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RESEARCH ARTICLE

Antibacterial activity of *Lactobacillus plantarum* BS25, *Pediococcus acidilactici* S3 crude, and partially-purified cell-free supernatants against methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* strains

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ABSTRACT

Background: The rising public health threat brought about by antibiotic resistance, such as of *Staphylococcus aureus*, opened doors of opportunities for natural products research to explore novel antimicrobial agents.

Objective: This study aimed to determine the antimicrobial activity of cell-free supernatants from *Lactobacillus plantarum* BS25 and *Pediococcus acidilactici* S3 against *Staphylococcus aureus* (ATCC# 25923) and methicillin-resistant S. aureus (ATCC# 33591).

Methodology: Cell-free supernatants (CFS) of *Lactobacillus plantarum* BS25 and *Pediococcus acidilactici* S3, isolated from fermented rice-fish mixture *balao-balao* and fermented spicy sausage *longganisa*, respectively, were tested against methicillin-susceptible (MSSA, ATCC 25923) and methicillin-resistant (MRSA, ATCC 33591) *Staphylococcus aureus* strains for antibacterial activity using the resazurin assay.

Results: Both BS25 and S3 CFS showed high activities against MSSA and partial inhibition against MRSA. Proteinaceous components of the CFS were extracted using ammonium sulfate precipitation with BS25 and S3 exhibited low activities against MSSA but partial inhibition was observed against MRSA. Other small molecules were extracted from the CFS through liquid-liquid extraction using ethyl acetate and tested in 100, 250, 500, 750, and 1000 ppm concentrations. The 1000-ppm concentrations of the BS25 and S3 ethyl acetate extracts achieved the highest antibacterial activity against MSSA and MRSA.

Conclusion: This study showed that the crude cell-free supernatants, ammonium sulfate precipitates, and ethyl acetate extracts of BS25 and S3 CFS exhibited potential in inhibiting Gram-positive MSSA and MRSA. However, the partially-purified samples require relatively high concentrations in order to produce significant inhibition activities and therefore require further purification.

Keywords: *antimicrobial activity, Lactobacillus plantarum BS25, Pediococcus acidilactici S3, MRSA, partial purification*

Introduction

Antibiotics have greatly benefited the health of society since its discovery by treating and preventing bacterial infections, consequently improving the quality of health especially in developing countries. However, antibiotic-resistant microorganisms have been emerging rendering most of these drugs ineffective. This has been a result of the overuse and misuse of antibiotics in the clinical or agricultural practices allowing pathogenic bacteria to adapt through the transfer of resistance genes. Antibiotic resistance is a serious global threat to public health which has already resulted in a drastic increase in morbidity rates, mortality rates, and healthcare costs [1]. More so, a group of pathogenic bacteria originating from hospitals termed ESKAPE which represent *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumanni*, *Pseudomonas aeruginosa*, and *Enterobacter* species are known for having multi-drug resistance [2]. A bacterium included in this group of urgent concern is the methicillin-resistant *S. aureus* (MRSA) which is responsible for most of the *S. aureus* bacteremia cases in the world. In the Philippines alone,

30.1% of *S. aureus* cases were caused by MRSA as per the study of the Asian Network for Surveillance of Resistant Pathogens [3]. Additionally, a localized study observed that 45.7% of S. aureus isolates from a tertiary hospital in the Philippines were MRSA [4]. With the onset of this crisis, the demand for novel antimicrobial agents has been higher.

Natural products have been of significant importance in identifying and developing antimicrobial agents such as those found in plants and microorganisms. Such microorganisms are found in fermented foods which can inhibit the growth of other microbes that can induce spoilage, thus prolonging the shelf life of these foods. One of the most important groups of microorganisms in food fermentation are gram-positive lactic acid bacteria which has lactic acid as a major metabolic end product and generally recognized as safe. They are also known to produce antimicrobial compounds such as organic acids, ethanol, hydrogen peroxide, enzymes, and protein or peptides called bacteriocins. Specifically, bacteriocin is a group of antibacterial peptides or proteins that can inhibit or eliminate closely-related species but not the producer strain. For instance, the bacteriocin nisin, from *Lactococcus lactis*, exhibits a broad spectrum of antimicrobial activity which is utilized in food preservation. This shows that novel antimicrobial can be potentially sourced from these organisms, especially lactic acid bacteria.

