Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2015, 7(4):1005-1010

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Antibacterial activity of probiotic mixed culture against MRSA and ESBL

***Isnaeni[1] and Ni Made Mertaniasih[2]**

[1]Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Universitas Airlangga, Indonesia
[2]Department of Microbiology, Faculty of Medicine, Universitas Airlangga, Indonesia

---

ABSTRACT

Faced with the methicilin resistant Staphilococcus aureus (MRSA) and the extended-spectrum beta-lactamase (ESBL) pandemic, this study reports the antibacterial activity of seven probiotics against two MRSA (Px1 and Px2) and six ESBL (Px3, Px4, Px5, Px6, Px7, and Px8) isolated from clinical patients. The mixed culture of probiotics was consisted of Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Bifidobacterium bifidum, Bifidobacterium animalis, Lactobacillus plantarum, Streptococcus thermophilus. The probiotics mixture was performed by mixing equally each probiotic starter in MRS broth medium and then incubated at 35^{o}C, 150 rpm for 48 hours. The anti bacterial activity of free cell supernatant (FCS) against each of the bacterial test was assayed by agar diffusion method by using hole as reservoir. The result showed that all the isolates exhibited very good inhibitory activity against target bacteria as indicated by the diameter of inhibition zone: ESBL (21-22 mm) and MRSA (19-20 mm). The potency ratio of the probiotics mixture was also evaluated against kanamycin, streptomycin, and ultra broad spectrum meropenem as standard.

Key words: Antibacterial activity, extended-spectrum beta-lactamase (ESBL), methicilin resistant *Staphylococcus aureus* (MRSA), mixed cultures, probiotics

---

INTRODUCTION

Probiotics are defined as live microorganisms, which when administered in adequate amounts, confer a health benefit on the host [1]. Health benefits have mainly been demonstrated for specific probiotic strains of the following genera: *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* [2]. Credibility of specific health claims of probiotics and their safety must be established through science-based clinical studies.

The antimicrobial activity of seven probiotics as starter cultures against methicilin resistant *Staphylococcus aureus* (MRSA) and extended-spectrum β-lactamase (ESBL) bacteria is the main subject of this research. The probiotics used in this research are the most widely used bacteria as starter cultures for the industrial processing of fermented dairy, meat, vegetable and cereal products. Evidence is presented that the antimicrobial activity of probiotics as starter cultures has been attributed to the production of metabolites such as organic acids (lactic and acetic acid), hydrogen peroxide, ethanol, diacetyl, acetaldehyde, other low molecular mass compounds with antimicrobial activity and bacteriocins [3]. The potential of using bacteriocins of lactic acid bacteria, primarily used as bio preservatives, represents a perspective, alternative antimicrobial strategy for continuously increasing problem with antibiotic resistance. Another strategy in resolving this problem is an application of probiotics for different gastrointestinal and urogenital infection therapies.

It has been shown that the probiotics active against Gram-positive and Gram-negative bacteria, moulds and yeasts. The rapid emergence of drug resistant strains of different microbial pathogens, especially those with multiple resistances, is a major health problem because of their high occurrence worldwide [4, 5]. Probiotics bacteria isolated from commercial dairy products were established to have antimicrobial activities against extended-spectrum β-lactamase (ESBL)-producing strains of *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Proteus mirabilis*, and *Escherichia coli* metallo-β-lactamase (MBL)-producing strain of *Pseudomonas aeruginosa*, and three strains of methicillin resistant *Staphylococcus aureus* (MRSA). The probiotics inhibited the growth of all the tested multiple drug resistant strains [6, 7].

The objective of this study was to investigate the inhibition activities of the mixed probiotics as starter cultures; which consisting of *Lactobacillus acidophilus, Lactobacillus bulgaricus, Lactobacillus casei, Bifidobacterium bifidum, Bifidobacterium animalis, Lactobacillus plantarum, Streptococcus thermophilus* against five strains of MRSA and six strains of ESBL producing bacteria isolated from clinical patients in the Dr Soetomo hospital Surabaya.

The mixed culture of probiotics as starter grown in the MRS agar media for 72 hours was inoculated in MRS broth medium and their activities were observed after incubating for 24, 48, and 72 hours. The activities were compared with meropenem; one of multi resistant drug of choice carbapenem antibiotic. It kills bacteria by blocking the growth of the bacteria's cell wall.

In order to obtain maximum activities, free cell supernatant (FCS) of fermentation broth was concentrated by using freeze dryer and the dry powder obtained was dissolved in a small volume of physiological or saline solution. The activities test was carried out by cylinder plate method. The assay was based on the inhibitory effect of the test solution upon the MRSA and ESBL isolated from clinical patients in DR Soetomo hospital used as test microorganisms. Diameter of zone inhibition (mm) was measured to visualize potency of the solution test.

