EFFECT OF BACTERIOCIN PRODUCED FROM ENTEROCOCCUS FAECIUM AGAINST DRUG RESISTANT BACTERIAL ISOLATES

Chandrashekhar Unakal*, Gizachew Yismaw, Amare Gebrehiwot, Mengistu Endris and Feleke Moges

*Department of Medical Microbiology, School of Biomedical and Laboratory Sciences, University of Gondar, Ethiopia
E-mail of Corresponding Author: cg.unakal@gmail.com

Abstract
A bacteriocin-producing strain isolated from Ethiopian honey was identified as Enterococcus faecium. The incidence of multidrug resistance has become a leading challenge in the treatment of deadly infectious diseases specifically of bacterial origin. In order to reduce the risk and spread of bacterial antibiotic resistance, various new drugs have been searched and introduced in the biomedical science. In this reference, bacteriocin could have been an important alternative for the control of certain infectious agents. The present study was carried out to check the effect of bacteriocin produced from Enterococcus faecium on multidrug resistant bacterial isolates. The isolates of Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus, were isolated from different clinical specimens and were screened for multidrug resistance by the standard Kirby-Bauer disk diffusion method. Bacteriocin was produced from Enterococcus faecium during growth in MRS broth at 37°C and was checked for antibacterial activity against above mentioned resistant bacterial isolates by agar well diffusion method. All the clinical isolates were confirmed to be multi drug resistant. The bacteriocin proved to be active against a narrow range of gram positive bacteria i.e. isolates of Staphylococcus aureus and it showed reduced activity against; isolates of Pseudomonas aeruginosa and Escherichia coli.

Keywords: Enterococcus faecium, antibacterial activity

1. Introduction
Antimicrobial peptides present new possibilities for combating infectious diseases. They inhibit the growth of pathogenic microorganisms, without affecting the host or the animals and plants that produce them, have a broad spectrum of antimicrobial activity. It is well known that bacteria, induced by stress, produce bacteriocin that may cure infectious diseases1. There is increasing concern about the resistance of microorganisms to various drugs and the perspective of continuous use of antibiotics is not yet well defined. Therefore, many measures to solve this problem need to be adopted e.g., the controlled use of antibiotics, expansion of research for the better understanding of resistance mechanisms, and continuing attempts to develop new synthetic and natural drugs2. Peptides, we believe, constitute a novel potential therapeutic agent against diseases caused by pathogenic organisms. Bacteriocins are antibacterial peptides or proteins with spectra of inhibition usually confined to strains closely related to the producing strain. Several bacteriocins produced by enterococci, especially by Enterococcus faecium strains of different origins (rumen contents, sausages, waste, olives) have been described3. To date, the following enterocins from E. faecium have been characterized: enterocin A4, enterocin B5, enterocin P6, enterocins L50A and L50B7, enterocin I (identical to enterocin L50A)8, enterocin Q9,10 and enterocin M11 (a new variant of enterocin P). In general, most enterocins belong to the class IIa bacteriocins, which are thermostable, pediocin-like bacteriocin. The use of bacteriocinogenic enterococci to control contamination in food, feed and in the digestive tract of animals has been reported10,12,13. The extensive use of antibiotics has contributed to limited array of effective drugs for treating multi-resistant bacteria. This problem remotivated research efforts to find alternatives towards increasingly limited antibiotic resources. Bacteriocins have been considered as potential antimicrobial agents to combat bacterial infections14. Enterococcal bacteriocins, often termed as enterocins, have been widely investigated, mainly because they are active against gram-positive food-borne pathogens, such as Listeria monocytogenes, Staphylococcus aureus, and Bacillus cereus15. However, some enterocins exhibit broad activity spectra and inhibit the growth of gram-negative...
This research work was carried out in order to determine the antibacterial activity of bacteriocin produced by Enterococcus faecium against multidrug resistance (MDR) bacterial species. Considering the current antibiotic resistance issue, the effort is made to isolate and purify the bacteriocin from above mentioned species.

2. Materials and Methods
2.1 16S rDNA sequencing for bacterial isolate: Enterococcus Spp. was used as bacteriocin producer previously isolated from Ethiopian honey and further subjected for identification by16S rRNA sequencing. The 16S rRNA gene sequence was used to carry out BLAST with the nrdatabase of NCBI Genbank database.

