ANTIBIOTIC RESISTANCE OF LACTIC ACID BACTERIA ISOLATED FROM PANGELANGAN COW'S MILK

B. Norman[1*], B.L. Roostita[2], R. Nia[3], S.B. Lia[4], P.S. Wendri[5], S. Puja[6], U.L. Gemilang[7]

[1]Department of Biotechnology, School of Postgraduate, University Padjadjaran, Bandung, Indonesia
[2]Department of Veterinary Medicine, Faculty of Medicine, University Padjadjara, Bandung, Indonesia
[3]Department of Biology, Faculty of Mathematic and Natural Science, University Padjadjaran, Bandung, Indonesia
[4]Department of Animal Production, Faculty of Animal Husbandry, University Padjadjaran, Bandung, Indonesia
[5]Department of Animal Product Technology, Faculty of Animal Husbandry, University Padjadjaran, Indonesia
[6]Faculty of Agriculture, University Padjadjaran, Bandung, Indonesia
[7]Department of Food Technology, Faculty of Agricultural Industrial Technology, University Padjadjaran, Bandung, Indonesia

Abstract

World Health Organization (WHO) has stated that antibiotic resistance is one of the global threats for humankind. The occurrence of antibiotic resistance will make antibiotics unable to fight against pathogenic bacteria as the leading cause of several diseases. Nowadays, antibiotic resistance has also been discovered in non-pathogenic bacteria like Lactic Acid Bacteria (LAB), including Lactobacillus sp isolated from milk. This research was used by some steps started from the isolation of lactic acid bacteria, identification of lactic acid bacteria, and antibiotic resistance test. The result has exhibited that Lactobacillus plantarum isolate from cow's milk is resistant to vancomycin (57%), ampicillin (29%), and intermediate resistance to ciprofloxacin (35%). As a result, Lactic Acid Bacteria isolated from cow's milk at Pangalengan show the dominant resistance to vancomycin. The mechanism of antibiotic resistance that happens in Lactobacillus plantarum is intrinsic resistance.

Key word: antibiotic, lactic acid bacteria, lactobacillus, resistance

INTRODUCTION

Cow's milk is white fluid generated from the mammary gland and has enormous nutrition like protein and lactose. That condition makes milk become the natural medium for the growth of some bacteria. One of the kinds of bacteria generally discovered in milk is lactic acid bacteria (LAB) [1], [2].

Lactic acid bacteria (LAB) are a group of gram-positive bacteria, non-spore, facultative anaerobes, cocci or rod-shaped, and produce lactic acid as a result of carbohydrate fermentation. Naturally, lactic acid bacteria can be found in milk and its production. LAB can grow optimally in habitats rich in nutrients such as milk, cheese, vegetables, and meat. In addition, LAB can also be isolated from soil, lakes, or the digestive tract of animals and humans [3]

Lactic acid bacteria (LAB) have long been used in the food fermentation process, and until now LAB in the food industry is still used as a starter [4]. Lactic acid bacteria (LAB) have long been known as biological agents with the ability to ferment dairy products very well so that they can enrich nutrition and increase health [5] [6]. In addition, many LAB species appear as contaminants in livestock raw materials or are intentionally added as starter cultures to them [7].

Antibiotic resistance has been one of the most crucial threats for global health in

[*]Corresponding author:
Normanbilli79@gmail.com
The manuscript was received: 01.10.2021
Accepted for publication: 18.02.2022

which the use of antibiotics has not been potent to pathogenic bacteria. The dissemination of antibiotic resistance can happen through food consumption like milk. Milk can act as the medium for the entry of bacteria in a large amount into the human body. Those bacteria will probably carry Antibiotic Resistance Gene (ARG). A recent study has explained that non-pathogenic bacteria known as probiotics also have the potency to be resistant to antibiotics [8]. Lactic acid bacteria (LAB) like *Lactobacillus* can play a role as a reservoir of antibiotic resistance gene and transfer that gene to other microorganisms in the body [9],[10] Additionally, *Lactobacillus* has been reported resistant to antibiotic quinolones, trimethoprim, and sulfonamide [11].