The Philippines, with its diversity in ethnic cultures, offers a wide variety of fermented food prepared from materials readily available in the local vicinity such as rice, seafood, coconut, sugarcane, fruits, and vegetables. Recently, lactic acid bacteria which include strains *Lactobacillus plantarum* BS25 and *Pediococcus acidilactici* S3 were found to be bacteriocinogenic [5]. BS25 was isolated from a rice-shrimp mixture (*balao-balao*) while S3 was from spicy fermented sausage (*longganisa*). A recent experiment was also performed at the Natural Products and Peptidomics Laboratory, University of the Philippines Manila, in which cell-free supernatants (CFS) of the two strains exhibited inhibitory activity against *Staphylococcus aureus* (ATCC# 25923) which make them a promising source of an antimicrobial agent. In this study, strains BS25 and S3 were tested against methicillin-susceptible and methicillin-resistant *S. aureus* strains for antibacterial activity.

The public health crisis brought about by the emergence of antibiotic-resistant and multidrug-resistant bacteria calls for the development of new antimicrobial agents in order to control the already increasing rates of morbidity and mortality. Lactic acid bacteria found in fermented foods are observed to have the potential in producing bacteriocin or other antimicrobial compounds.

Since antibiotic resistance has remained a constant threat to public health and there is a growing interest in lactic acid bacteria as a source of possible antibacterial agents, this study focused on the inhibitory activity of cell-free supernatants of strains *Lactobacillus plantarum* BS25 and *Pediococcus acidilactici* S3 against methicillin-resistant *S. aureus*. It aimed to determine the activity of crude cell-free supernatants of BS25 and S3 against MSSA and MRSA using the resazurin assay; separate the peptidic components and other small molecules of the crude cell-free supernatants and evaluate the activity of the separated components against the SA strains. Through this study, novel antibacterial agents can be eventually found which will greatly improve the management and treatment of diseases brought about by MRSA.

Methodology

Microorganisms and Reagents

Strains of *Lactobacillus plantarum* BS25 and *Pediococcus acidilactici* S3, isolated from rice-shrimp mixture "balao-balao" and fermented sausage "longganisa", respectively, were provided by Dr. Marilen P. Balolong, Department of Biology, University of the Philippines Manila. Bacterial test strain *S. aureus* (ATCC# 25923) and MRSA (ATCC# 33591) was provided by the Natural Products and Peptidomics Laboratory, University of the Philippines Manila. All reagents used are of analytical grade.

Culture of Lactic Acid Bacteria

Revival of the bacterial strains was done in Lactobacilli de Man, Rogosa, and Sharpe (MRS) broth; and incubated for 24 hours at 37°C in a microaerophilic container. After incubation, the strains were then subjected to Gram staining and catalase test to confirm the purity of the cultures and identity of the bacteria. Cultures were plated on MRS agar prior to inoculation in MRS broth media and downstream experimentation.

Preparation of Cell-Free Supernatants

Each of the two lactic acid bacterial strains was inoculated in MRS broth by transferring a single colony to 10 mL of broth with an inoculating loop before incubating at 37°C for 24 hours in a modified anaerobic chamber. The broth culture

was then transferred to 990 mL of MRS and incubated for 72 hours with the same growing conditions. After incubation, the broth cultures were subjected to refrigerated centrifugation (4°C) at 12,000 rpm for 10 minutes, in 25 mL conical tubes once. The supernatant was separated from the pelleted cells through decantation and filtered with a PES 0.22 µm syringe filter. The cell-free supernatants were then pooled into 500-mL containers and stored at 4°C before use.

Partial Purification

To 500 mL aliquot of the cell-free supernatant of each strain, at 4°C, 358 grams of ammonium sulfate was added slowly while stirring to reach a saturation of 100%. The suspension was then centrifuged at 8000 rpm for 10 minutes. The supernatant was discarded and the precipitates were pooled and subjected to freeze-drying. Samples were stored at 4°C before use.

The dried precipitates were dissolved in ammonium acetate buffer (25 mM, pH 6.5) to a 100-ppm concentration and then tested against the two bacterial strains.

