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Use of enterocin MSW5 in combination with antibiotics to increase the antimicrobial and antibiofilm efficacy against foodborne pathogens

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ABSTRACT

The development of resistance to conventional antibiotics by pathogens is one of the major global health issues. To solve this problem, antimicrobial combinatorial therapy can be one of the alternatives. In this work, we have used an antimicrobial peptide in combination with antibiotics against foodborne pathogens. An antimicrobial peptide (AMP) (Enterocin MSW5) was produced by *Enterococcus faecalis* MSW5. To determine *in vitro* antimicrobial combinatorial therapy, five different antibiotics (oxacillin, tetracycline, vancomycin, doxycycline, and linezolid) were selected. Their minimum inhibitory concentration (MIC) was examined against three different foodborne pathogens. The MIC of Enterocin MSW5 was determined to be 0.36, 0.36, and 0.72 mg/mL against *Staphylococcus aureus, Salmonella* ser. Typhimurium, and *Listeria monocytogenes,* respectively. Further, the synergistic effect of antibiotics with Enterocin MSW5 was determined using a checkerboard assay. The fraction inhibitory concentration index (FICI) values were <0.5 for all combinations, which indicated synergism between any two antimicrobials tested. Among all the combinations, the best synergism was observed for Enterocin MSW5 in combination with linezolid (FICI 0.255) against *S. aureus*, tetracycline (FICI 0.249) against *Salmonella* Typhimurium, and linezolid (FICI 0.125) against *L. monocytogenes*. Furthermore, to assess the antibiofilm potential of such antimicrobial combinatorial content, firstly biofilm potential of three test organisms was determined using a 96-well microtiter plate assay. A significant biofilm formation was detected with a 10% inoculum size in Tryptic Soy Broth for *S. aureus* and *L. monocytogenes* while for *S.* Typhimurium inoculum size was 12%. Further, Enterocin MSW5 in combination with antibiotics were analyzed at their MIC and sub-MIC value for biofilm eradication purposes. Maximum biofilm eradication was observed with the combination of Enterocin MSW5 with linezolid (96.97%) against *L. monocytogenes*. These results showed that the combinations have better antimicrobial effects than the individual effect of antibiotics.

1. INTRODUCTION

From the past decades, the increase and spread of drug resistance to antibiotics is much more due to their misuse. As per the data of the Global Antimicrobial Resistance and Use Surveillance System of the WHO in 2020, more than 2,800,000 cases of anti-microbial resistance (AMR) infection and 35,000 demises were recorded each year in the United States. AMR strains such as *Staphylococcus aureus*, *Pseudomonas aeruginosa, Acinetobacter baumannii*, *Listeria monocytogenes, Salmonella* Typhimurium, and many more are a major threat to human health, globally. Among them, due to antibiotic resistance, the human foodborne pathogen *S. aureus* has become resistance towards antibiotics such as aminoglycosides, macrolides, tetracycline, chloramphenicol, lincosamides and even higher class of antibiotics such as methicillin and oxacillin [1]. Certain strains of *S. aureus* have also been earlier responsible for normal skin infections which can also lead to severe diseases such as meningitis, pleuropulmonary, pneumonia, bacteremia, nosocomial infections endocarditis, and post-surgical wound infections. [2] In addition, these methicillin-resistant *S. aureus* (MRSA) strains are strong biofilm formers and are very difficult to remove. Another Gram-positive pathogen that is responsible for the contamination of foods is *L. monocytogenes* which can colonize the surface of gastrointestinal tracts. It mainly infect spinal cord membranes followed by the bloodstream [3]. *L. monocytogenes* is a psychotropic foodborne pathogen with high risk due to its maximum mortality rates in individuals compared to other foodborne pathogens [4,5]. An illness caused by *L. monocytogenes* is called listeriosis, which covers mostly newborns, pregnant women, and individuals with compromised immune systems [6,7]. It has the maximum (90%) hospitalization rate and 20% of fatality rate in 2016 in European countries [8-10].

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Furthermore, *S.* Typhimurium is also one of the major gram-negative foodborne pathogens which cause widespread contamination in food industries. It is responsible for causing a larger range of host tropism and that is one of the major reasons for salmonellosis all over the world in humans. Many research on the distribution pattern of antibiotic resistance in *S.* Typhimurium is helping in the selection of suitable antibiotics for *Salmonella* infections [7,11-17].

Another global concern related to AMR is biofilm formation by pathogenic microbes. Biofilm is a bunch of microorganisms that attach to surfaces and produce extracellular polysaccharides. It can be composed of different kinds of microorganisms such as bacteria, protozoa, fungi, and algae. They are generally formed on both living as well as nonliving surfaces and are observed in industrial, natural, and hospital settings. The main purpose of biofilm formation is to protect the microorganisms from a hostile environment or to act as a trap for nutrient acquisition [18]. In this study, we have focused on bacterial biofilm eradication. Bacteria are mostly found in a free-floating form in sessile states. The sessile stage of bacteria leads to biofilm formation. The biofilms on internal and external medical devices can be responsible for causing infection in patients and it is very much difficult to remove because of the increased resistance to biofilm-forming pathogens to antimicrobial agents [19-21]. In humans, 80% of microbial infections are caused by the formation of biofilms such as cystic fibrosis, endocarditis, periodontitis, osteomyelitis, rhinosinusitis, meningitis, prosthesis, kidney infections, and implantable device-related infections. A major hurdle faced in treating biofilms is their diagnosis, unavailability of biomarkers, and difficult to remove because of their high tolerance toward antibiotics [22].

