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ISOLATION AND MOLECULAR IDENTIFICATION OF INDIGENOUS BACTERIOCIN-PRODUCING *WEISSELLA CIBARIA*

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ABSTRACT

Globally, over 6.22 million deaths are associated with antibiotic resistance. Bacteriocins, a set of antimicrobial peptides synthesized on the ribosomes, are widely viewed as a potential answer to this issue. This is due to their pore-forming ability and antimicrobial activity against antibiotic-resistant pathogens. The aim of this study is to isolate bacteriocin-producing *Weissella cibaria* and evaluate its antimicrobial activity against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Proteus mirabilis*, *Streptococcus sp*., *Candida sp*. and *Rhizopus* stolonifer. *Weissella cibaria man1* was isolated by inoculating deMan Rogosa Sharpe (MRS) broth with small pieces of ripe *Mangifera indica* (mango), 24-hour incubation at 37^0C, 10-fold serial dilution and plating on MRS agar. Molecular identification was achieved by DNA extraction, amplification of the 16S rRNA gene through polymerase chain reaction (PCR), agarose gel electrophoresis, gene sequencing, and BLASTN homology searches in the National Center for Biotechnology Information (NCBI). Antimicrobial activity of the bacteriocin was determined by agar well diffusion assay. *Mangifera indica* (mango) was found to harbor bacteriocin-producing *Weissella cibaria man1*. The bacteriocin (weissellicin man1) exhibited a broad spectrum of antimicrobial activity. Weissellicin man1 suppressed the growth of several target pathogens (*Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Proteus mirabilis*, *Candida sp.* and *Rhizopus stolonifer*) but had no inhibitory action against *Escherichia coli*, *Streptococcus sp.*, *Staphylococcus aureus.* In conclusion, weissellicin man1 from *Weissella cibaria man1* has a broad-spectrum of antimicrobial action. These findings will facilitate further evaluation of the antimicrobial potency of weissellicin man1.

Keywords: Lactic acid bacteria, *Weissella cibaria*, Bacteriocins, *Pseudomonas aeruginosa*, *Candida sp.*

INTRODUCTION

According to world health organization (WHO), *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Proteus mirabilis*, *Streptococcus sp.*, *Candida sp.* are priority pathogens requiring urgent attention (World Health Organization, 2022, 2024). Further, *Rhizopus stolonifer* is a causative agent for mucormycosis, also known as zygomycosis (World Health Organization, 2020). These pathogens contribute to global human mortality. Moreover, the emergence and spread of antibiotic resistance among them exacerbates the problem. WHO reported that antibiotic resistance contributes to over 6.22 million deaths globally (World Health Organization, 2022, 2023). Bacteriocins are a promising solution to this problem (Nisa et al., 2023).

Bacteriocins are a group of peptides synthesized on the ribosomes (Jain et al., 2024). The capacity of bacteriocins to eliminate pathogens that have developed resistance to traditional antibiotics has led many to believe that they could one day replace these drugs (Wayah et al., 2022). Since their identification nearly a century ago, a wide range of bacteriocins have been unraveled and characterized (Sugrue et al., 2024). These include nisin (da Silva Oliveira et al., 2024), pediocin PA-1 (Thu et al., 2024), and fermencin SA715 (Wayah & Philip, 2018a). Bacteriocins have been reported to inhibit various pathogens (Soltani et al., 2022). They act against pathogens through various mechanisms including pore formation, inhibition of nucleic acid biosynthesis, impeding ATP synthesis, and prevention of cell wall formation (Li et al., 2023; Sharma et al., 2021).

Lactic acid bacteria (LAB) is a group of bacteria that produce lactic acid during fermentation of carbohydrates. In addition to lactic acid formation, some strains LAB are also capable producing bacteriocins (Castrejón-Jiménez et al., 2024; Pujato et al., 2024). *Weissella spp.* are a group of lactic acid bacteria. Apart from their acknowledged function in conventional fermentations, several strains of *Weissella spp.* have been found to exhibit probiotic attribute. *Weissella cibaria* is one of the groups of *Weissella spp.* receiving attention because of their probiotic potential (Kang et al., 2023).