EXPERIMENTAL SECTION

Bacterial strains and growth conditions

Each probiotic strain: *Lactobacillus acidophilus* (provided by Universitas Gadjah Mada), *Lactobacillus bulgaricus, Lactobacillus casei, Bifidobacterium bifidum, Bifidobacterium animalis, Lactobacillus plantarum, Streptococcus thermophilus* from stock culture was cultivated in the basic growth media (broth) for Lactic Acid Bacteria (LAB) Man-Rogosa-Sharpe (MRS; Difco), incubated in shaking incubator for 72 hours at 37^{o}C and 150 rpm to gain starter culture. Five of ESBL and six MRSA clinical patient's isolates (derived from DR. Soetomo Hospital) as well *Staphylococcus aureus* ATCC 6538 were inoculated on the Nutrient (broth and agar) media. The mixed culture probiotics starter was prepared by mixing one mL of the each suspension culture. Six of ESBL and five of MRSA clinical patient's isolates (derived from DR. Soetomo Hospital) as well *Staphylococcus aureus* ATCC 6538 (provided by Assessment and community Services Unit of Faculty of Pharmacy Airlangga University) were inoculated on the nutrient (broth and agar) media and incubated for 24 hours at 37^{o}C.

Anti bacterial activity

Antibacterial activity of the probiotics mixed starter was assessed using modified procedures of agar diffusion method on solid medium [3, 8, 9]. The viability of cell in the culture suspension was calculated using modified standard procedure as described by Sugiyartono et al. [10]. The pre-culture preparation consisted in creating optimal conditions for the mixed culture starter to express their capacity to produce anti-*S.aureus*, MRSA, and ESBL components. Furthermore, free cell supernatant (FCS) was harvested at 2, 4, and 6 days after incubation in shaking incubator 150 rpm at 35^{o}C by centrifugation and separation of the suspension broth culture. Further tests were carried out to observe the time to be the maximum active components production. Based on these results, before being tested for antibacterial activities, the mixed culture probiootics had been sub cultured in MRS medium in the 150 rpm shaking incubator at 35^{o}C for optimally time incubation. In order to obtain maximum activities, 50 mL of the FCS was concentrated by using freeze dryer and dissolved the powder in the saline solution up to 5 mL. The free cell supernatant concentrate (FCSC) was investigated to observe the MIC against the bacterial tests, using kanamycin, streptomycin, and meropenem as standard solutions.

Specifically, the agar diffusion test was divided into three steps: (1) pre-culture preparation of the bacterial tests by pour plate method, (2) agar hole was prepared as a reservoir of test solution, and (3) the test and standard solution

were filled in the agar hole. Meropenem, kanamycin and streptomycin were used as standard solution representatively sensitive for *S.aureus*, ESBL and MRSA bacteria.

The detailed procedure of agar diffusion test was as follows: a 5 mL volume of 48 hours (150 ppm, 35 ^{o}C) probiotics mixed culture starter raised on MRS broth medium was transferred to 50 mL fresh MRS broth medium, incubated in 150 rpm shaking incubator at 35^{o}C for six days. The FCS and FCSC were prepared as method above used as test solution. The two layers agar plate media were prepared by pour the seed layer media on the surface of 10 mL base layer agar plate media. The seed layer media was consisted of 5 µL inoculums of overnight (24 h at 37 ^{o}C) bacterial test inoculated in 8 mL nutrient agar media. The next step was filling the each agar hole by 50 µL test or standard solution. The plates were incubated for 24h at 37^{o}C under aerobic conditions. The growth inhibitory effect of test and standard solutions comprised of different concentrations of FCS and FCSC at were tested on the *Staphylococcus aureus*, clinical ESBL and MRSA isolates. The bacterial growth inhibitions were observed and measured, noted as zone diameters (mm).

RESULTS AND DISCUSSION

The genus *Lactobacillus* used in this research is a major part of the lactic acid bacteria (LAB) group (including *Lactobacillus* and *Streptococcus* species) that can convert hexose sugars to lactic acid thus producing an acid environment; which inhibits the growth of several species of harmful bacteria [9]. The genera *Streptococcus* is also part of the lactic acid bacteria and contain several strains associated with severe health-care associated infections such as: *Streptococcus pyogenes*, *Streptococcus pneumoniae*, vancomycin-resistant *Enterococcus faecium* [11]. *Bifidobacterium* species together with other probiotics have been proven to treat constipation [12], travellers' diarrhoea [13], antibiotic-associated diarrhoea [14], maintaining remission of disease activity of gut inflammation and moderate ulcerative colitis [15], cholesterol-lowering capacities [16].