The tremendous increase in AMR toward antibiotics suggests that, without taking a most crucial step, we are entering a "post-antibiotic era," which means the therapeutic strategies which were used previously are now no longer applicable for infectious diseases. Therefore, current research is focused on investigating new and non-conventional therapy for the treatment of AMR infections such as vaccines, adjunctive, probiotics, and AMPs [23]. The major components of the innate immune system are AMPs and they play a crucial role in the host defense system against microorganisms; they are produced by bacteria, fungi, protozoa, algae, animals, and plants. Among them, a heterogeneous group of ribosomally synthesized AMPs from bacteria are known as bacteriocins and they can kill closely related microbes known as a narrow spectrum or a diverse range of microbes known as a broad spectrum. Bacteriocin has rapid action and a wide range of antimicrobial activity against both Gram-negative and Gram-positive bacteria which makes it important as a therapeutic agent. Furthermore, antibiotic resistance mechanisms create hindrance to the penetration of antibiotics which do not affect the AMPs [24,25]. Many of the AMPs are also active against bacterial biofilms [21,26-29]. Moreover, several researches have demonstrated a synergistic relationship between bacteriocin and antibiotics [30]. Therefore, we tried to determine whether the Enterocin MSW5 in combination with antibiotics has *in vitro* antimicrobial and antibiofilm activities alone or combined against *S. aureus, L. monocytogenes,* and *S.* Typhimurium.

2. MATERIALS AND METHODS

2.1. Materials

Nutrient Broth (NB), Luria Broth (LB), and Tryptic Soy Broth (TSB) were purchased from Himedia Laboratories (Mumbai, India). All other reagents and media were procured from Himedia Laboratories (Mumbai, India). The 96-well microtiter plate was purchased from Axiva Sichem Biotech (Delhi, India).

2.2. Bacterial Strains and Culture Conditions

The bacteriocin-producing strain *Enterococcus faecalis* MSW5 with accession number MW672393. *S. aureus* ATCC 6538, *L. monocytogenes* ATCC 13932, and *Salmonella enterica subsp. enterica serovar* Typhimurium ATCC 6539 indicator organisms were procured from the American Type Culture Collection and Gene Bank.

2.3. Antimicrobial Agents

Tetracycline, vancomycin, oxacillin, doxycycline, and linezolid were obtained from Hi-media, Mumbai, India. All five antibiotics were prepared in distilled water freshly as per Clinical and Laboratory Standards Institute (CLSI) guidelines. Enterocin MSW5 was produced from *E. faecalis* MSW5. Enterocin is purified using cold acetone extraction followed by ion-exchange chromatography using SP Sepharose fast flow cation exchanger (Sigma Aldrich Chemicals Pvt Ltd, Bangalore, India). The protein concentration of Enterocin MSW5 was determined in triplicate by Lowry methods using BSA (Hi-media, Mumbai, India) as a standard [31].

2.4. Determination of Minimum Inhibitory Concentration (MIC) of Enterocin MSW5

MIC of Enterocin MSW5 was determined using 96 well microtiter plate assay as suggested by Fugaban *et al.* [32] with slightly modifications against *S. aureus* ATCC 6538, *L. monocytogenes* ATCC 13932, and *S.* Typhimurium ATCC 6539 indicator organisms. For that, Sterile 100 µL of TSB was inoculated with 10% of actively grown cultures of indicator organisms. After that, Enterocin MSW5 was 2-fold serially diluted from 5.8 to 0.09 mg/mL. Further, 50 µL of these diluted Enterocin MSW5 samples were added in wells. The untreated well was considered as a positive control. Plates were incubated at 37°C for 24 h and cell density was measured at 595 nm in ELISA plate reader.

2.5. Determination of MIC of Antibiotics

MIC of antibiotics was determined using broth dilutions method as per CLSI guidelines against *S. aureus* ATCC 6538, *L. monocytogenes* ATCC 13932, and *S.* Typhimurium ATCC 6539 indicator organisms. For that, Sterile 100 µL of TSB was inoculated with 10% of actively grown cultures of indicator organisms. After that, five antibiotics (Doxycycline, Tetracycline, Vancomycin, Linezolid, and Oxacillin) were 2-fold serially diluted from 16 to 0.0156 µg/mL. Further, 50 µL of these diluted antibiotics samples were added in wells. The untreated was considered as a positive control [32]. Plates were incubated at 37°C for 24 h and cell density was measured at 595 nm in ELISA plate reader.

2.6. Determination of MIC of Enterocin MSW5, Antibiotics, and their Combinations by Checkerboard Assay

Synergy measurement using checkerboard analysis was used for the determination of the impact of the potency of the combination of two antimicrobial agents in comparison to their activities. The checkerboard assay was performed using a 96-well microtiter plate to find the fractional inhibitory concentration (FIC) index of antibiotics in combination with Enterocin MSW5 against each indicator organism. In microtiter plates, 100 µL of TSB was distributed into each well. In this method, 2-fold serially diluted antimicrobial agents were added in each well of the microtiter plate in the X- and Y-axis of an 8×8 matrix. Whereas Compound A was considered an antibiotic and Compound B

was considered Enterocin MSW5. For each combination (A × B), each well of columns having 25 µL of antibiotics (A) which was serially double diluted in a basal medium along the X-axis, and rows of the same plates contained 25 µL of Enterocin MSW5 which was also serially double diluted in the same basal medium along the Y-axis. In each well, a 10% of inoculum size of *S. aureus, L. monocytogenes*, and *S.* Typhimurium with 1×10^8 CFU/ml cell density was added in separate plates. The plates were incubated at 37°C for 24 h and the next day cell density was measured at 595 nm in an ELISA reader (Multiskan Go, Thermo Scientific). The same procedure was repeated in triplicate sets for all five antibiotics against three indicator strains as mentioned above. MIC of antibiotics, Enterocin MSW5, and their combinations had been determined [2].