Pangalengan is the center of milk production and farm of dairy cattle in West Java. It is proved that in 2019 according to the Central Bureau of Statistics, the population of dairy cattle in Indonesia is up to 15.052. Moreover, the high frequency of mastitis cases in Pangalengan has caused the increase of overuse of antibiotics until it leads to the emerging of antibiotic resistance in bacteria both pathogenic and non-pathogenic. Waskita et al., [12] has reported that pathogenic bacteria like *Staphylococcus aureus* isolated from milk in Pangalengan had been resistant to antibiotic Ampicillin, Oxytetracycline, and Sulfamethoxazole. However, there is no report about the emerging antibiotic resistance in non-pathogenic bacteria. Thus, this study is carried out to investigate antibiotic resistance in Lactic Acid Bacteria from milk in Pangalengan.

MATERIAL AND METHOD

Sampling

The sample used in this study was 35 samples of cow's milk taken from two villages in the Pangalengan area, Cipanas and Los-Cimaung villages. Both were selected based on the highest total milk production. This is because if the milk was found resistant to lactic acid bacteria, it would cause a huge impact on the community. A total of 35 milk samples were put into bottles and stored in the cooling box.

Media Preparation

The preparation of MRSA (Man Rogosa Sharpe Agar) media was carried out by weighing 176 grams of MRSA and dissolved in 2 liters of distilled water. Then, it was heated to be boiling while stirred until dissolved. After that, the media was put into the tube, and it was sterilized in an autoclave at a temperature of 121ºC, with the pressure of 15 psi for 15 minutes. A total of 15 ml of that media was put into a petri dish and allowed to solidify [13]. The preparation of MHA (Muller Hinton Agar) media was carried out by weighing 28 grams of MHA and dissolved in 1 liter of distilled water. Then, it was heated to be boiling while stirred until dissolved. After that, the media was put into the tube, and it sterilized in an autoclave at a temperature of 121ºC, with a pressure of 15 psi for 15 minutes. A total of 15 ml of that media was put into a petri dish and allowed to solidify.

Isolation of Lactic Acid Bacteria

Isolation of Lactic Acid Bacteria was done in 1 ml fresh milk diluted in 9 ml physiological NaCl. The dilution was done up to 10-2 (the second dilution). 1 ml sample was taken from the second dilution then grown in MRS agar using the pour plate method. Then it was incubated at 37ºC for 24 hours. The grown isolate was inoculated in another MRS agar using a streak plate to obtain pure culture, and it was incubated at 37ºC for 24 until 48 hours.

Identification of Lactic Acid Bacteria

Identification of Lactic Acid Bacteria was carried out based on the morphological characteristic, physiology, and biochemistry [14], [15]. All isolates were tested using some test procedures such as morphological observations, gram staining, and catalase test.

Morphological Observation

The observation of morphological colony was done after obtaining the culture. Macroscopic observation included shape, color, margin, and elevation of bacterial colony. The surface of the colony could be observed from the side, and the edge of the colony could be observed from the top of dish [16].

Gram Staining

The smear sample was made on a glass object and fixed over a Bunsen burner. That sample was dripped with carbon gentian violet, allowed to stand for 60 seconds, washed with running water, and dried. Then, it was dripped with lugol, allowed to stand for 2 minutes, washed with running water, and dried. After that, it was dripped with alcohol 96% until the purple color was fade. Furthermore, drip fuchsin water into that sample and allowed to stand for 3 minutes, washed with running water, and dried. Finally, the sample was observed with a microscope and dripped immersion oil. Gram-positive bacteria were marked with purple color, and gram-negative bacteria were marked with pink color [17].

Catalase Test

The catalase test was carried out by dripping 3% H_2O_2 on LAB isolates at a glass object. A positive reaction in the catalase test was indicated by the existence of air bubbles on the isolate after 3% H_2O_2 had been added. The formation of air bubbles in the isolate indicates the presence of catalase enzymes produced by bacteria that can convert H_2O_2 into H_2O and O_2 [17].

Cell colony with morphology cocci or bacilli, gram-positive, and negative catalase was considered as LAB colony. All colonies were identified and characterized in the genus level based on Bergey's Manual of Determinative Bacteriology [19]. Further, the identification in the species level used instrument Vitec with two compacts with a procedure based on manufacture.

Antibiotic Susceptibility Test

Isolates of Lactic Acid Bacteria (LAB) with turbidity according to the standard McFarland 0.5 were tested for antibiotic sensitivity by disc diffusion method, using Mueller Hinton Agar (MHA) medium. The sample was swabbed using a sterile cotton bud on the entire surface of the MHA media. After 5 minutes, put antibiotic disk ampicillin (10 mcg), vancomycin (30 mcg), gentamicin (10 mcg), and ciprofloxacin (5 mcg) (oxoid). The result would emerge after sample had incubated at 37ºC for 24 hours. The determination of antibiotic resistant based on diameter of inhibition zone.