The mixed culture probiotics in this research; that consisting of seven probiotics belonging to Lactobacillus, Bifidobacterium, and Streptococcus species has been proven to inhibit *Staphylococcus aureus*, MRSA, and ESBL microorganisms (Fugure 1, Figure 2, and Figure 3). The mixed culture was performed as starter with Total Viability Count (TVC) of 10^{6} cfu/mL and then grown in the MRS broth media for 6 days. The obtained results showed that the free cell supernatant (FCS) of fermentation broth exhibited growth inhibition activity against all microbial tests (Table 1). The highest activities were achieved after 4 days incubation (Figure 4) and generally decreased at 6 days, except against ESBL-2, to which the highest activity was recorded at two days.

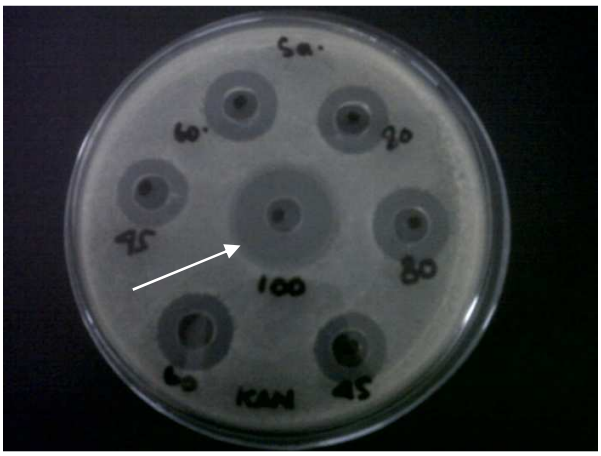

Figure 1. Inhibition activity of concentrate of free cells probiotic fermentation liquid (100) against *Staphylococcus aureus* growth on the nutrient agar medium compared with kanamycin at concentration of 45, 60, and 80 ppm

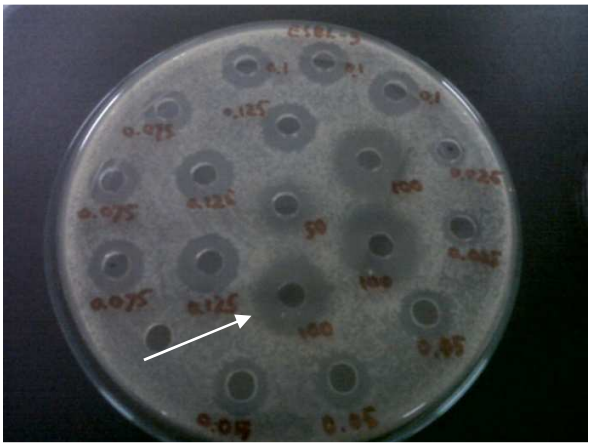

Figure 2. Inhibition activity of concentrate of free cells probiotic fermentation broth (100), meropenem at concentration of 0.05, 0.075, 0.10, 0.125 ppm against *ESBL* growth on the nutrient agar medium

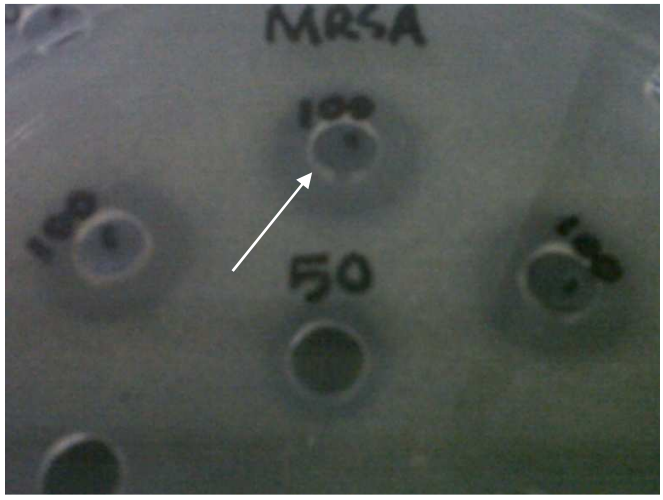

Figure 3. Inhibition activity of 100% (100) and 50% (50) concentrate of free cells probiotic fermentation broth against MRSA growth on the nutrient agar medium