2.7. Determination of Fractional Inhibitory Concentration Index (FICI) of Enterocin MSW5 in Combination with Antibiotics

To check the synergistic effect of five different antibiotics and bacteriocin fractional inhibitory concentration (FIC) was determined. The formulas used to calculate the FICI are as follows:

$$\text{FIC A}=\frac{\text{MIC of A in presence of B}}{\text{MIC of A individually}} \quad (1)$$

$$\text{FIC B}=\frac{\text{MIC of B in presence of A}}{\text{MIC of B individually}} \quad (2)$$

The Fractional Inhibitory Concentration Index (FIC Index) for antibiotics and Enterocin MSW5 can be determined by the equation

$$\text{FIC index}=\text{FIC A}+\text{FICB} \quad (3)$$

FIC results were interpreted, if FIC ≤0.5 was recognized as a synergistic effect, 0.5 < FIC ≤ 1 was assigned as an additive effect, 1 < FIC ≤ 4 indicate no interactive effect, and FIC >4 antagonistic effects in between two antimicrobial agents [33].

2.8. Determination of Inoculum Size for Potential Biofilm Formation of Pathogens

Biofilm formation of pathogens was determined using microtiter plate assay in triplicates [34]. Test organisms *S. aureus, L. monocytogenes,* and *S.* Typhimurium were activated in a sterile TSB medium at 37°C for 24 h. Next day, activated cultures were centrifuged and cell pellets were dissolved in sterile distilled water. The cell density of pathogens was adjusted to 1×10^8 CFU/mL. In a sterile microtiter plate, wells were filled with 200 µl of sterile TSB medium and indicator organisms were added in variable inoculums size ranges from 2% to 12% of the total media volume in respective wells, and plates were incubated for 24 h at 37°C. Further, crystal violate staining was carried out, microtiter plate was decanted gently followed by the addition of 200 µL methanol for biofilm fixation and allowed for 1 min to react. After that, 200 µL D/W was added for washing purposes. Then, 200 µL of crystal violet (0.1% w/v) stain was added and allowed to react for 2–3 min. Further, the plates were decanted gently, and cell density was measured at 595 nm using an ELISA reader by adding 200 µL of 33 % glacial acetic acid.

2.9. Determination of Medium for Biofilm Potential of Pathogens

Test organisms *S. aureus, L. monocytogenes,* and *S.* Typhimurium were activated in a sterile TSB medium and incubated overnight at 37°C. Further, the cultures were centrifuged and cell pellets were dissolved in sterile distilled water. The cell density of pathogens was adjusted to 1×10^8 CFU/mL. In a sterile microtiter plate, 200 µL sterile NB, Luria Bertani broth (LB), and Tryptic Soybean casein Broth (TSB) medium were added in triplicates [34]. Media-containing wells were inoculated with 10% activated indicator cultures and plates were incubated for 24 h at 37°C. The next day, as described earlier staining procedure was carried out and cell density was measured at 595 nm using an ELISA reader.

2.10. Use of Enterocin MSW5 in Combination with Antibiotics for Eradication of Biofilm

In a sterile Microtiter plate, sterile TSB medium (100 µL) was added and each well was inoculated with 10% of activated indicator organisms with cell density of 1×10^8 CFU/mL in each separate plate. In each well 25 µL of filter-sterilized Enterocin MSW5, antibiotics, and their combinations were added as per their MIC and sub-MIC values. The untreated well was considered a positive control. This same procedure was carried out for Oxacillin, Vancomycin, Tetracycline, Doxycycline, and Linezolid with Enterocin MSW5. Plates were then overnight incubated at 37°C. The next day staining procedure was carried out as described earlier and cell density was measured at 595 nm using an ELISA reader. Percent Biofilm eradication was calculated as per the given formula [21].

$$\text{Biofilm eradication \%}=\frac{\text{Initial OD-Test OD}\times 100}{\text{Initial OD}}$$

Where, Initial OD: OD of positive control; Test OD: OD of treated wells with antimicrobials.

2.11. Statistical Analysis

All the experiments were performed in triplicate. Data were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's test using GraphPad Prism 8.0. Differences between mean values were considered significant at $P \leq 0.05$.

3. RESULTS

3.1. Determination of the MIC of Enterocin MSW5

The MIC of Enterocin MSW5 was determined by microdilution method using 96 well microtiter plate. The MIC of Enterocin MSW5 was 0.362 ± 0.00 mg/mL for both *S. aureus* and *S.* Typhimurium while 0.725 ± 0.00 mg/mL for *L. monocytogenes*. This implies that *S. aureus* and *S.* Typhimurium were comparatively more susceptible to Enterocin MSW5 than *L. monocytogenes.*

3.2. Determination of the MIC of Antibiotics

In the present study, we have selected broad spectrum antibiotics based on their mode of action; oxacillin and vancomycin can inhibit cell wall synthesis of Gram-positive bacteria whereas tetracycline, doxycycline, and linezolid can inhibit the protein synthesis. As per CLSI guidelines, the MICs of five different antibiotics such as tetracycline, vancomycin, oxacillin, doxycycline, and linezolid were determined against *S. aureus, L. monocytogenes,* and *S.* Typhimurium and are tabulated in Table 1. Tetracycline and doxycycline were the most potent antibiotics among five antibiotics with 1 ± 0.00 µg/mL MIC whereas oxacillin was comparatively less potent antibiotic with 4 ± 0.00 µg/mL MIC against *S. aureus.* Similarly, antibiotics inhibiting protein synthesis work well against another Gram-positive pathogen *L. monocytogenes,* where the MIC of tetracycline and linezolid was low

(1 ± 0.00 µg/mL). Furthermore, tetracycline antibiotic also works well against gram-negative pathogen *S.* Typhimurium, where the MIC of tetracycline was low (1 ± 0.00 µg/mL). However, the MIC of oxacillin and linezolid was more (8 ± 0.00 µg/mL) against *S.* Typhimurium.