2.2 Bacterial strains for susceptibility testing: Species used as indicator microorganisms were three clinical isolates of Pseudomonas aeruginosa (N=30), Escherichia coli (N=30) and Staphylococcus aureus (N=30) were collected from surgical wounds whereas; E. coli were collected from urine samples. All strains were kept frozen in Brain Heart Infusion with 20% (v/v) glycerol at -20°C.

2.3 Morphological and biochemical Identification of bacterial strains: All isolates were then subjected for morphological and biochemical characterization using conventional methods and the isolates were identified up to species level.

2.4 Disc diffusion method: Antimicrobial susceptibility testing was carried out by the standard Kirby-Bauer disk diffusion method following guidelines provided by ‘‘National Committee for Clinical Laboratory Standards, 2002. The antibiotic impregnated disks (Oxoid) used which contained doxycycline (30 µg), trimethoprim (5 µg), streptomycin (10 µg), gentamicin (10 µg), ampicillin (25 µg), cefoperazone (30 µg), ticarcillin+clavulanic acid (85 µg), ciprofloxacin (5 µg), chloramphenicol (30 µg), aztreonam (30 µg), erythromycin (15 µg), rifampicin (5 µg), vancomycin (30 µg), piperacillin+tazobactam (110 µg), methicillin (5 µg), meropenem (10 µg), and ceftriaxone (30 µg). Cell suspension of E. coli, S. aureus, P. aeruginosa was made in sterile saline (0.85%) to obtain the turbidity optically comparable to that of the 0.5 Mc-Farland standards (containing 1 x 108 cfu/ml) for each isolate. The dried surface of a Mueller-Hinton agar (MHA) plate was inoculated by streaking the swab over the entire sterile agar surface. The antibiotic impregnated disks used were placed individually, on the inoculated agar plate surface and incubated at 37°C for 18-24 hrs. Diameters of the zones were measured to mm. According to the diameter, isolates were classified into resistant, intermediate and susceptible.

2.5 Bacteriocin production: Bacteriocin producing strain was tested for the presence of bacteriocin production using the stab-overlay method. Enterococcus faecium was used as sensitive indicator strain. Bacteriocin was produced from Enterococcus faecium after cultivation in 100 ml de Man, Rogosa and Sharpe (MRS) medium at 37°C for 24 hrs. The culture was centrifuged at 10,000 rpm for 20 minutes at 4°C, and the supernatant was filtered through 0.22 µm membranes. This material was used as a crude bacteriocin preparation.

2.6 Detection of antimicrobial activity: The agar well-diffusion method was performed to determine the antimicrobial activity of cell free supernatant. MRS soft agar (7 ml) was seeded with 70 µl of overnight culture of indicator strain and was poured into a Petri dish. Wells (8-mm diameter) were cut with sterilized cork-borer from the indicator-seeded agar and 100 µl of cell free supernatant fluid (CFS) was poured in to each well. After diffusion of CFS in to agar medium, plates were incubated upside down for 24 hrs. at 37 °C. After 24 Hrs. zones of inhibition of the indicator species around the wells were measured in mm. Similarly, the antimicrobial activity of supernatant was checked against different multidrug resistant bacterial isolates i.e. Pseudomonas aeruginosa (N=15), Escherichia coli (N=15), and Staphylococcus aureus (N=15) (Table 2).

2.7 Physico-chemical characterization: CFS containing bacteriocin was taken from overnight culture of E. faecium grown in MRS broth. CFS at pH 7.0 was treated with proteinase K, lysozyme and lipase at a final concentration of 0.5 mg/ml and the activity was assayed by agar well diffusion method. To determine the effect of pH and temperature, CFS was adjusted to pH values from 4-10 and heat stability was assessed at 60°C, 80°C and 100°C for 30 minutes in water bath and autoclaving the bacteriocin at 121°C for

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15 minutes. Stability of CFS at temperatures of -4, 4 and 30°C was also determined by preserving it up to 3 months. Samples were withdrawn at different time intervals and the activity was checked by the same method against indicator strain.

