0.1 mL of the dadih LAB culture in a test tube containing 5 mL of MRS broth. It was then shaken evenly and incubated at 37°C for 18 hours. The pathogenic bacteria was activated by inoculating 0.1 ml of the test bacteria into 5 mL of the nutrient broth before it was shaken evenly and incubated at 37°C for 18 hours.

### Antimicrobial activity of the dadih LABs in the in-vitro test

The antimicrobial activity of the supernatant obtained from the 12 strains of dadih LAB was determined using the paper disk diffusion method as described by Saranya and Hemashenpagam (2011) and Syukur et al. (2014). The cultures of dadih LAB were incubated aerobically at 37°C for 24 hours. The indicator bacterium, *L. monocytogenes*, was grown in nutrient broth at 37°C for 24 hours. Following this, 100 µL of pathogenic microorganisms were placed and spread using glass hockey sticks on the surface of MRS agar. The sterile paper disc (6mm) is dipped into the LAB supernatants and into sterile MRS broth as a negative control. The discs are then put onto the surface of MRS agar plates that have been previously seeded with the indicator bacteria. The plates were incubated at 37°C for 24 hours. After incubation, the diameter of the zone of growth inhibition was measured.

### Characterization of bacteriocin

#### Effect of pH

In order to determine the effect of pH, 0.5 mL of purified bacteriocin was added to 4.5 mL of nutrient broth with different pH values (3 to 11) and incubated for 30 min at 37°C. Each of the bacteriocin samples treated at different pH values was assayed against the indicator bacteria through the agar diffusion method (Saranya and Hemashenpagam 2011; Syukur et al. 2014).

#### Effect of temperature

Purified bacteriocin (0.5 mL) was added to 4.5 ml of nutrient broth in the test tube. Each test tube was then overlaid with paraffin oil to prevent evaporation before being heated at different temperatures (30, 40, 50, 60, 70, 80, 90, 100 and 121°C) for 10 minutes. The preparations containing nutrient broth (4.5 mL) and bacteriocin (0.5 mL) were plugged with non-absorbent cotton, covered with aluminum foil and kept in an autoclave at 121°C for 10 minutes to check its activity at very high autoclaving

temperature. The bacteriocin activity of the above different heat-treated specimens was measured using the agar diffusion method (Saranya and Hemashenpagam 2013; Syukur et al. 2014).

#### Effect of amylase and proteolytic enzymes

The effect of amylase and proteolytic enzymes on the activity of crude bacteriocin was studied as described by Zhou et al. (2014). Crude bacteriocin was treated with amylase (5 mg/mL), trypsin (5 mg/mL), and proteinase (5 mg/mL) respectively and a phosphate buffer (0.5M, pH 7.0) was used as a control. The effect of the enzymes on bacteriocin activity was studied through the agar diffusion method (Saranya and Hemashenpagam 2013; Syukur et al. 2014) using the above preparations against LM as the indicator bacteria.

### Production of crude bacteriocin

Dadih LABs were propagated in MRS broth (1000 mL) seeded with a 10% inoculum of overnight culture before being incubated at 37°C for 24 hours. After incubation, the whole broth was centrifuged at 10,000×g for 15 minutes before the cell-free supernatant was collected. The cell-free culture supernatant was saturated with 70% ammonium sulfate and stored at 4°C to precipitate the proteins out. The crude bacteriocin was collected after centrifugation at 10,000 rpm at 4°C for 30 minutes (Ogunbanwo et al. 2003; Sankar et al. 2012).

## RESULTS AND DISCUSSION

In the present study, 12 lactic acid bacteria strains from the genus of *Streptococcus* sp. and *Leuconostoc* sp. isolated from dadih were evaluated for their antimicrobial activity against *Listeria monocytogenes* (Table 1). The inhibition zone produced by the cell-free supernatant indicated the secretion of the antibacterial compounds into the extracellular environment. *St. lactis* subsp. *diacetylactis* R-43, *Leu. paramesenteroides* R-45 and *St. faecalis* subsp. *liquefaciens* R-19 have the highest antimicrobial properties, while *Leu. paramesenteroides* R-8, *St. cremoris* R-14 and *Leu. paramesenteroides* R-31 have the lowest antimicrobial activity.