Notwithstanding the extensive evaluation of the characteristics of many strains of *Weissella spp.*, only a few have been observed to produce bacteriocins (Singh et al., 2024). Based on available literature, the named *Weissella cibaria* bacteriocins are as follows: weissellicin L (from *Weissella hellenica* 4-7) (Leong et al., 2013), weissellicin D (from *Weissella hellenica* D1501) (Chen et al., 2014a; Chen et al., 2014b), weissellicin 110 (from *Weissella cibaria* 110) (Srionnual et al., 2007), weissellicin Y and M (from *Weissella hellenica* QU 13) (Masuda et al., 2012), weissellin A (from *Weissella paramesenteroides* DX) (Papagianni & Papamichael, 2011; Papagianni & Sergelidis, 2013), weissellicin MBF, Bac1, Bac2, and Bac3 (from *Weissella confusa* MBF8-1) (Malik et al., 2020), bacteriocins 7293A and 7293B (from *Weissella hellenica* BCC 7293) (Woraprayote et al., 2015). Additionally, some unnamed *Weissella spp.* bacteriocins have been reported. These include bacteriocins from *Weissella confusa* A3 (Goh & Philip, 2015), *Weissella confusa* LM85 (Kaur & Tiwari, 2018), *Weissella cibaria* FMF4B16 and *Weissella paramesenteroides* LC11 (Ndagano et al., 2011), *Weissella paramesenteroides* DFR-8 (Pal & Ramana, 2010), *Weissella cibaria* N23 (Pringsulaka et al., 2012), and *Weissella cibaria* KMITL-QU 21 (Singh et al.,

2024). In spite of the potential of *Weissella cibaria* bacteriocins as a possible replacement to traditional antibiotics, not much progress has been made. In Nigeria for example, no study has been done to evaluate the bacteriocins from *Weissella cibaria*. Consequently, there is a need to identify and evaluate indigenous *Weissella cibaria* capable of producing bacteriocins. Therefore, the overall objective of this study is to isolate bacteriocin-producing *Weissella cibaria* and evaluate its antimicrobial activity against *Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Proteus mirabilis*, *Streptococcus sp*., *Candida sp*. and *Rhizopus* stolonifer.

MATERIALS AND METHODS

Bacterial strains and growth media

Escherichia coli, *Streptococcus sp*., *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Proteus mirabilis*, and *Candida sp*. were obtained from the culture collection of Barau Dikko teaching hospital while *Rhizopus* stolonifer was obtained from the culture collection of Microbiology Department of Kaduna State University. *Weissella cibaria man1* was isolated in this study and maintained on De man Rogosa Sharpe (MRS) agar (Merck, Germany). *Escherichia coli*, *Streptococcus sp*., *Staphylococcus aureus*, and *Pseudomonas aeruginosa* were maintained on Mueller-Hinton agar (Becton, United States). *Klebsiella pneumoniae*, *Salmonella typhi*, and *Proteus mirabilis*, were maintained on nutrient agar (Thermo Fisher Scientific, United States). *Candida sp*. and *Rhizopus stolonifer* were maintained on sabouraud dextrose agar (Merck, Germany), and potato dextrose agar respectively (Merck, Germany).

Isolation of lactic acid bacteria

Lactic acid bacteria (LAB) were isolated as described by Wayah and Philip (Wayah & Philip, 2018b). Samples of Ripe *Mangifera indica* (mango) were obtained from central Market Kaduna, put in sterile sampling bottles and taken to the Microbiology Laboratory of Kaduna State University. Thereafter, it was in cut into small pieces using sterilized knife, added to freshly made MRS broth and incubated for 24 hours at 37°C. Ten-fold serial dilution using peptone water, followed by inoculation of MRS agar and overnight incubation at 37°C enabled isolation of LAB.