3. Results
The bacteriocin producing strain previously isolated from honey was reconfirmed as *E. faecium* by the morphological, cultural and biochemical characteristics such as colony morphology on MRS agar was; off white, round, with smooth edges and raised from center, Gram positive cocci with cells arranged singly or in clusters. Biochemically the strain was catalase and oxidase negative. The final pH in glucose broth was 4±2, and acid was produced from L-arabinose, grew in 6.5% NaCl broth at 9.6 pH. It was non-hemolytic on blood agar plates containing 5-7% (v/v) of sterile human blood. By 16s rDNA gene sequencing it was observed that, the sequence of strain displayed the highest identity with the 16s rDNA gene of *Enterococcus faecium* based on nucleotide homology and phylogenetic analysis (Fig. 1).

![Phylogenetic tree for honey isolate](image)

The drug resistance pattern of *Pseudomonas aeruginosa* against antibiotics like ticarcillin+clavulanic acid, ceftriaxone, gentamicin and doxycycline, was (90%), (83%), (73%) and (70%) respectively. In case of *Escherichia coli*, resistant pattern was observed to Aztreonam, doxycycline, gentamicin and Ticarcillin + clavulanic acid, 93%, 90%, 84% and 80% respectively. Isolates of *Staphylococcus aureus* showed increased resistance to antibiotics tested. Maximum numbers of resistance i.e. 100% were noted against ticarcillin+clavulanic and ampicillin, while they were 96% against ciprofloxacin and piperacillin+tazobactam and 90% against gentamicin and meropenem. (Table 1).

| Antibiotics     | *P. aeruginosa* (n=30) | *E. coli* (n=30) | *S. aureus* (n=30) |
|-----------------|------------------------|-----------------|-------------------|
| AMP             | ND                     | ND              | 100               |
| ATM             | 40                     | 93              | 47                |
| C               | 16                     | 36              | 68                |
| CIP             | 46                     | 73              | 96                |
| CN              | 73                     | 84              | 90                |
| CRO             | 83                     | ND              | 20                |
| DO              | 70                     | 90              | 40                |
| E               | ND                     | ND              | 86                |
| MEM             | 36                     | 13              | 90                |
| MET             | ND                     | ND              | 86                |
| RD              | ND                     | ND              | 75                |
| S               | ND                     | 63              | 80                |
| TIM             | 90                     | 80              | 100               |
| TZP             | 50                     | 53              | 96                |
| VA              | ND                     | ND              | 20                |
| W               | ND                     | ND              | ND                |

AMP: Ampicillin, ATM: Aztreonam, C: Chloramphenicol, CIP: Ciprofloxacin, CN: Gentamicin, CRO: Ceftriaxone, DO: Doxycycline, E: Erythromycin, MEM: Meropenem, MET: Methicillin, RD: Rifampicin, S: Streptomycin, TIM: Ticarcillin + clavulanic acid, TZP: Piperacillin+tazobactam, VA: Vancomycin, W: Trimethoprim, ND: Not determine.
Enterococcus faecium was screened for bacteriocinogenic potential by two different methods i.e. stab-overlay test and agar-well diffusion assay. The supernatant of sample created a zone of inhibition of 14 mm against indicator strain after 24 hrs. incubation. Antibacterial activity of E. faecium bacteriocin was checked against MDR bacterial isolates. It was found to be increased activity against Gram positive Staphylococcus aureus (89%) strains. Escherichia coli isolates showed 67% while Pseudomonas aeruginosa isolates showed 56% of sensitivity to crude bacteriocin respectively (Table 2).

Table 2: Antimicrobial activity of bacteriocin against isolates

| Isolates | P. aeruginosa | E. coli | S. aureus |
|----------|---------------|---------|-----------|
| 1        | 0             | 06      | 14        |
| 2        | 10            | 0       | 16        |
| 3        | 07            | 10      | 12        |
| 4        | 09            | 11      | 14        |
| 5        | 0             | 09      | 13        |
| 6        | 0             | 0       | 0         |
| 7        | 05            | 07      | 12        |
| 8        | 0             | 14      | 14        |
| 9        | 10            | 0       | 16        |
| 10       | 0             | 12      | 15        |
| 11       | 08            | 0       | 0         |
| 12       | 10            | 12      | 12        |
| 13       | 0             | 0       | 14        |
| 14       | 0             | 0       | 13        |
| 15       | 0             | 0       | 0         |

Bacteriocin preparation was also subjected to different heat treatments and was found stable at temperatures; 60, 80, 90°C for 30 minutes. It was observed that bacteriocin was stable in pH range of 4 to 10 for 1 hour. The bacteriocinogenic activity of bacteriocin was completely lost when it was treated with enzyme proteinase K, while it remained unaffected when treated with enzymes lysozyme and lipase at a final concentration of 1 mg/ml.