The last three BAL strains were therefore not used in the next test. Some researchers have previously reported on the ability of LABs such as *Enterococcus faecium*, *Pediococcus pentosaceus*, and *Lactococcus lactis* subsp. *lactis* to inhibit the growth of *L. monocytogenes* (Pinto et al. 2009; Cosentino et al. 2012; Barman et al. 2014). Because of *Leu. paramesenteroides* R-8, *St. cremoris* R-14 and *Leu. paramesenteroides* R-31 have the lowest antimicrobial activity, these three BAL strains were not used in the next test.

To find out the antimicrobial compounds of the 9 dadih LABs, the testing was continued to evaluate the supernatant's sensitivity to various pH treatments. The results are as shown in Table 2.

**Table 2.** Sensitivity of antimicrobial activity of supernatant from dadih's lactic acid bacteria against *Listeria monocytogenes* at various pH

Dadih's LAB	Inhibition zone (mm)					
	Control	pH				
		3	5	7	9	11
<i>St. faecalis</i> subsp. <i>liquefaciens</i> R-19	10.30	10.03	13.95	8.20	7.87	10.30
<i>St. lactis</i> subsp. <i>diacetylactis</i> R-22	1.90	2.20	1.53	0.00*	0.00*	0.00*
<i>St. faecalis</i> subsp. <i>liquefaciens</i> R-32	10.40	9.60	13.60	9.00	10.33	12.30
<i>St. lactis</i> subsp. <i>diacetylactis</i> R-41	5.10	4.07	4.63	7.10	7.53	8.07
<i>St. lactis</i> subsp. <i>diacetylactis</i> R-43	6.17	3.67	5.17	7.10	9.20	9.40
<i>Leu. paramesenteroides</i> R-45	5.65	4.85	5.50	4.63	4.10	4.86
<i>Leu. paramesenteroides</i> R-49	2.43	1.30	0.43	0.00*	0.00*	0.00*
<i>St. faecalis</i> subsp. <i>liquefaciens</i> R-55	5.10	5.77	3.45	9.10	6.77	1.30
<i>St. faecalis</i> subsp. <i>liquefaciens</i> R-56	2.43	3.00	2.77	0.00*	0.00*	0.00*

Note: \*Lost antimicrobial activity

**Table 1.** Antimicrobial activity of cell-free supernatant from dadih's lactic acid bacteria against *Listeria monocytogenes*

Dadih's LAB	Inhibition zone (mm)
<i>Leu. paramesenteroides</i> R-8	2.10
<i>St. cremoris</i> R-14	1.60
<i>St. faecalis</i> subsp. <i>liquefaciens</i> R-19	5.40
<i>St. lactis</i> subsp. <i>diacetylactis</i> R-22	8.10
<i>Leu. paramesenteroides</i> R-31	2.80
<i>St. faecalis</i> subsp. <i>liquefaciens</i> R-32	3.30
<i>St. lactis</i> subsp. <i>diacetylactis</i> R-41	3.83
<i>St. lactis</i> subsp. <i>diacetylactis</i> R-43	6.20
<i>Leu. paramesenteroides</i> R-45	5.97
<i>Leu. paramesenteroides</i> R-49	3.63
<i>St. faecalis</i> subsp. <i>liquefaciens</i> R-55	5.07
<i>St. faecalis</i> subsp. <i>liquefaciens</i> R-56	4.30

Different kinds of antimicrobial compounds from LAB are derived from organic acids, diacetyl, hydrogen peroxide and proteinaceous bacteriocins (Daeschel 1989; Saranya and Hemashenpagam 2013). *St. lactis* subsp. *diacetylactis* R-22, *Leu. paramesenteroides* R-49 and *St. faecalis* subsp. *liquefaciens* R-56 lost their antimicrobial activity after the supernatants pH was set to 7 through to 11. This indicates that the antimicrobial compounds from these three LABs are derived from organic acids, especially the lactic acid produced during their growth (Table 2). Similar results were reported by Pato et al. (2017) for *Lb. casei* subsp. *casei* R-68 isolated from dadih, Shokryazdan et al. (2014) for the *L. casei* Shirota, *L. casei* BF strain and *L. acidophilus*, *L. fermentum*, Neal-McKinney et al. (2012) for Lactobacilli, Keersmaecker et al. (2006) for *L. rhamnosus*, and GG, Vätuiu and Popa (2015) for *L. delbrueckii* subsp. *bulgaricus* and *Lac. lactis* to inhibit various pathogens including *Listeria monocytogenes* by organic acids. The antimicrobial components of the other 6 dadih LAB strains, namely *St. faecalis* subsp. *liquefaciens* R-19, *St. faecalis* subsp. *liquefaciens* R-32, *St. lactis* subsp.