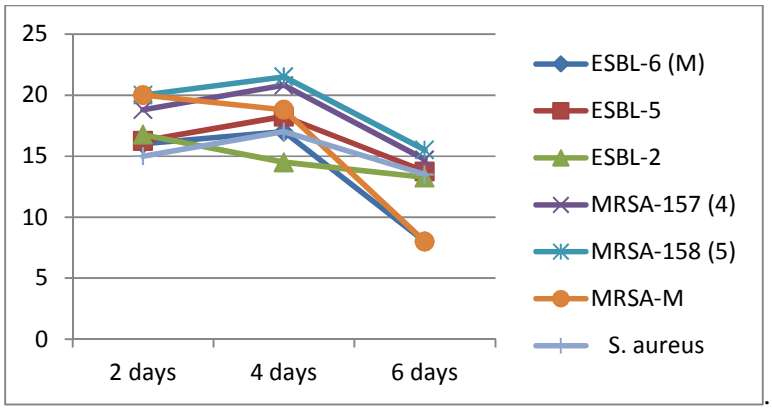

Figure 4. Inhibition activity of 100% (100) and 50% (50) concentrate of free cells probiotic fermentation broth against MRSA growth on the nutrient agar medium

The Minimum Inhibition Concentration (MIC) of the FCS concentrate (FCSC) by diffusion agar method was 10 %, 50% and 50% against *Staphylococcus aureus*, ESBL (1-5), and MRSA (1 and 147) respectively.

Table 1. The growth inhibition activity of the FCSC against *Staphylococcus aureus*, ESBL and MRSA

Bacterial tests	Φ (mm) of inhibition zone of FCSC at concentration of					
	10%	20%	25%	30%	50%	100%
S.aureus	9.75	11.4	12.1	13.38	15.25	20.25
MRSA-1	0	0	0	0	13	16.5
MRSA-147	0	0	0	0	13.5	16.5
ESBL-1	0	0	0	0	14	21
ESBL-2	0	0	0	0	14.8	19.7
ESBL-3	0	0	0	0	16	24
ESBL-4	0	0	0	0	13.5	20
ESBL-5	0	0	0	0	16	23

Comparing the FCSC and meropenem, one of the multi resistant drugs of choice carbapenem antibiotic, the FCSC (100%) potency against ESBL (2, 5, 6) and MRSA (157 and 158) was equal to 0.5-0.5 ppm and more than 2 ppm meropenem respectively.

Tabel 2. Comparing growth inhibition activity of FCSC and meropenem standard Against ESBL and MRSA

Bacterial test	Φ (mm) of meropenem at Conc. (ppm)					Equation of chart	R2	Φ of FCSC
	0.125	0.25	0.5	1	2			100%
ESBL-2	16.1	18.25	19.5	23.75	26	y = 5.068x + 16.79	0.905	19.7
ESBL-5	-	15.25	16.5	21.5	24.5	y = 5.382x + 14.39	0.928	23
ESBL-6	16.25	17	18.5	21.5	24	y = 4.155x + 16.22	0.951	18
MRSA-157	-	-	13	19.25	22.5	y = 5.892x + 11.37	0.868	24
MRSA-158	-	-	20	22	24	y = 2.571x + 19.00	0.964	26

Comparing the FCSC (100%) and kanamycin (200 ppm), by which *Staphylococcus aureus* growth is inhibited, the FCSC activity was relatively lower. On the other hand, the FCSC activity against ESBL (6) and (MRSA-158) was relatively higher than that amino glycoside antibiotic (Figure 5).

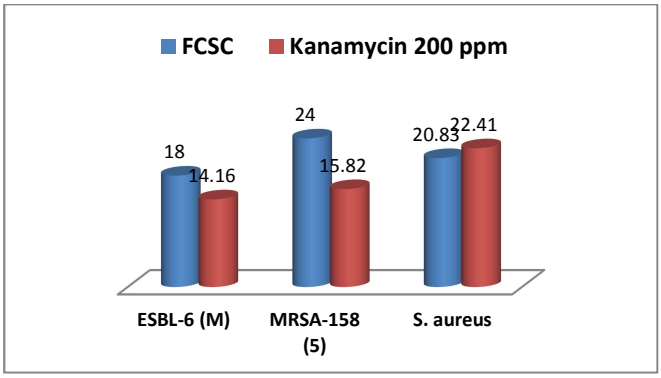

Figure 5. Inhibition activity of FCSC (100%) and kanamycin (200 ppm) against ESBL-6 (M), MRSA-158 (5), and *S.aureus*

The potential of dosage form that developed by using probiotics as mixed culture in a microparticle or nanoparticle preparation for inhalation, topical and other dosage forms would be an alternative antimicrobial strategy for continuously resolving problem with antibiotic resistance. Another strategy in resolving infectious problems in the future is an application of those probiotics in mixed culture for different gastrointestinal, urogenital, and other infection therapies. Characterization of lactic acid bacteria and their beneficial mechanisms allows progress in their use in the food industry and their potential in promoting human and animal health and nutrition.