RESULT AND DISCUSSION

Bacterial isolation is a technique for obtaining a single bacterial colony [18]. The media used in bacterial isolation is selective media de Man Rogosa Sharpe Agar (MRSA) as the specific media to grow lactic acid bacteria (LAB). MRSA contains polysorbate, acetate, magnesium, and manganese, known as LAB-growth factors. In this research, the colony is grown in 20 Petri dishes.

Isolation of Lactic Acid Bacteria

The LAB isolation was carried out using the dilution method and grown in MRS agar using the pour plate method at 37ºC. The incubation result exhibites the growth of the bacterial colony in MSRA. The second phase of bacterial isolation was continued using the streak plate method for getting a single culture [13].

Identification of Lactic Acid Bacteria

Isolates that have grown are then carried out in the identification process. Identification can be carried out morphology traits of colony and cell, and biochemistry [20]. In this research, identification of lactic acid bacteria was done by observing morphology structure, gram staining, and catalase test. Table 1 below presents the identification result of lactic acid bacteria.

Based on the table, 20 isolates have been identified as *Lactobacillus sp* because it has characteristic of rod-shape, gram positive, and catalase negative. It is in line with Tserovska et al., [29] who has stated that the characteristic of *Lactobacillus sp* is Gram-positive, nonspore-forming rods, catalase-negative, microaerophylic, with fermentative metabolism. Identification at the species level was carried out using the Vitek 2 compact. LAB species identified are *Lactobacillus plantarum*.

Table 1 The observation of morphology, gram staining, catalase test for each isolate

No.	Sample Code	Morphology	Gram Staining	Catalase
1	LC-9	Rod	Positive	(Negative)
2	CI-11	Rod	Positive	(Negative)
3	CI-15	Rod	Positive	(Negative)
4	LC-8	Rod	Positive	(Negative)
5	CI-17	Rod	Positive	(Negative)
6	LC-10	Rod	Positive	(Negative)
7	LC-15	Rod	Positive	(Negative)
8	CI-2	Rod	Positive	(Negative)
9	C1-20	Rod	Positive	(Negative)
10	LC-13	Rod	Positive	(Negative)
11	CI-9	Rod	Positive	(Negative)
12	CI-4	Rod	Positive	(Negative)
13	C1-18	Rod	Positive	(Negative)
14	CI-13	Rod	Positive	Negative)
15	C1-14	Rod	Positive	(Negative)
16	CI-8	Rod	Positive	(Negative)
17	CI-9	Rod	Positive	(Negative)
18.	LC-1	Rod	Positive	(Negative)
19.	LC-2	Rod	Positive	(Negative)
20.	CI-3	Rod	Positive	Negative)

LC = Los Cimaung
CI = Cipanas

Antibiotic Resistance on Lactic Acid Bacteria

In this study, 14 Isolates of *Lactobacillus plantarum* in milk were tested for antibiotic susceptibility by disc diffusion method. Then, it was incubated for one night, and the result was obtained in the form of which antibiotics were resistant. The result of this test is presented in the form of picture and graph.

A total of 14 LAB isolates have proven to be at least resistant to one of the tested antibiotics. The determination of resistance is according to the inhibition zone formed around antibiotics. Antibiotic with the high level of resistance was vancomycin with a resistance percentage of 57%, ampicillin with a resistance percentage of 29%, while gentamicin and ciprofloxacin had the lowest resistance level of 7% and 8%.

Vancomycin is a glycopeptide antibiotic and is narrow spectrum antibiotic that attacks a gram-positive bacteria. Vancomycin is one the kind of antibiotics which is frequently resistant to *Lactobacillus sp.* According to Campedelli et al., [21] a total of 141 from 182 (77%) strain *Lactobacillus sp* were resistant to vancomycin.). Shao et al.,[22] stated that 15 isolates of *Lactobacilus plantarum* had been resistant to vancomycin with the presence of gene vanA, vanB, and vanX. Vancomycin mechanism is destruct peptidoglycan synthesis as the main component of gram-positive bacteria [23].