*diacetylactis* R-43, *Leu. paramesenteroides* R-45, *Leu. paramesenteroides* R-49 and *St. faecalis* subsp. *liquefaciens* R-55, are most likely proteinaceous bacteriocin.

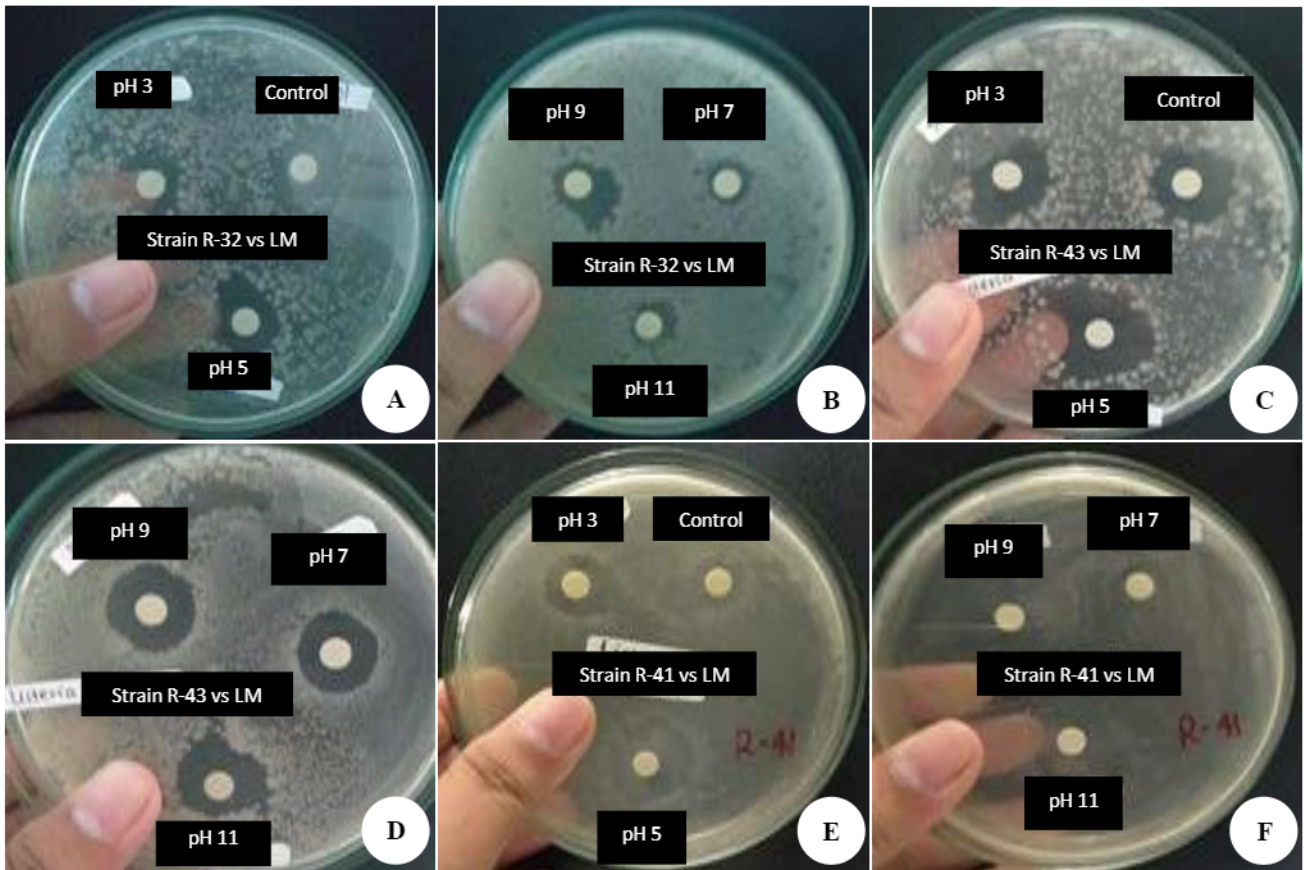
This is characterized by no loss in the inhibitory zones even though the pH of the supernatant is adjusted to pH 11 (Figure 2). Generally speaking, bacteriocin is a peptide or protein compound that is resistant to high heat as shown in Table 3. The antimicrobial potential of heat-killed supernatant supports the thermostable nature of the antimicrobial compound(s).