Liquid-liquid extraction with ethyl acetate was done to the remaining CFS. To 50 mL of the aqueous CFS, 50 ml of ethyl acetate was added, gently mixed, and incubated for at least an hour. This was repeated three times for the same 50 mL of the CFS. The organic layers were pooled and the aqueous layers were discarded. Ethyl acetate was removed from the organic layer through a rotatory vacuum evaporator. Samples were stored in a 4°C refrigerator before use.

Resazurin Cell Viability Assay

The crude cell-free supernatants were tested with the test strains without further dilution. The crude and fractionated peptide precipitates were dissolved in the ammonium acetate buffer solution. The organic fractions were dissolved in DMSO with the total concentration of DMSO in the assay not exceeding 2%. A stock solution of 0.02% resazurin dye in ultrapure water was prepared for the assay. A CLSI-standard oxacillin was used as a positive control against *S. aureus* and MRSA. A 1000-ppm stock solution of the antibiotic in ultrapure water was prepared. As for the negative controls: filtered MRS broth, ultrapure water, ammonium acetate buffer, and 2% DMSO were used. Sterile MHB broth was also prepared as a sterility control.

The MSSA and MRSA strains were inoculated in Mueller-Hinton broth before incubating at 37°C for 16-24 hours. The concentration of the incubated bacteria was measured at 625 nm using a UV/Vis spectrometer. The inoculum was diluted with sterile MHB to reach a McFarland standard of 0.5 (1.5x108 CFU/mL).

Three controls set for the assay were used: sterility control, positive control, and negative controls. The sterility control contained 100 µL of sterile MHB. For the negative controls, 80 µL of the inoculum was added to 20 µL of solution (filtered MRS broth, ultrapure water, ammonium acetate buffer, and 2% DMSO) corresponding to the type of sample. The positive control contained 80 µL of the inoculum and 20 µL of the oxacillin solution. The sample solution contained 80 µL of the inoculum and 20 µL of the crude cell-free supernatant, peptide, and organic extract solutions. Each of the controls and the samples were dispensed in a black 96-well microplate with at least three replicates. The concentration of the antibiotic control was kept at 100 ppm. After placing the solutions, the plate was incubated at 37°C for 5 hours. After incubation, 20 µL of the resazurin solution was added to all solutions in the wells before incubating again at 37°C for an hour. Fluorescence of the solutions was measured at 560 nm excitation and 590 nm at emission using a BMG Labtech FLUOstar® Omega Microplate Reader and data collection was done by the MARS Data Analysis software. Percentage inhibition was calculated for all solutions with the following equation.

$$\%inhibition = \frac{F_{negative\ control} - F_{sample}}{F_{negative\ control}} * 100\%$$

Statistical Analysis

Data from the resazurin assay was processed using Microsoft® Office Excel 2016 and subjected to statistical analysis using the One-way Analysis of Variance (ANOVA) and Post-hoc Tukey test of GraphPad Prism® 6 with a p-value less than 0.05 considered as statistically significant.

Discussion

Antibacterial Screening of Cell-free Supernatants

Cell-free supernatants of *L. plantarum* BS25 and *P. acidilactici* S3 were screened against methicillin-susceptible *Staphylococcus aureus* ATCC 25923 (MSSA) and methicillin-resistant *Staphylococcus aureus* ATCC 33591 (MRSA) through the resazurin assay with 100 ppm of oxacillin as the positive control. Both of the CFS were observed to have

inhibitory activity towards the two strains of *S. aureus* as shown in Figure 1. The BS25 CFS exhibited the highest inhibition activity of 84.99 2.61% against MSSA which was significantly higher than the activity of S3 CFS at 71.92 1.90% and oxacillin at 80.61 1.30% ($p<0.05$). Similarly, the CFS of BS25 had a higher antimicrobial activity at 54.22 1.49% compared to S3 CFS with an activity of 49.23 0.94% and oxacillin with 25.99 7.74 ($p<0.05$) against MRSA. A negative control of filtered MRS broth was used in this evaluation to remove possible activities included in the broth. However, it does not completely reflect the behavior of the matrix in the supernatants as some of its contents might have already been transformed by the lactic acid bacteria. With this, it is recommended that a more detailed study of the effect of the matrix on the inhibition activities samples (crude or partially purified) be done. This evaluation only served as an overview of the potential activities of the supernatants.