The mechanism of resistance of *Lactobacillus sp.* against the antibiotic vancomycin was occurred intrinsically in which vancomycin resistance of *Lactobacillus* was generally considered as intrinsic due to the presence of D-Ala-D-lactate instead of natural D-Ala-D Ala dipeptide in their peptidoglycan [22] D-Ala-D-lactate has a low affinity to vancomycin. Generally, *Lactobacillus sp* that is resistant to vancomycin cannot disseminate its resistant gene horizontally [23]. It is because bacteria that receive antibiotic resistance genes through intrinsic mechanisms are unable to spread them horizontally.

Resistance of lactic acid bacteria against ampiclin tend to have a fairly high frequency up to 29%. This is in line with Dec et al., [24] in which *Lactobacillus* isolated from chicken had been resistant 26% on ampicillin. Kmet and Piatnicova [25] exhibited that *Lactobacillus* isolated from chicken gastrointestinal tract was 100% resistant to

ampicillin. The mechanism of resistance *of Lactobacillus* to ampicillin still cannot be explained in detail. However, Kim et al., [26] stated that *Lactobacillus casei* and *Lactobacillus plantarum* produce penicillin g tolerance which causes cell membrane impermeability.

Ciprofloxacin is a class of fluoroquinolone antibiotics. In this study, *Lactobacillus* isolates showed 8% resistance to ciprofloxacin and 35% intermediate to ciprofloxacin. The intermediate-range indicates that the administration of ciprofloxacin has not been effective in the normal dose so that it needs to be increased. Ciprofloxacin works by inhibiting topoisomerase that is an enzyme involved in bacterial DNA synthesis. Topoisomerase is essential in the DNA replication process [26]. According to Danielsen M, dan Wind A [27] *Lactobacillus* have generally been resistant against antibiotic which has a role as an inhibitor of nucleic acids such as enoxacin, ciprofloxacin, and cotrimoxazole. The resistance of ciprofloxacin occurs intrinsically. The resistance of ciprofloxacin is caused by the change of molecular targets like the alteration of DNA topoisomerase and the decrease of intracellular accumulation [28]. *Lactobacillus* isolates are still susceptible to gentamicin with a sensitivity level of 93%. It indicates that gentamicin is still safe to use with the proper regulation.

CONCLUSION

This study showed that *Lactobacillus plantarum* isolates from cow milk in Pangalengan were 57% resistant to vancomycin, 29% resistant to ampicillin, and 35% intermediate resistance to ciprofloxacin.. The high resistance case on *Lactobacillus plantarum* will contributes to the dissemination antibiotic resistance to other bacteria such as enteric pathogen and commensal bacteria. Therefore, the administration of antibiotic in accordance with rule is necessary to prevent the emergence of antibiotic resistance in the future.

ACKNOWLEDGEMENT

The author would like to thank the Academic Leadership Grant research scheme to fund this project under supervision Prof. drh. Roostita L. Balia, M. App. Sc, Ph.D

REFERENCES

[1] Delavenne E, Mounier J, Déniel F, Barbier G, Le Blay G., Biodiversity of Antifungal Lactic Acid Bacteria Isolated from Raw Milk Samples from Cow, Ewe and Goat over One-Year Period, International Journal of Food Microbiolog , 2012, p 185-190

[2] Wouters J. T, Ayad E. H, Hugenholtz J, Smit G., Microbes from Raw Milk for Fermented Dairy Products, International Dairy Journal, 2002, p 91-109.

[3] Tserovska L, Steno S, Yordaufa T., Identification of Lactic Acid Bacteria Isolated from Katyk Goat milk and Cheese, J. Cult collection, Bank Industri Mic Cell Cult., 2004, p 48-52

[4] Boonmee M, Leksawasdi N, Bridge W, Rogers, 2003, P Batch and continuous culture of Lactococcus lactic NZ133: experimental data and model development, Biochem. Eng. J., 2003, p 127-35

[5] Sharma R, Sanodiya B. S, Bagrodia D, Pandey M, Sharma A, Bisen P. S., Efficacy and Potential of Lactic Acid Bacteria Modulating Human Health, International Journal of Pharma and Bio Sciences., 2012, p 935-948

[6] Steele J, Broadbent J, Kok J., Perspective on the Contribution of Lactic Acid Bacteria to Cheese Flavor Development, Current Opinion in Biotechnology., 2013, p 135-141

[7] Sharma R, Sanodiya B. S, Bagrodia D, Pandey M, Sharma A, Bisen P. S., Efficacy and Potential of Lactic Acid Bacteria Modulating Human Health, International Journal of Pharma and Bio Sciences, 2012, p 935-948.