Determination of antimicrobial spectrum of bacteriocin from lactic acid bacteria

Since the main interest of the study is to identify bacteriocin-producing LAB strain, 15 single colonies from the 24-hour old MRS agar LAB culture were separately inoculated into MRS broth and aerobically incubated at 37°C for 24 hours. Screening of the LAB isolates for bacteriocin production was conducted by agar well diffusion assay as described by Goh and Philip (2015) with slight modifications. The 24-hour MRS broth cultures were centrifuged at 10,000 x g for a period of 20 minutes to collect the supernatant which was filtered using 0.2 µm membrane to obtain cell-free supernatant (CFS). The pathogens (*Pseudomonas aeruginosa*, *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Proteus mirabilis*, *Streptococcus sp.*, *Candida sp*. and *Rhizopus* stolonifer) were cultured in their respective broth at 37°C (30°C for *Candida sp*. and *Rhizopus stolonifer*) for 24 hours. The cultures were centrifuged at 5000 rpm for 5 minutes to collect cell pellet which was then, resuspended in 0.85% saline solution and the optical density at 600 nm was adjusted to 0.1. Afterwards, appropriate agar plates (enriched with 0.1% $CaCO_3$ to counteract the acidity caused by organic acids) were seeded with these resuspended pathogens and wells were made. To these wells, 50 µl of CFS was added. Uninoculated broth without bacteriocin was used as the control. Inoculated plates were incubated at 37°C (30°C for *Candida sp.* and *Rhizopus stolonifer*) for 24 hours and zones of inhibition were measured.

Molecular identification of bacteriocin-producing lactic acid bacteria

Since the principal focus of the research is bacteriocin, only LAB isolate that displayed antimicrobial activity against at least one of the target pathogens was identified using molecular approaches. DNA was extracted using the AccuPrep genomic DNA extraction kit as recommended by the manufacturer. Colonies from overnight MRS agar culture of the bacteriocin-producing LAB isolate were added to 2 ml Eppendorf tube after which 20 µl of proteinase K and 10 µl of RNase A were added. Next, 200 µl of GB buffer was added to the sample, and the mixture was vortexed immediately to ensure complete resuspension for maximum lysis efficiency. The sample was incubated for 10 minutes at 60°C. Following incubation, 400 µl of absolute ethanol was added to the sample, and the mixture was pipetted to combine. The lysate was carefully moved to the upper reservoir of the Binding column tube (fitted in a 2 ml tube) while avoiding the rim from getting wet. The tube was closed and centrifuged at 8,000 rpm for 1 minute. The solution in the collection tube was discarded, and the tube used for collection was used again.

Subsequently, 500 µl of Washing buffer 1 (W1) was added in such a manner that the rim did not get wet. After closing the tube, it was centrifuged for 1 minute at 8,000 rpm. The solution in the collection tube was discarded, and the collection tube was used again. The solution from the 2 ml tube was then poured into a disposal bottle. Next, 500 µl of Washing buffer 2 (W2) was carefully added without wetting the rim, and the tube was closed and centrifuged at 8,000 rpm for 1 minute. The solution in the collection tube was discarded again, and the collection tube was reused. The sample was centrifuged once more for 1 minute at 13,000 rpm to remove any remnant ethanol. In order to obtain the purified genomic DNA, the Binding column tube was then put inside a new 1.5 ml tube and 200 µl of Elution buffer was added. The sample was left at room temperature (15–25°C) for at least 1 minute until the elution was complete. Finally, the sample was centrifuged at 8,000 rpm for 1 minute to elute the genomic DNA, which was stored at 4°C for later analysis.

Polymerase chain reaction (PCR) was carried out as previously described by Goh and Philip (2015) with modifications. This involved amplifying the 16S rRNA gene using PCR with the universal primers 27F [5′-AGAGTTTGATC(A/C)TGGCTCAG-3′] and 1492R [5′-ACGG(C/T)TACCTTGTTACGACTT-3’]. For PCR, a total reaction volume of 20 µl was prepared, comprising of 10 µl Taq Master Mix, 1 µl each, of the forward and reverse primer, 5 µl of DNA template and 3 µl of nuclease-free water. PCR condition consisted of initial denaturation for 5 minutes at 94°C, followed by 35 cycles of denaturation for 1 minute at 94°C, annealing for 1 minute at 52°C, extension for 1.5 minutes at 72°C, and afterwards, final extension for 10 minutes at 72°C.

The 16S rRNA gene was sequenced and similarity searches was conducted using NCBI BLAST available at https://blast.ncbi.nlm.nih.gov/Blast.cgi. Subsequently, the sequence of the PCR product was deposited in the GenBank.

Phylogenetic analysis

Sequence of gene encoding 16S rRNA of the bacteriocin-producing lactic acid bacteria isolated in this study in addition to 6 other lactic acid bacteria obtained from the NCBI data base were used for multiple sequence alignment using MEGA11 software (Tamura et al., 2021). Phylogenetic tree was constructed using MEGA11 software. Evolutionary relationship was established using the Maximum Likelihood method and Kimura 2-parameter model. Appearance of the phylogenetic tree was enhanced using iTOL software.