3.3. Evaluation of the Synergistic Effect of Antibiotics and Enterocin MSW5

The Fractional Inhibitory Concentration Index (FICI) was determined for all the combinations of Enterocin MSW5 and antibiotics. In this study, the FICI value was <0.5 for all the antibiotics in combination with Enterocin MSW5 against *S. aureus, L. monocytogenes,* and *S.* Typhimurium which indicated synergism was observed between all the five antibiotics and Enterocin MSW5. In the case of *S. aureus,* the best synergism was observed with linezolid in combination with Enterocin MSW5 with FICI, 0.25 followed by doxycycline (FICI 0.263), vancomycin (FICI 0.279), tetracycline (FICI 0.310), and oxacillin (FICI 0.498) as mentioned in Table 2. Likewise, tetracycline-Enterocin MSW5 combination has also shown potential synergism against *S.* Typhimurium. The FICI value for this combination was 0.249, which was less compared to the other four antibiotic combinations. While, other four antibiotics also showed synergism with enterocin MSW5 in sequence doxycycline (FICI 0.251), vancomycin (FICI 0.255), linezolid (FICI 0.263), and oxacillin (FICI 0.507) [Table 3]. Furthermore, best synergism was observed with linezolid in combination with Enterocin MSW5 with FICI 0.125 for *L. monocytogenes*. Furthermore, tetracycline, doxycycline, and vancomycin have shown synergism with enterocin MSW5 with FICI 0.139 followed by oxacillin (FICI 0.155) for *L. monocytogenes* [Table 4]. Hence, these results depict that the MIC of both Enterocin MSW5 as well as antibiotics were decreased when utilized in combinations compared to individual. Moreover, the efficacy of Enterocin MSW5 increased toward indicator pathogens when utilized in combinations with antibiotics.

3.4. Determination of Biofilm Potential

3.4.1. Determination of inoculum size for potential biofilm formation

To determine inoculum size for potential biofilm formation, 96 well microtiter plate assay was performed in triplicates. In the case of *S. aureus* and *L. monocytogenes*, maximum biofilm formation was observed at 10% inoculum size while in case of *S.* Typhimurium, maximum biofilm formation was observed at 12% inoculum size [Figure 1].

3.4.2. Determination of biofilm potential using LB, NB, and TSB media

Different growth media such as LB, NB, and TSB have been assessed for potential biofilm formation using microtiter plate assay. Among them, at 10% inoculum size with TSB media, maximum biofilm formation was observed for indicator organisms *S. aureus,* and *L. monocytogenes* while in case of *S.* Typhimurium, maximum biofilm formation observed in same TSB media but at 12 % of inoculum size [Figure 2].

3.5. Eradication of Biofilm using Antibiotics, Enterocin MSW5, and their Combination

The biofilm eradication study was carried out using different antimicrobials (antibiotics and Enterocin MSW5) by microtiter plate assay. They were applied individually and in combination. When antimicrobials were utilized individually at their MIC and sub-MIC concentration, less biofilm eradication was observed. When antibiotics were used alone, biofilm eradication was 27.86 ± 0.78% for tetracycline against *S. aureus*, 24.31 ± 0.81% for tetracycline against *S.* Typhimurium and 38.58 ± 0.96% for linezolid against *L. monocytogenes*. Similarly, when biofilm eradication was studied alone with Enterocin MSW5, eradication was 53.21 ± 0.75% at MIC; 30.72 ± 0.99% at sub-MIC concentration for *S. aureus,* it was 30.02 ± 0.81% at MIC; 21.50 ± 0.70% at sub-MIC

Table 1: MIC of antibiotics against indicator organisms.

Antibiotics	MIC of antibiotics (µg/mL)		
	***Staphylococcus aureus* ATCC 6538**	***Salmonella* typhimurium ATCC 6539**	***Listeria monocytogenes* ATCC 13932**
Tetracycline	1±0.00^{a}	1±0.00^{A}	1±0.00^{I}
Oxacillin	4±0.00^{c}	8±0.00^{D}	8±0.00^{III}
Doxycycline	1±0.00^{ab}	2±0.00^{B}	2±0.00^{II}
Linezolid	2±0.00^{ab}	8±0.00^{D}	1±0.00^{I}
Vancomycin	4±0.00^{c}	4±0.00^{C}	8±0.00^{III}

Mean±SD. Different letters above the columns denote statistically significant differences at $P<0.05$ by Tukey's test. SD: Standard deviation, MIC: Minimum inhibitory concentration, ATCC: American Type Culture Collection

Table 2: Fraction inhibitory concentration of enterocin MSW5 in combination with antibiotics against *Staphylococcus aureus* American Type Culture Collection 6538.

Enterocin MSW5 in combination with antibiotics	MIC of A in presence of B (µg/mL)	MIC of B in presence of A (mg/mL)	FIC A^{a}	FIC B^{b}	FICI^{c}	Activity^{d}
Enterocin MSW5+Tetracycline	0.062±0.00^{a}	0.090±0.00^{A}	0.062	0.248	0.310	Synergic effect
Enterocin MSW5+Oxacillin	1±0.00^{d}	0.090±0.00^{A}	0.250	0.248	0.498	Synergic effect
Enterocin MSW5+Doxycycline	0.062±0.00^{a}	0.090±0.00^{A}	0.015	0.248	0.263	Synergic effect
Enterocin MSW5+Linezolid	0.012±0.00^{b}	0.090±0.00^{A}	0.007	0.248	0.255	Synergic effect
Enterocin MSW5+Vancomycin	0.125±0.00^{c}	0.090±0.00^{A}	0.031	0.248	0.279	Synergic effect

^{a}FIC A=MIC of A in presence of B (µg/mL)/MIC of A alone (µg/mL), ^{b}FIC B=MIC of B in presence of A (mg/mL)/MIC of B alone (mg/mL), ^{c}FICI=FIC A+FIC B), ^{d}Activity=S: FICI≤0.5, AD: 0.5≤FIC≤1, No interaction (I): FIC≤4, Antagonistic effect (A): FIC>4. Mean±SE. Different letters above the columns denote statistically significant differences at $P<0.05$ by Tukey's test. A: Antibiotics, B: Enterocin MSW5, SE: Standard error, MIC: Minimum inhibitory concentration, FIC: Fractional inhibitory concentration, FICI: FIC index, AD: Additive effect

Table 3: Fraction inhibitory concentration of enterocin MSW5 in combination with antibiotics against *Salmonella typhi* American Type Culture Collection 6539.