CONCLUSION

Our results, demonstrated that mixed culture of probiotics that consisted of *L. acidophilus, L. bulgaricus, L. cassei, L. plantarum, B. bifidum, B. animalis, and S. thermophilus* as both free cell supernatant (FCS) and its concentrate (FCSC) exhibited growth inhibition activities against *Staphylococcus aureus* ATCC 6538, six ESBL and five MRSA bacteria isolated from clinical patients. Generally, the maximum time for active components production from FCS was 4 days after incubation at the observation condition. The MIC of the FCSC was 10% and 50% against *S.aureus* and ESBL and MRSA bacteria respectively. The potency of the FCSC against ESBL (2, 5, 6) and MRSA (157 and 158) bacteria were equal to 0.5-0.5 ppm and more than 2 ppm meropenem respectively. The activities of the FCSC produced by the probiotic mixed culture were claimed as very diverse and range beneficial for antibacterial agents to overcome multi-drug resistant bacteria related problems; by which important health-care associated infections.

Further studies are required in order to isolate and identify the chemical nature of active substances and their mechanism of the antibacterial activities. The strategic issues that could be developed are toxicity effect of the FCSC before suggesting the FCSC as raw material for a dosage form formulation.

Acknowledgements
The authors are grateful for probiotic microorganisms, ESBL, MRSA, and *Staphylococcus aureus* provided by Universitas Gadjah Mada, Institute Tropical Diseases Universitas Airlangga, Faculty of Science, Assessment unit of Faculty of Pharmasi Universitas Airlangga, and Technology Universitas Airlangga, and DR Soetomo Hospital Surabaya respectively.

REFERENCES

[1] Food and Agriculture Organization of the United Nations/World Health Organization FAO/WHO. *Guidelines for the Evaluation of Probiotics in Food*; Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food: London, Ontario, Canada, **2002**.
[2] S Fijan; **2014**. *Int. J. Environ. Res. Public Health***. 2014**, (11), 4745-4767.
[3] CN Jacobsen; V Rosenfeldt-Nielsen; AE Hayford; PL Moller; KF Michaelsen; A Paerregaard; et al. **1999**. *Applied and Environmental Microbiology*;65: 4949–56.
[4] *Jagoda [u{kovi}, Bla`enka Kos, Jasna Beganovi}, Andreja Lebo{ Pavunc, Ksenija Habjani~* and *Sre}ko Mato{i}. Food Technol. Biotechnol.* **2010**, *48* (3) 296–307.
[5] B Rouveix, *J. Antimicrob. Chemother*, **2007**, 59(6), 1208-1209.
[6] KW Barbara; B Mari; S Wanda, *Microbiol. Res*., **2010**, 165, 674—686
[7] CAM Mary; CC Esperanza, *Philippine Science Letters* **2011**, 4 (2)| 2011
[8] CCGO Lopes and HRN Salgado. *Talenta*, **2010**. 82, 918-922.
[9] Agil Antono, Dike Bagus Pamuji,Sugiyartono, Isnaeni. **2012**. *PharmaScientia*, **2012**, 1 (2),
[10]Sugiyartono, Dike Bagus Pamuji, Agil antono, Idha Kusumawati, Isnaeni. *Int J. Pharm. Pharm. Sci*, **2014**. (6), Suppl 2, 296-298.
[11] Z Hossain, *Food Safety* **2014**, (1), 535–545.
[12] A Chmielewska; H Szajewska, *World J. Gastroenterol*. **2010**, (16), 69–75.
[13] LV McFarland, *Travel Med.Infect. Dis*. **2007**, *(5)*, 97–105.
[14] S Hempel; SJ Newberry; AR Maher; Z Wang; JN Miles, R Shanman; B Johnsen; PG Shekelle, *JAMA* **2012**, (307), 1959–1969.
[15] I Aloisio; C Santini; B Biavati; G Dinelli; A Cencič; W Chingwaru; L Mogna; L D Gioia, *Appl. Microbiol. Biotechnol*., **2012**, (*96)*, 561–576.
[16] L Ruiz; A Margolles; B Sánchez, *Front Microbiol*., 2013, (*4)*, 396, doi:10.3389/fmicb.**2013**.00396.