[8] Gazzola, S., Fontana, C., Bassi, D. And Cocconcelli, P.S., Assessment of tetracycline and erythromycin resistance transfer during sausage fermentation by culture-dependent and -independent methods. Food Microbiol, 2012, p 348–354.

[9] Gevers, D.; Huys, G., Swings, In vitro conjugal transfer of tetracycline resistance from *Lactobacillus* isolates to other Gram-positive bacteria. FEMS-Microbiol. 2003, p 125-130

[10] Teuber, M., Spread of antibiotic resistance with food-borne pathogens. Cellular and Molecular Life Sciences, 1999, p 755–763.

[11] Nawaz, M., Wang, J., Zhou, A., Ma, C., Wu, X., Moore, J. E., … Xu, J ., Characterization and transfer of antibiotic resistance in lactic acid bacteria from fermented food products. Current Microbiology, 2011, p 1081–1089.

[12] Waskita, P. T., Balia, R., Joy, B., Utama, G. L., & Novianto., A. Antibiotic Resistance Analysis Of Staphylococcus Aureus As A Main Cause Of Mastitis In The Southern Region Of Bandung Regency. Lxiii(1)2020, p 240–246.

[13] Ismail, Y. S.; Yulvizar, C. dan Putriani., Isolasi, Karakterisasi dan Uji Aktivitas Antimikroba Bakteri Asam Laktat dari Fermentasi Biji Kakao (Theobroma cacao L.), J. Bioleuser, 2017, p 45-53
[14] Harrigan W. F, McCance M. E., Laboratory Methods in Food and Dairy Microbiology. 1st Edn., Academic Press, London, 1976, p 25-29.
[15] Kimaryo V, Massawe G, Olasupo N, Holzapfel W., The use of a starter culture in the fermentation, 2012, p 935-948.
[16] Hadioetomo, R.S., 1993, Mikrobiologi Dasar Dalam Praktek, Teknik dan Prosedur Dasar Laboratorium,, PT. Gramedia, Jakarta.
[17] Hadioetomo, R. S., 1985, Mikrobiologi Dasar-dasar Praktik, Gramedia, Jakarta
[18] Delvia, F., Fridayanti, A., & Ibrahim, A., Isolasi Dan Identifikasi Bakteri Asam Laktat (Bal) Dari Buah Mangga (Mangifera indica L.), 2015, p 114–120.
[19] Holt J, Krieg R, Senath A, Staley T, Williams S, Bergey's manual of determinative bacteriology. Hensly RW and Forlifer E L (eds). 9th ed. Williams and Wilkins. Baltirnore, USA, 1994, p 787.
[20] Purwoko, T, 2007, Fisiologi Mikroba. Bumi Aksara, Jakarta.
[21] Campedelli I, Mathur H, Salvetti E, Rea MC, Torriani S, Ross RP, et al. crossm Genus-Wide Assessment of Antibiotic Resistance in. Appl Enveronmental Microbiol. 2019,p 1–21.
[22] Shao, Yuyu; Zhang, Wenyi; Guo, Huiling; Pan, Ling; Zhang, Heping; Sun, Tiansong., Comparative studies on antibiotic resistance in Lactobacillus casei and Lactobacillus plantarum. Food Control,2015,p 250–258.
[23] Li, Ting; Teng, Da; Mao, Ruoyu; Hao, Ya; Wang, Xiumin; Wang, Jianhua ., A critical review of antibiotic resistance in probiotic bacteria. Food Research International, 2020, 109571–.
[24] Dec, M., Urban-Chmiel, R., Stępień-Pyśniak, D., & Wernicki, A., Assessment ofantibiotic susceptibility in Lactobacillus isolates from chickens. Gut Pathogens,2017, p 1–16
[25] Kmeť, V., & Piatnicová, E, Antibiotic resistance in commensal intestinal Microflora. Folia Microbiologica, 2010, p 332–335.
[26] Appelbaum PC, Hunter PA, The fluoroquinolone antibacterials: past, present and future perspectives. Int J Antimicrob Agents., 2000, p 5-15.
[27] Danielsen M, Wind A. Susceptibility of *Lactobacillus spp*. to antimicrobial agents. Int J Food Microbiol. 2003, p 1–11.
[28] Di, D., & Internazionale, R. Study of fluoroquinolone resistance in *Lactobacillus spp*, 2012,