Enterocin MSW5 in combination with antibiotics	MIC of A in presence of B (µg/mL)	MIC of B in presence of A (mg/mL)	FIC A[a]	FIC B[b]	FICI[c]	Activity[d]
Enterocin MSW5+Tetracycline	0.015±0.00[a]	0.090±0.00[A]	0.001	0.248	0.249	Synergic effect
Enterocin MSW5+Oxacillin	0.062±0.00[b]	0.181±0.00[B]	0.007	0.500	0.507	Synergic effect
Enterocin MSW5+Doxycycline	0.015±0.00[a]	0.090±0.00[A]	0.003	0.248	0.251	Synergic effect
Enterocin MSW5+Linezolid	0.015±0.00[a]	0.090±0.00[A]	0.015	0.248	0.263	Synergic effect
Enterocin MSW5+Vancomycin	0.015±0.00[a]	0.090±0.00[A]	0.007	0.248	0.255	Synergic effect

[a]FIC A=MIC of A in presence of B (µg/mL)/MIC of A alone (µg/mL), [b]FIC B=MIC of B in presence of A (mg/mL)/MIC of B alone (mg/mL), [c]FICI=FIC A+FIC B, [d]Activity=S: FICI≤0.5, AD: 0.5≤FIC≤1, No interaction (I): FIC≤4, Antagonistic effect (A): FIC>4. Mean±SE. Different letters above the columns denote statistically significant differences at $P<0.05$ by Tukey's test. A: Antibiotics, B: Enterocin MSW5, MIC: Minimum inhibitory concentration, FIC: Fractional inhibitory concentration, FICI: FIC index, AD: Additive effect, SE: Standard error

Table 4: Fraction inhibitory concentration of enterocin MSW5 in combination with antibiotics against *Listeria monocytogenes* American Type Culture Collection 13932.

Enterocin MSW5 in combination with antibiotics	MIC of A in presence of B (µg/mL)	MIC of B in presence of A (mg/mL)	FIC A[a]	FIC B[b]	FICI[c]	Activity[d]
Enterocin MSW5+Tetracycline	0.015±0.00[a]	0.090±0.00[A]	0.015	0.124	0.139	Synergic effect
Enterocin MSW5+Oxacillin	0.25±0.00[c]	0.090±0.00[A]	0.031	0.124	0.155	Synergic effect
Enterocin MSW5+Doxycycline	0.030±0.00[b]	0.090±0.00[A]	0.015	0.124	0.139	Synergic effect
Enterocin MSW5+Linezolid	0.015±0.00[a]	0.090±0.00[A]	0.001	0.124	0.125	Synergic effect
Enterocin MSW5+Vancomycin	0.015±0.00[a]	0.090±0.00[A]	0.015	0.124	0.139	Synergic effect

[a]FIC A: MIC of A in presence of B (µg/mL)/MIC of A alone (µg/mL), [b]FIC B: MIC of B in presence of A (mg/mL)/MIC of B alone (mg/mL), [c]FICI: FIC A+FIC B, [d]Activity=S: FICI≤0.5, AD: 0.5≤FIC≤1, No interaction (I): FIC≤4, Antagonistic effect (A): FIC>4. Mean±SE. Different letters above the columns denote statistically significant differences at $P<0.05$ by Tukey's test. A: Antibiotics; B: Enterocin MSW5, AD: Additive effect, SE: Standard error, MIC: Minimum inhibitory concentration, FIC: Fractional inhibitory concentration, FICI: FIC index

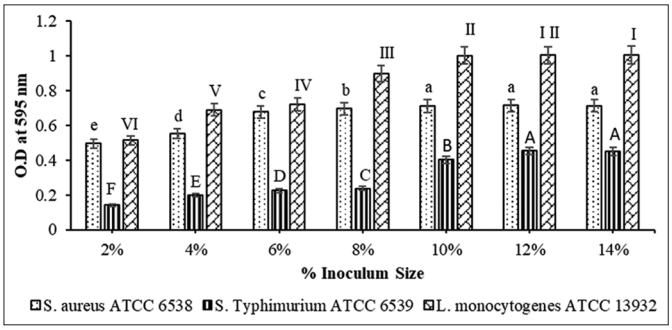

Figure 1: Determination of inoculum size of pathogens for potential biofilm formation. Different letters above the bars denote statistically significant differences at $P < 0.05$ by Tukey's test.

for *S.* Typhimurium and 42.18 ± 0.75% at MIC; 28.05 ± 0.87% at sub-MIC for *L. monocytogenes* [Figure 3]. Then, biofilm eradication was studied using five different antibiotics combined with Enterocin MSW5, the maximum biofilm eradication was observed in the case of tetracycline (68.08 ± 0.75%) against *S. aureus* and (53.76 ± 0.70%) against *S.* Typhimurium, and 96.97 ± 0.90% against *L. monocytogenes* in case of linezolid. Further, potential biofilm eradication was also observed at the sub-MIC concentration of these antimicrobials when they were applied in combination. The biofilm eradication was 40.19 ± 0.67% for tetracycline against *S. aureus*, 33.99 ± 0.72% for tetracycline against *S.* Typhimurium, and 88.36 ± 0.90% for linezolid against *L. monocytogenes* [Figure 4]. Thus, the overall results depict that the combinatorial effect of antibiotics and Enterocin MSW5 is significant for biofilm eradication in comparison to individual antimicrobial agents.