Partial Purification

Possible free proteins and peptides were then extracted from the cell-free supernatants through ammonium sulfate precipitation. The amount of solid ammonium sulfate added to the cell-free supernatants was determined using a calculator by EnCor Biotechnology [6]. The precipitates were dissolved in the ammonium acetate buffer then tested with the resazurin assay. The samples and oxacillin standard were added with the inoculum with each having a final concentration of 100 ppm. The percentage inhibition of oxacillin, against MSSA with 91.82 1.72 and against MRSA with 90.99 1.36 ($p<0.05$), was significantly higher than that of the protein extracts (Figure 2). It was also noted that the inhibition of BS25 and S3 protein extracts against MRSA (47.88 3.10% and 47.05 0.79%) was higher compared to that of against MSSA (12.88 4.84% and 10.28 8.63%) but not greater than 50%. This could indicate that MRSA is more susceptible to the proteinaceous components of the CFS of *L. plantarum* and *P. acidilactici*.

The low inhibition of the extracts, however, may have been because of the presence of ammonium sulfate in the precipitates which in turn, decreased the concentration of active compounds in the prepared sample. A succeeding desalting step could improve the activity of the samples after precipitation with ammonium sulfate. These methods include dialysis, ion-exchange chromatography, affinity chromatography, gel-filtration chromatography, and hydrophobicity interaction chromatography but are usually time-consuming and require special equipment. In a study by Hu *et al.* (2013), ammonium sulfate precipitates from *L. plantarum* 163 strain were partially purified using gel-filtration chromatography with Sephadex G25 and Sephadex LH20; and the resulting fraction exhibited high activity (>10mm diameter) against MSSA 25923 through the agar well-diffusion assay [7]. Tiwari *et al.* (2008) used both cation-exchange and gel-filtration chromatography in partially purifying ammonium sulfate precipitates from L. plantarum LR14 strain and the resulting fraction had almost a three-fold increase in specific activity against Micrococcus luteus MTCC106 through a microtiter plate-based assay [8]. The assay measured the growth of Micrococcus luteus MTCC106 at 630 nm. In the case of pediocin, produced by *P. acidilactici*, the study of Elegado *et al.* (1997) utilized the pH-adsorption/desorption method instead of ammonium sulfate

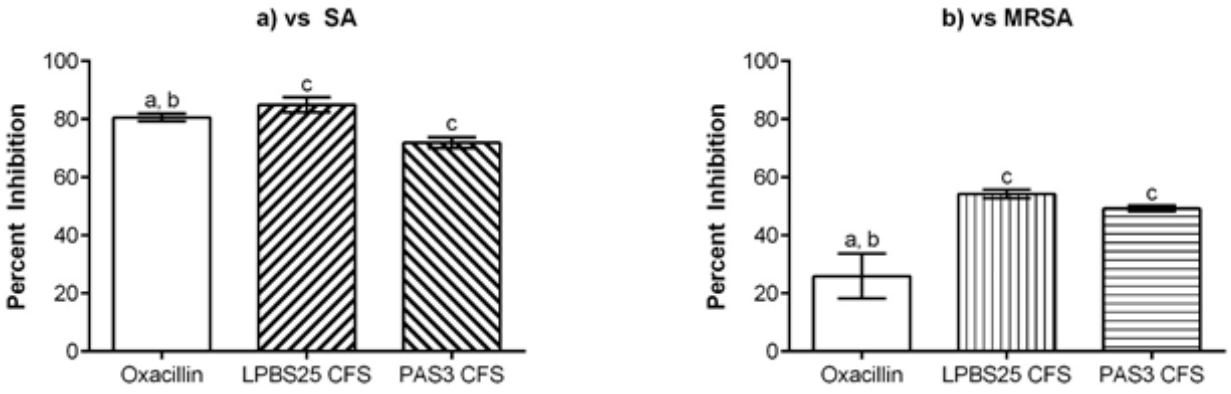

Figure 1. *Inhibition of LPBS25 CFS and PAS3 CFS against methicillin-susceptible S. aureus (a) and methicillin-resistant S. aureus (b). The pH of LPBS25 CFS and PAS3 CFS was measured at 4.6 and 4.4, respectively. LPBS25 CFS exhibited significantly higher inhibition of 84.99 2.61% and 54.22 1.49% against oxacillin for both (a) and (b), respectively. Note: ap>0.05 compared to LPBS25 CFS, bp>0.05 compared to PAS3 CFS, cp>0.05 compared to oxacillin, based on the Post-hoc Tukey Test.*