Data analysis

Experiments were done in three (3) replications from which mean values and standard deviations were calculated. One-way analysis of variance, using IBM SPSS statistics software version 29 was carried out to compare mean values for significant difference at 95% confidence level.

RESULTS AND DISCUSSION

Molecular identification of bacteriocin-producing lactic acid bacteria

Only one (1) of the LAB isolates displayed antimicrobial activity against at least one of the target pathogens. Therefore, molecular identification of this isolate was carried out. Image of agarose gel electrophoresis of PCR product revealed an estimated PCR product size of 700 bp (Figure 1). After sequencing of the PCR product, it was observed to have a size of 666 bp which is close to the estimated size. In order to confirm the identify of the bacteriocin-producing LAB isolate, homology search of the sequenced PCR product was performed. This search revealed that the bacteriocin producer is 96.74% similar to *Weissella cibaria*. This finding was consolidated by the results of phylogenetic analysis (Figure 2) which revealed that the isolated bacteriocin producer belong to the same clade as *Weissella cibaria 110*, *Weissella cibaria II-I-59*, and *Weissella cibaria CAG14a*. Therefore, the bacteriocin producer was named *Weissella cibaria* man1. Following the deposition of the 16S rRNA gene sequence in GenBank, an accession number, PQ282392.1 was assigned. Subsequently, the bacteriocin was named weissellicin man1. Four bacteriocin-producing strains of *Weissella cibaria* have been previously reported. These are, *Weissella cibaria* 110 (Srionnual et al., 2007), *Weissella cibaria* FMF4B16 (Ndagano et al., 2011), *Weissella cibaria* N23 (Pringsulaka et al., 2012), and *Weissella cibaria* KMITL-QU 21 (Singh et al., 2024; Zendo et al., 2008).

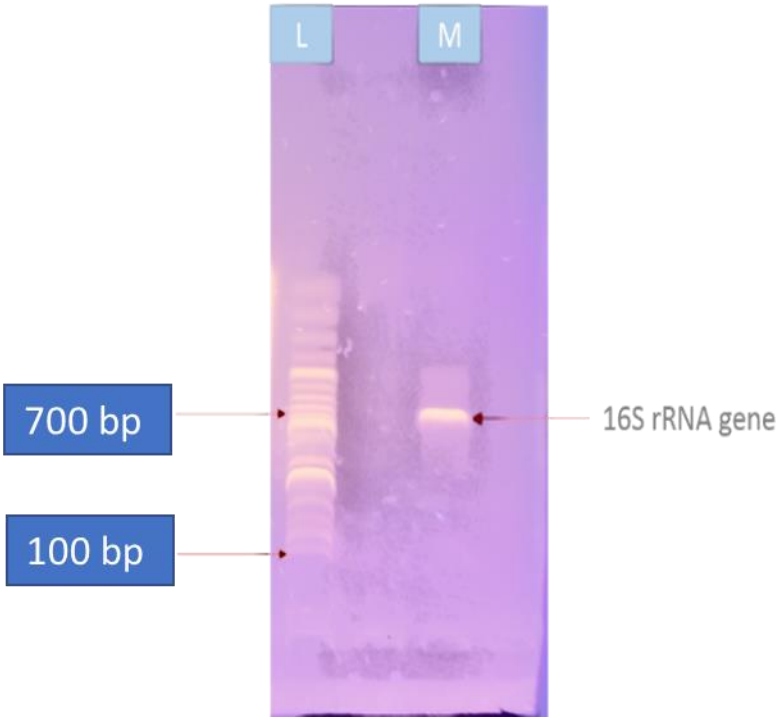

Figure 1: Agarose gel electrophoresis image of the PCR product. L: 1 Kb molecular ladder; M: PCR product

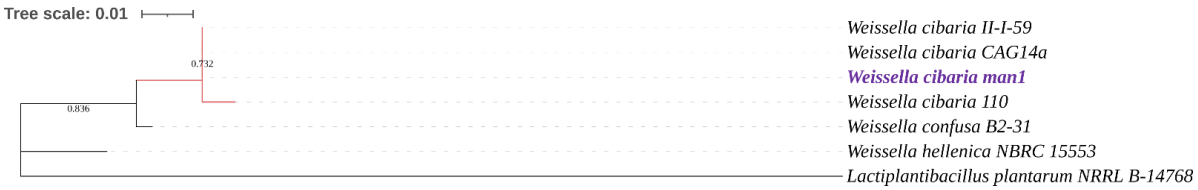

Figure 2: Phylogenetic tree based on 16S rRNA gene sequence. *Weissella cibaria man1*, highlighted in purple was isolated in this study