4. DISCUSSION

In the present work, we studied the *in vitro* activities of conventional antibiotics and Enterocin MSW5 alone and in combinations against various pathogenic strains. We used purified Enterocin MSW5 which was obtained from isolate *E. faecalis* MSW5. Enterocin is a small molecular weight AMP which have ability to inhibit closely related Gram-positive bacteria and also in some cases Gram-negative bacteria. It is basically cationic heat stable peptide synthesized by ribosomes and contains 20–60 amino acids. Enterocin is stable over a wide range of pH and heat. They are classified in to four classes such as lantibiotic (Class I), non-lantibiotic (Class II), cyclic Enterocin (Class III), and Enterocin with high molecular weight (Class IV). The antimicrobial mode of action of Enterocin is different than antibiotics. They inhibit the growth of bacteria by different mechanisms like perforating on target cells, inhibiting protein synthesis by interacting with ribosomes or tRNA, inhibiting peptidoglycan synthesis, and directly degrading target cell DNA [35]. The MIC of this Enterocin MSW5 was same for *S. aureus* and *S.* Typhimurium, respectively, while slightly higher for *L. monocytogenes*. Similar kind of result was noted by another group of researchers and Enterocin TJUQ1 isolated from pickled Chinese celery, MIC was 5.26 µg/ml against *L. monocytogenes* CMCC 1595 and 46.50 µg/mL against *S. aureus* [36]. Similarly, other researchers have also found that Enterocin RM6 from *E. faecalis* has potential activity against foodborne pathogens like *B. cereus* ATCC 14579, *L. monocytogenes*, and MRSA *S. aureus*, but it has no activity against Gram-negative bacteria such as *Yersinia enterocolitica, S.* Typhimurium, and *Escherichia coli* [37]. These results indicate bacteriocins produced by Gram-positive bacteria can significantly inhibit the growth of closely related strains [38].

Further, the synergism between Enterocin MSW5 and five different antibiotics was studied using a checkerboard assay, and their FICI value was <0.5 for all the combinations. Among them all the antibiotic combinations with Enterocin MSW5, linezolid has shown maximum two-way synergism against Gram-positive pathogens *S. aureus* (FICI = 0.25) and *L. monocytogenes* (FICI = 0.125). Hanchi *et al.* [2] established the two-way synergy for durancin and vancomycin against *S. aureus* ATCC 700699 with FICI 0.3. Likewise, combination studies reported for the cationic peptide Enterocin CRL35, FICI was <0.5 for antibiotics, erythromycin, chloramphenicol, and tetracycline but it was not for cefalexin, ciprofloxacin, nalidixic acid, ampicillin, or vancomycin against *Listeria innocua* 7 [39]. A similar set of experiments using *L. monocytogenes* FBUNT was accomplished by researchers, where the MIC of Enterocin CRL35 alone was observed at 1.6 ng/ml that was reduced to 0.8 ng/mL when the Enterocin CRL35 was utilized in combination with bacitracin, gramicidin, and monensin antimicrobials [40]. In our study, we also observed a similar kind of observation, the MIC of five different antibiotics was high but when they were used in combination with Enterocin MSW5, MIC was reduced and FICI value was 0.255 in case of combination of tetracycline and Enterocin MSW5 against *S.* Typhimurium. Likewise, Sharma *et al.* [41], have determined the synergy effect of ampicillin, penicillin, and ceftriaxone with Enterocin E20c and it has given <0.5 FICI against *Salmonella enterica.* Similarly, the use of bacteriocin produced by *Pediococcus pentosaceus* ST44AM in combination with ciprofloxacin have shown synergism with each other against *Listeria ivanovii* subsp. *ivanovii* ATCC 19119 [42]. These results revealed that bacteriocin may create a pore in the cell membrane which allow the penetration of antibiotic within the cell and shows its inhibitory effect [42]. Bacteriocin can bind with cell receptors such as lipid II and other cell wall precursors, the mannose phosphotransferase system, undecaprenyl pyrophosphate phosphatase, the maltose ABC transporter, and Zn-dependent metallopeptidase which can creates pore in cell membrane of bacteria [35]. Thus, the benefit of use of

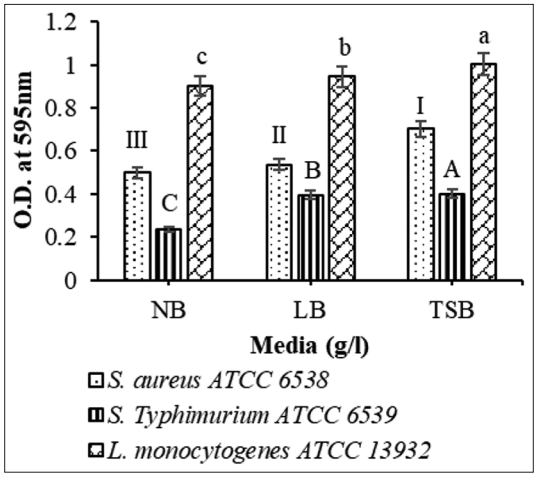

Figure 2: Determination of media for potential biofilm formation of pathogens (where NB: Nutrient Broth, LB: Luria Broth, TSB: Tryptic Soy Broth). Different letters above the bars denote statistically significant differences at $P < 0.05$ by Tukey's test.