The data in Table 3 shows that the compounds that are likely to be bacteriocin presented in the supernatant were very resistant to temperatures up to 121°C. Because the supernatants of the 6 dadih LABs were shown to have antimicrobial activity that was resistant to various heating temperatures and pH, the study continued to isolate the crude bacteriocin from the LABs.

The antimicrobial activity of the crude bacteriocin against *Listeria monocytogenes* has been presented in Table 4. The crude bacteriocin from the 6 dadih LABs was able to inhibit the growth of *Listeria monocytogenes* with different inhibition zones.

The highest antimicrobial activity was obtained in *St. faecalis* subsp. *liquefaciens* R-55 and *St. lactis* subsp. *diacetylactis* R-41. The lowest activity was found in *St. faecalis* subsp. *liquefaciens* R-32. The inhibition of *Listeria monocytogenes* using bacteriocin-producing LAB strains has been reported by several researchers. *P. acidilactici* and *Lac. lactis* subsp. *lactis* MM217 produce bacteriocin that can inhibit the growth of *Listeria monocytogenes* in some dairy products (Pucci et al. 1988), cheddar cheese (Buyong et al. 1998), ready-to-eat meat products (Amezquita and Brashears 2002) and meat sausage model systems (Díaz-Ruiz et al. 2012).

Furthermore, the crude bacteriocin was evaluated for its sensitivity at various heating temperatures related to the growth of *Listeria monocytogenes* and the results have been presented in Table 5.



**Figure 2.** Antimicrobial activity of supernatants for some dadih's lactic acid bacteria against *Listeria monocytogenes*. A. Strain R-32 vs LM, B. Strain R-32 vs LM, C. Strain R-43 vs LM, D. Strain R-43 vs LM, E. Strain R-41 vs LM, F. Strain R-41 vs LM. Control = supernatant without pH adjustment, pH 3 = supernatant adjusted to pH 3, pH 7 = supernatant adjusted to pH 7, pH 9 = supernatant adjusted to pH 9, pH 11 = supernatant adjusted to pH 11

**Table 3.** Sensitivity of antimicrobial activity of supernatant from dadih's lactic acid bacteria against *Listeria monocytogenes* at various heat temperatures

Dadih's LAB	Inhibition zone (mm)						
	Heat temperatures (°C)						
	Control	30	50	70	90	100	121
<i>St. faecalis</i> subsp. <i>liquefaciens</i> R-19	8.90	11.77	9.77	5.10	6.77	6.77	4.20
<i>St. faecalis</i> subsp. <i>liquefaciens</i> R-32	9.47	8.10	8.97	12.23	8.87	7.97	5.33
<i>St. lactis</i> subsp. <i>diacetylactis</i> R-41	6.30	7.23	7.10	9.33	8.67	7.10	4.10
<i>St. lactis</i> subsp. <i>diacetylactis</i> R-43	8.33	7.43	8.90	5.47	6.10	6.57	3.10
<i>Leu. paramesenteroides</i> R-45	8.20	9.23	9.33	6.00	8.00	7.00	3.47
<i>St. faecalis</i> subsp. <i>liquefaciens</i> R-55	6.67	5.85	6.00	10.53	8.00	8.67	5.00

**Table 5.** Sensitivity of antimicrobial activity of crude bacteriocin from dadih's lactic acid bacteria against *Listeria monocytogenes* at various heat temperatures