Antimicrobial spectrum of bacteriocin from *Weissella cibaria man1*

Nine (9) pathogens were exposed to weissellicin man1, out of which 6 were found to be susceptible to the bacteriocins, namely, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Proteus mirabilis*, *Candida sp.* and *Rhizopus stolonifer* (Table 1). Typical zone of inhibition produced by weissellicin man1 against these pathogens is shown in Figure 3.

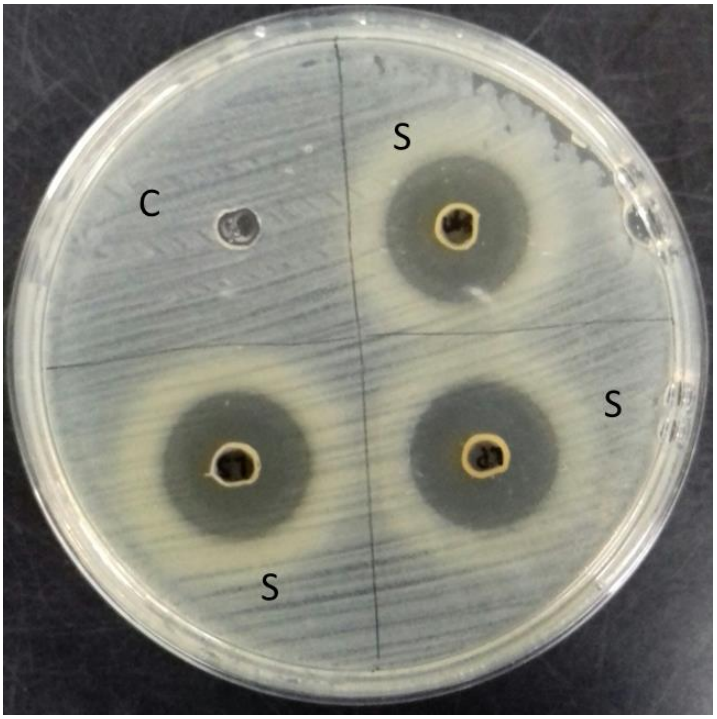

Figure 3: Typical zone of inhibition produced by bacteriocin from *Weissella cibaria man1*. C: control (uninoculated broth without bacteriocin), S: 3 replicates of cell-free supernatant from 24-hour MRS broth culture of *Weissella cibaria man1*

It was observed that *Candida sp.* was the most sensitive to weissellicin man1 with a zone of inhibition (ZOI) of 17.0 ± 0.51 mm while the ZOI for *Rhizopus stolonifer*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella typhi*, and *Proteus mirabilis* were 10.5 ± 0.43 mm, 8.5 ± 0.24 mm, 9.0 ± 0.22 mm, 9.5 ± 0.22 mm, and 10.5 ± 0.22 mm respectively (Table 1). Although 4 bacteriocin-producing strains of *Weissella cibaria* (*Weissella cibaria 110, Weissella cibaria FMF4B16*, *Weissella cibaria N23*, *Weissella cibaria KMITL-QU 21*) have been reported in literatures, antimicrobial activity of their bacteriocins against *Rhizopus stolonifer*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella typhi*, and *Proteus mirabilis* have not been evaluated (Ndagano et al., 2011; Pringsulaka et al., 2012; Srionnual et al., 2007; Zendo et al., 2008). However, bacteriocin from *Weissella cibaria FMF4B16* showed antimicrobial activity *Candida albicans* (Ndagano et al., 2011). *Pseudomonas aeruginosa* infections have become a genuine worry in hospital-acquired infections, especially in critically ill and immunocompromised patients. The main problem leading to high mortality lies in the emergence of drug-resistant forms (Bassetti et al., 2018). Candida species are responsible for around 80% of the invasive fungal infections that are causing an increasing burden on intensive care units. The presence of invasive Candida infection (ICI) is linked to a significant risk of death, with an attributable mortality rate of 49%. However, this risk may rise to 98% in patients with septic shock who get delayed antifungal administration (Thomas-Rüddel et al., 2022). *Rhizopus stolonifer* has rapid growth, extensive distribution, and pronounced aggressiveness. This pathogen has the ability to infect a diverse range of hosts, such as humans, cherry tomato, strawberry, grape, peach, sweet cheerful, sweet potato, and several other fruits, and vegetables (Liu et al., 2024; World Health Organization, 2020). *Klebsiella pneumoniae* is a major Enterobacteriaceae recognized as one of the opportunistic infections generating broad spectra of disorders and demonstrating increasingly common acquisition of resistance to drugs (Effah et al., 2020). On a yearly basis, an estimated 10.9 million new infections and 116,800 deaths are attributed to typhoid fever, a systemic infection caused by *Salmonella typhi* (Marchello et al., 2020). In many cases, catheter-associated urinary tract infections are caused by *Proteus mirabilis* (Armbruster et al., 2018). The ability of weissellicin man1from *Weissella cibaria man1* to suppress the growth of the aforementioned pathogens suggests its potential for treatment of diseases associated with them.