precipitation. Specifically, pediocins are adsorbed to the cell wall of the producer cells at pH 6 and 0.05 M NaCl and then desorbed at pH 2 and 1 M NaCl [9]. Wang *et al.* (2007) also described a quick and cost-effective method which used phenol for desalting [10]. In the process of precipitating proteins out of the solution using ammonium sulfate, it is also suggested that protein quantitation be done to the desalted precipitate and supernatant. This should be done to evaluate the completeness of protein precipitation. The Bradford assay is a viable method as it is quick to perform and is compatible with most salts and solvents.

Increasing final concentrations (250, 500, 750, 1000 ppm) of the extracts were tested against *S. aureus* and methicillin-resistant *S. aureus*. *L. plantarum* and *P. acidilactici* extracts. The increasing concentrations exhibited increasing inhibition activity against MSSA (Figure 3) and MRSA (Figure 4). However, even with a concentration of 1000 ppm the extracts did not achieve a 100% inhibition of either S. aureus strains. The highest concentration of *L. plantarum* ethyl acetate extracts only exhibited 84.73 5.22 percent inhibition against MSSA and 76.50 1.98 percent inhibition against MRSA. For the *P. acidilactici* extract,

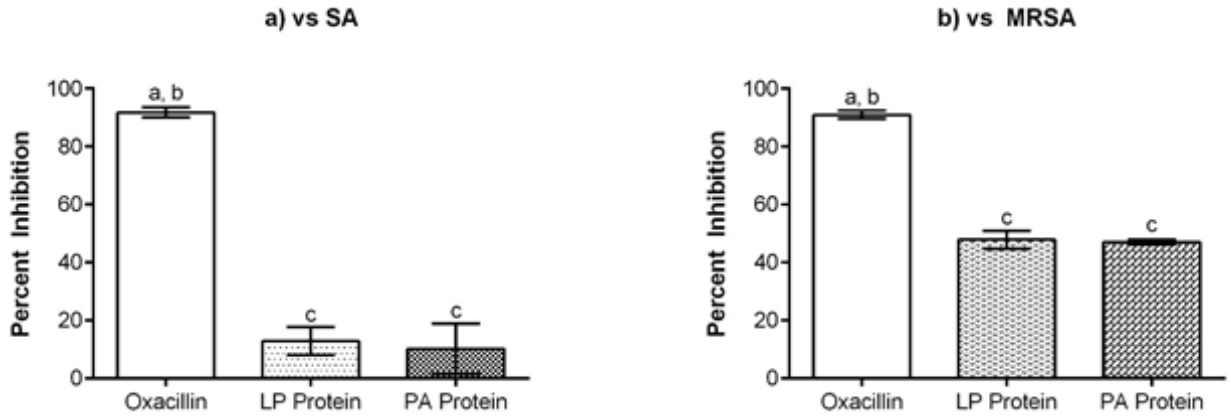

Figure 2. *Inhibition of protein precipitates from L. plantarum BS25 (LP protein) and P. acidilactici S3 CFS (PA Protein) against S. aureus (a) and methicillin-resistant S. aureus (b). The concentration of oxacillin and samples were at 100 ppm per well. The oxacillin standard exhibited inhibitions significantly higher than that of their respective protein extracts for both (a) and (b). Note: ap>0.05 compared to LP Protein, bp>0.05 compared to PA Protein, cp>0.05 compared to oxacillin, based on the Post-hoc Tukey Test.*