Dadih's LAB	Inhibition zone (mm)						
	Heat temperatures (°C)						
	Control	30	50	70	90	100	121
<i>St. faecalis</i> subsp. <i>liquefaciens</i> R-19	4.01	3.22	4.67	9.22	6.55	5.12	3.21
<i>St. faecalis</i> subsp. <i>liquefaciens</i> R-32	4.75	4.77	3.33	5.77	8.21	5.11	1.10
<i>St. lactis</i> subsp. <i>diacetylactis</i> R-41	5.99	10.87	6.2	5.43	4.63	4.43	1.30
<i>St. lactis</i> subsp. <i>diacetylactis</i> R-43	5.66	4.76	5.86	4.88	3.99	4.88	3.30
<i>Leu. paramesenteroides</i> R-45	5.32	7.32	5.77	4.67	4.99	4.67	1.50
<i>St. faecalis</i> subsp. <i>liquefaciens</i> R-55	8.67	6.63	7.09	7.43	5.99	6.55	1.22

**Table 6.** Antimicrobial activity of crude bacteriocin from dadih's lactic acid bacteria against *Listeria monocytogenes* treated with various enzymes

Dadih's LAB	Inhibition zone (mm)			
	Control	Amylase	Trypsin	Proteinase K
<i>St. faecalis</i> subsp. <i>liquefaciens</i> R-19	8.97	10.36	0.0*	0.0
<i>St. faecalis</i> subsp. <i>liquefaciens</i> R-32	6.45	11.23	0.0	0.0
<i>St. lactis</i> subsp. <i>diacetylactis</i> R-41	5.09	13.80	0.0	0.0
<i>Leu. paramesenteroides</i> R-43	6.76	15.76	0.0	0.0
<i>Leu. paramesenteroides</i> R-45	5.99	10.33	0.0	0.0
<i>St. faecalis</i> subsp. <i>liquefaciens</i> R-55	7.43	11.23	0.0	0.0

Note: \*Lost antimicrobial activity

**Table 4.** Antimicrobial activity of crude bacteriocin from dadih's lactic acid bacteria against *Listeria monocytogenes*

Dadih's LAB	Inhibition zone (mm)
<i>St. faecalis</i> subsp. <i>liquefaciens</i> R-19	5.57
<i>St. faecalis</i> subsp. <i>liquefaciens</i> R-32	5.00
<i>St. lactis</i> subsp. <i>diacetylactis</i> R-41	13.57
<i>St. lactis</i> subsp. <i>diacetylactis</i> R-43	6.10
<i>Leu. paramesenteroides</i> R-45	9.32
<i>St. faecalis</i> subsp. <i>liquefaciens</i> R-55	13.87

The antimicrobial activity of crude bacteriocin was relatively resistant to temperatures up to 121°C. These results confirm the resistance of the bacteriocin present in supernatants (Table 3) heated to the same temperature. To

ensure that the antimicrobial component was bacteriocin, the sensitivity was tested using various enzymes, and the results have been presented in Table 6.

The antimicrobial activity was not lost after the treatment with amylase, indicating that the antimicrobial component is not starch but protein. The loss of antibacterial activity after treatment with proteolytic enzymes such as trypsin and proteinase K demonstrates the proteinaceous nature of the antibacterial metabolite(s). It has already been reported by several researchers that the bacteriocin produced by lactic acid bacteria is a highly thermostable proteinaceous compound that shows antimicrobial activity even after autoclaving at 121°C (Mandal et al. 2013). Bacteriocin is protein compound that is biologically active when it comes to inhibiting bacterial growth in a group of related bacteria (Deraz et al. 2005). Most bacteriocins of LAB are peptide compounds with

small molecular weights (<10 kDa), in addition to being heat-resistant, cationic and amphiphilic (Saeed et al. 2014; Zacharof and Lovitt 2012).

In conclusion, all 12 LABs isolated from dadih can inhibit the growth of *Listeria monocytogenes*. However, 9 strains were found to have an inhibition zone of more than 3.5 mm. The antimicrobial compound of strains R-8, R-14, and R-49 derived from organic acids (mainly lactic acid) and the other 6 strains, namely R-19, R-32, R-41, R-43, R-45 and R-55 derived from bacteriocin, was based on their sensitivity to pH, enzymes and heat treatments. Crude bacteriocin derived from 6 LAB strains inhibited the growth of *Listeria monocytogenes*, and the highest antimicrobial activity was found in *St. faecalis* subsp. *liquefaciens* R-55.

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