Since weissellicin man1 inhibits pathogens outside its genus, it can be referred to as a broad spectrum bacteriocin. A study revealed that bacteriocin from *Weissella cibaria* FMF4B16 had broad spectrum of antimicrobial activity as shown by its ability to suppress the growth of *Aspergillus niger*, *Candida albicans*, *Penicillium crustosum*, and *Aspergillus tubingensis* (Ndagano et al., 2011). In another study, bacteriocin from *Weissella cibaria KMITL-QU* 21 was reported to possess antimicrobial activity against bacteria outside its genus this include *Micrococcus luteus*, *Bacillus circulans*, and *Leuconostoc mesenteroides* (Zendo et al., 2008). Other broad spectrum bacteriocins from *Weissella spp.* include Weissellicin D (inhibits *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*, yeasts and molds) (Chen, et al., 2014a; Chen et al., 2014b), weissellin A and class 11a bacteriocin (inhibit *Listeria innocua*, *Listeria monocytogenes*, and *Clostridium sporogenes*) (Papagianni & Papamichael, 2011; Papagianni & Sergelidis, 2013).

Despite the broad inhibitory activity of weissellicin man1, it was not inhibitory against *Escherichia coli*, *Streptococcus sp*., *Staphylococcus aureus*. A study observed that weissellicin 110 from *Weissella cibaria 110* was also not inhibitory against *Escherichia coli*, and *Staphylococcus aureus* (Srionnual et al., 2007). Another study reported that bacteriocin from *Weissella cibaria KMITL-QU* 21 did not inhibit the growth of *Escherichia coli* (Zendo et al., 2008).

Table 1: Inhibitory spectrum of bacteriocin from *Weissella cibaria man1*

Pathogen	Zone of inhibition (mm)
Escherichia coli	0.0 ± 0.00^{f}
Streptococcus sp.	0.0 ± 0.00^{f}
Staphylococcus aureus	0.0 ± 0.00^{f}
Pseudomonas aeruginosa	8.5 ± 0.24^{e}
Klebsiella pneumoniae	9.0 ± 0.22^{d}
Salmonella typhi	9.5 ± 0.22^{c}
Proteus mirabilis	10.5 ± 0.22^{b}
Candida sp.	17.0 ± 0.51^{a}
Rhizopus stolonifer	10.5 ± 0.43^{b}

Values are means of 3 replications ± standard deviation; means that differ significantly at 95% confidence level were assigned different alphabet.

CONCLUSION

Bacteriocin-producing *Weissella cibaria* man1 was isolated from ripe *Mangifera indica* (mango). This bacteriocin (weissellicin man1) has a broad spectrum of antimicrobial activity. Weissellicin man1 was inhibitory against pathogens including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Proteus mirabilis*, *Rhizopus stolonifer* and *Candida sp*. However, weissellicin man1 did not inhibit the growth of *Escherichia coli*, *Streptococcus sp*., and *Staphylococcus aureus*. The antimicrobial activity of weissellicin man1 against human pathogens suggests its potential in the treatment of diseases associated with the pathogens. These findings will enhance further *in vitro* and *in vivo* evaluation of the antimicrobial potency of weissellicin man1 against the target pathogens.

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