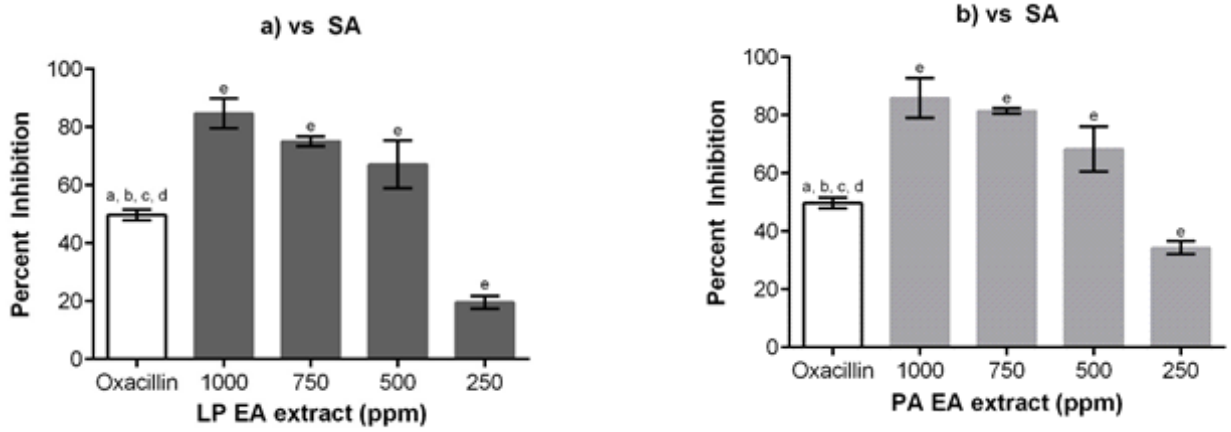

Figure 3. *Inhibition of ethyl acetate extracts LPBS25 and PAS3 CFS against SA. The 1000-ppm concentration of the sample exhibited the highest percentage inhibition at 84.73 5.22% (LPBS25) and 85.88 6.82% (PAS3) which are both significantly higher than their respective oxacillin standards. Note: ap>0.05 compared to 1000-ppm extract, bp>0.05 compared to 750-ppm ext., cp>0.05 compared to 500-ppm ext., dp>0.05 compared to 250-ppm ext., ep>0.05 compared to oxacillin based on the Post-hoc Tukey Test.*

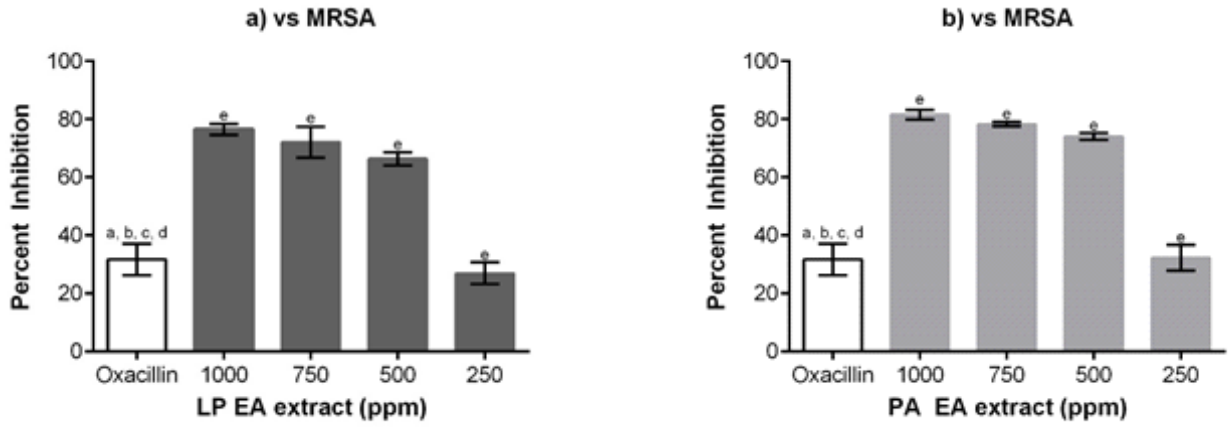

Figure 4. *Inhibition of ethyl acetate extracts LPBS25 and PAS3 CFS against MRSA. The 1000-ppm concentration of the sample exhibited the highest percentage inhibition at 76.50 1.98% (LPBS25) and 81.58 1.64% (PAS3) which are both significantly higher than their respective oxacillin standards. Note: ap>0.05 compared to 1000-ppm extract, bp>0.05 compared to 750-ppm ext., cp>0.05 compared to 500-ppm ext., dp>0.05 compared to 250-ppm ext., ep>0.05 compared to oxacillin based on the Post-hoc Tukey Test.*