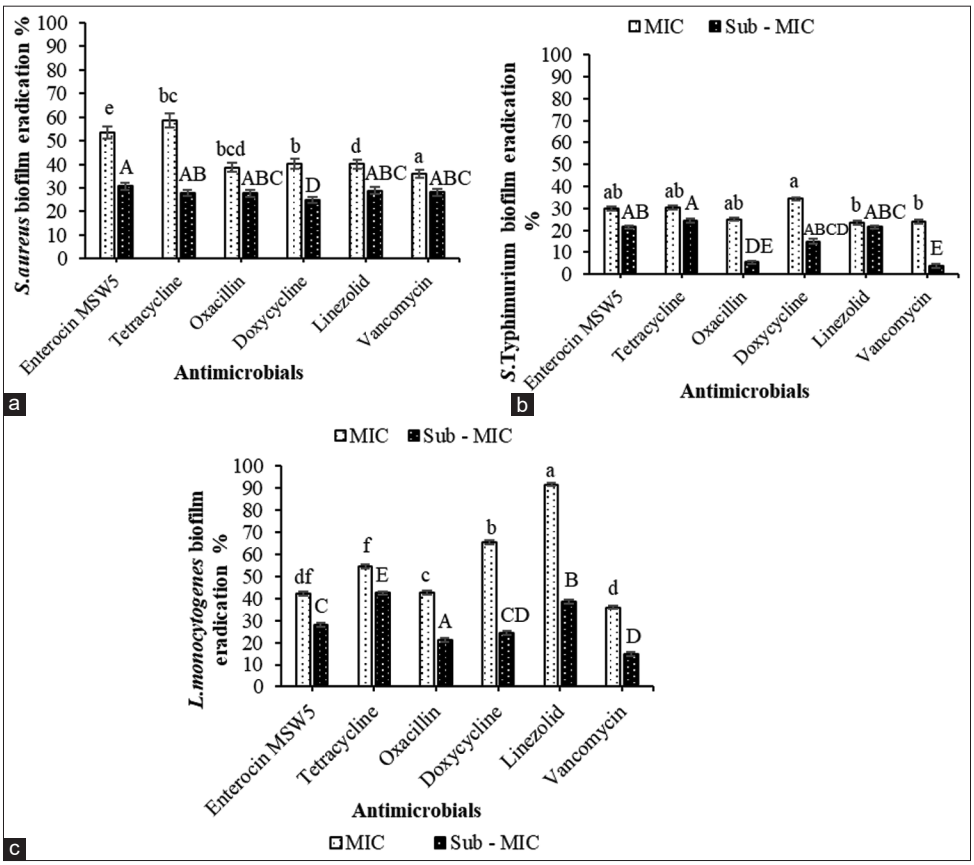

Figure 3: Biofilm eradication of pathogens by antimicrobials (Enterocin MSW5 and antibiotics alone) at their minimum inhibitory concentration (MIC) and sub-MIC values. (a) *Staphylococcus aureus* biofilm eradication by antimicrobials (b) *Salmonella* Typhimurium biofilm eradication by antimicrobials (c) *Listeria monocytogenes* biofilm eradication by antimicrobials. Different letters above the bars denote statistically significant differences at $P < 0.05$ by Tukey's test.

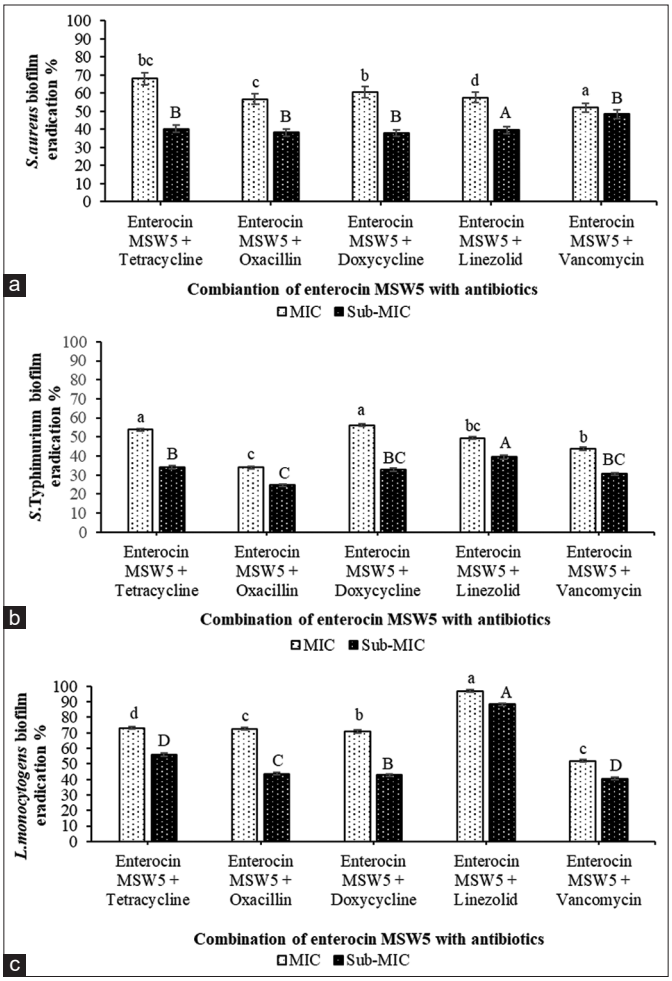

Figure 4: Biofilm eradication of pathogens by a combination of Enterocin MSW5 and antibiotics at their minimum inhibitory concentration (MIC) and sub-MIC values. (a) *Staphylococcus aureus* biofilm eradication by a combination of antimicrobials (b) *Salmonella* Typhimurium biofilm eradication by a combination of antimicrobials (c) *Listeria monocytogenes* biofilm eradication by a combination of antimicrobials. Different letters above the bars denote statistically significant differences at $P < 0.05$ by Tukey's test.

the combinatorial therapy of two antimicrobials with a different mechanism of action may result in a more lethal activity against pathogenic microorganisms compare to individual use. Moreover, the use of antibiotics with Enterocin might enhance each other's inhibitory effects, thereby possibly decreasing the probability of the development of resistance either to the Enterocin or antibiotics [43-45].