activity of 85.88 6.82% was achieved against MSSA and 81.58 1.64% inhibition for MRSA. These activities were all significantly higher than the positive control oxacillin. It was also noted that between the concentrations of 500 and 250 ppm, 50% inhibition can be achieved for both extracts against both bacterial strains.

Further purification could remove nonactive compounds in the extracts and in turn, increase the activity of the samples at lower concentrations and help in isolating specific active compounds. A study by Lei *et al.* (2020) compared activities of ammonium sulfate (40-80% saturation, desalted), ethyl acetate, n-butanol, n-hexane, dichloromethane, and trichloromethane extracts from *L. plantarum* zrx03 CFS; and found that the ethyl acetate extract, pH 7.0 PBS, had the highest activity against Gram-positive (MSSA ATCC 25923, *Bacillus anthracis* CICC 20443), Gram-negative bacteria (*E. coli* JM109 ATCC 67387, *Salmonella* CMCC 541), and fungus (*Saccharomyces*) [11]. It was also found that the extract had proteinaceous characteristics indicated by a decrease in activity after protease treatment. Similarly, in a study by Lin *et al.* (2019), *L. plantarum* NTU 102 CFS showed significant antimicrobial activity against multiple bacterial strains which includes *S. aureus*, *S. mutans*, *K. pneumoniae*, and *P. aeruginosa*. Fractions, from partial purification of the *L. plantarum* NTU 102 CFS through ethyl acetate extraction and silica gel column chromatography, also exhibited antimicrobial activity. By subjecting the active fractions to high-performance column chromatography and NMR analysis, it was narrowed down to a compound with an IUPAC name of 2-(2-1 mino-1-hydroxyethoxy) ethyl 2-methylpropanoate but its mechanism of action was not explored in the study [12]. Although lactic acid bacteria are known for their production of bacteriocins, they can also produce other antimicrobial compounds that do not have proteinaceous characteristics.

Conclusion

Both crude cell-free supernatants, without dilution, from *L. plantarum* BS25 and *P. acidilactici* S3 exhibited percent inhibitions greater than 50 percent against methicillin-susceptible *S. aureus* (ATCC 25923) and only partial inhibitions against methicillin-resistant *S. aureus* (33591). Partial purification of BS25 and S3 CFS for peptidic components and other small molecules was performed through ammonium sulfate precipitation and ethyl acetate liquid-liquid extraction, respectively. Both protein precipitates did not have significant activity against MSSA but had partial inhibitions against MRSA. Ethyl acetate extracts of BS25 and S3 CFS only exhibited partial inhibitions against MSSA and MRSA at concentrations greater than 250 parts per million. It is to be noted that the oxacillin positive controls in the resazurin assays did not have consistent percentage inhibitions against both *S. aureus* strains and so, it is recommended to always use freshly prepared standards.

The ammonium sulfate precipitates and ethyl acetate extracts of BS25 and S3 CFS thus exhibited potential in inhibiting Gram-positive MSSA and MRSA. However, the partially-purified samples require relatively high concentrations in order to produce significant inhibition activities and therefore require further purification. For the ammonium sulfate precipitates, it is

recommended to desalt the samples through dialysis, ion-exchange chromatography, affinity chromatography, gel-filtration chromatography, hydrophobicity interaction chromatography, or phenol extraction (Wang, Liu, & Cui, 2007). Alternatively, pH-adsorption/desorption method (Elegado, Kim, & Kwon, 1997) can be used in order to avoid using ammonium sulfate. Further purification of the ethyl acetate extracts is recommended through the use of chromatographic methods, specifically thin-layer chromatography for component analysis; and column chromatography to obtain larger volumes of samples for testing. Upon achieving purer samples, minimum inhibitory concentrations of the samples can be evaluated. Testing the samples against other resistant bacterial strains can also be performed.

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