Another major global concern of antimicrobial resistance is biofilm formation by AMR bacteria. Both types of bacteria, Gram-negative and positive such as *S. aureus*, *L. monocytogenes, S.* Typhimurium, *E. faecalis*, *E. coli, Staphylococcus epidermidis, Staphylococcus viridans*, *Proteus mirabilis, Klebsiella pneumoniae*, and *P. aeruginosa* can form biofilms. Therefore, we have studied the biofilm formation assay of three foodborne pathogens, *S. aureus*, *L. monocytogenes,* and *S.* Typhimurium using a microtiter plate assay. At 10% inoculum size of *S. aureus* and *L. monocytogenes* maximum biofilm formation whereas, *S.* Typhimurium had shown maximum biofilm formation at 12% inoculum size. The ability of formation of biofilm was considered as OD570 < 0.120, there is no biofilm-formation, 0.120 < OD570 < 0.240, weak biofilm formation, and OD570 > 0.240, strong biofilm formation [46,47]. According to these studies, *S. aureus, S.* Typhimurium, and *L. monocytogenes* pathogens were found to be strong biofilm formers. Medium composition is perhaps the most imperative factor which can influence biofilm formation by bacteria under laboratory *in vitro* conditions. For this work, we selected three different media such as LB, NB, and TSB for the investigation of potential biofilm formation by *S. aureus, S.* Typhimurium, and *L. monocytogenes*. Among them, TSB media showed maximum biofilm formation than LB and NB media. A similar kind of study was carried out by Cruz *et al.* [48], *S. aureus* biofilm formation was observed in Muller Hinton (MH) broth, Tryptic Soy Glucose (TSG), TSB, brain heart infusion glucose, and brain heart infusion (BHI). Among them, significant biofilm formation was observed with TSB. Likewise, a study was carried out for *S.* Typhimurium and *L. monocytogenes* by Ranin *et al.* [49], they observed that diluted TSB (1/20-TSB) has shown maximum biofilm formation of *Salmonella* sps.(0.51 ± 0.177) and BHI (0.326 ± 0.06) for *L. monocytogenes.* All these results revealed that the presence of carbohydrates plays a significant role in biofilm formation [48] and the potential biofilm formation observed in the TSB medium might be due to the amino acids present in the media as a main nutrient component, as well as, it contains enough amount of glucose (2.5 g/l) as a carbohydrate source [50].

Antibiofilm activities of clinically available antibiotics are becoming a significant part of treating infections that occur due to biofilms, such as microbial infections, including cystic fibrosis, endocarditis, periodontitis, osteomyelitis, rhinosinusitis, non-healing chronic wounds, kidney infections, meningitis, prosthesis and infections related to an implantable device, catheter-associated infections, or wound infections. However, due to AMR development in bacteria, it becomes hard to eradicate the biofilm using antibiotics on the bases of their MIC. As a solution, we studied the *in vitro* activities of antibiotics (Tetracycline, vancomycin, oxacillin, doxycycline, and linezolid) and Enterocin MSW5 alone and in combinations against *S. aureus, S.* Typhimurium, and *L. monocytogenes*. Enterocin MSW5 has shown 53.51 ± 0.75%, 30.02 ± 0.81%, and 42.18 ± 0.75% eradication of *S. aureus, S.* Typhimurium, and *L. monocytogenes* biofilm, respectively at their MIC value. However, with the use of Enterocin MSW5 in combination with antibiotics, eradication was increased for all the combinations. Moreover, Enterocin MSW5 and antibiotics were also used in combination at their sub-MIC concentration which has shown potential biofilm eradication of all the three pathogens. Similarly, Field *et al.* [51] have found synergistic relationship between nisin + chloramphenicol and nisin + penicillin against *S. aureus* biofilm but in contrast, no synergistic effect was found for nisin and vancomycin combinations. Whereas, Dosler and Gerceker [52] have confirmed synergism between nisin and vancomycin against MRSA and MSSA biofilms. Similar results were also observed when vancomycin paired with bacteriocins from *E. faecalis* ST651ea, ST7119ea, or ST7319a, all the combinations have shown synergism against *Enterococcus faecium* VRE19 biofilm; while in the case of *L. monocytogenes,* only ST651ea and ciprofloxacin combination has shown synergism [32]. Along similar lines, the combination of micrococcin P1, garvicin KS, and penicillin G has also significantly eradicated the biofilm of *S. aureus* by causing severe cell damage [53].

5. CONCLUSION

A combination of Enterocin MSW5 with five different antibiotics has shown a synergistic effect against three food borne pathogens, *S. aureus, S.* Typhimurium, and *L. monocytogenes*. Their combinations have also shown potential antibiofilm activity and can significantly

eradicate the biofilm of three food borne pathogens at their MIC and sub–MIC concentrations. Therefore, in the future, this combinatorial therapy can be used in the therapeutic sector and might help to solve the problem of multidrug resistance across a wide range of bacterial populations.

6. AUTHORS' CONTRIBUTIONS

All authors have equally contributed to this manuscript. Material preparation, data collection, and analysis were performed by Mansi Shukla and Dhruv Valand. Shilpa Gupte analyzed these data and necessary inputs were given for the design of the manuscript. The first draft of the manuscript was written by Mansi Shukla and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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8. CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

10. DATA AVAILABILITY

Data and material described in this study are available from the authors upon request and availability.

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