4. Discussion

Multidrug resistance is common nowadays in bacteria isolated from clinical samples. According to Rahman et al., resistance of pathogenic bacteria toward antibiotics is increasing due to decline in the development of new and effective antibiotic compounds. Still, there is growing research with reference to development of new drugs and therapeutic agents like bacteriocins; though with their limited application has been thought to be effective for treating certain resistant pathogenic bacterial species. The present study was conducted to collect multidrug resistant bacterial species from clinical samples of surgical wounds and urine and screened for their sensitivity against bacteriocin produced by bacterial strain Enterococcus faecium. The bacteriocin producing strain was Gram-positive coccus, catalase negative, ability to grow at pH 9.6, gave a positive Voges Proskauer reaction and acid was produced from L-arabinose. The three bacterial species in the present study were initially screened for resistance against different antibiotics. The isolates showed variable resistance to a variety of antibiotics, however, Pseudomonas aeruginosa showed resistant to ticarcillin+clavulanic acid (90%), doxycycline (70%), ceftiraxone (83%) and gentamicin (73%). A study revealed that the increasing resistance in Pseudomonas aeruginosa to antibiotics such as gentamycin (35.1%), piperacillin+tazobactam (67.8%) while meropenem (28%), whereas; resistance to tetracycline was (70%) while ceftiraxone resistance was observed (63%). It was concluded that with the passage of time antibiotics resistance in P. aeruginosa is increasing. In case of E. coli, almost 93% isolates were resistant to azetronam, while 90% isolates were resistant against doxycycline. Khan and Zaman, also reported that 90% isolates of E. coli were resistant to ampicilin and 60-79% isolates were resistant against chloramphenicol. In case of chloramphenicol there was observed less resistance as reported earlier. Resistance in S. aureus to different antibiotics was observed high i.e. isolates of Staphylococcus aureus showed maximum numbers of resistance i.e. 100% were noted against ticarcillin+clavulanic and ampicillin in S. aureus, while they were; 96% against ciprofloxacin and piperacillin+tazobactam, 90% against gentamicin and meropenem, 86% against erythromycin, and 83% against methicillin. Similar studies also demonstrated a high resistance in S. aureus against ampicillin (92%), gentamycin (58%), ciprofloxacin (59%), cephalosporin (27%), erythromycin (44%) and meropenem (45%), which is quite low as compare to recent study. So with the passage of time, an increase was seen in antibiotics resistance in S. aureus.

Due to emergence of multidrug resistant bacterial species in clinical samples, a further step was taken to produce an alternative to antibiotics, in order to treat these MDR bacterial species. So, a bacteriocin producing strain, Enterococcus
faecium, previously isolated from honey, was used to produce bacteriocin. Gupta and Malik also isolated enterococci from a total number of 68 dairy products and were screened for bacteriocin production. The bacteriocin produced by Enterococcus strain was checked for its antimicrobial potential by using stab-overlay and agar well diffusion assay. It produced 14mm zone of inhibition against indicator strain of Staphylococcus aureus. According to Achemchem et al. revealed that the bacteriocin was active against many Gram positive bacteria but have no antimicrobial activity against Gram negative bacteria. Therefore, it was concluded that the bacteriocin of E. faecium has a narrow spectrum activity against Gram positive bacteria.

Thermostability of bacteriocin was monitored and the bacteriocin was found stable at 60, 80 and 100°C for 30 minutes and at 121°C for 15 minutes by autoclaving. These findings matched to a report that lactocin LC-09 were stable at 80 and 100°C and autoclaving the bacteriocin at 121°C for 15 minutes. The bacteriocin was found quite stable to a wide range of pH (4-10) for 1 hour. But it lost its activity when treated with enzyme proteinase K which indicates the proteinaceous nature of bacteriocin. Line et al., observed and demonstrated the proteinaceous nature of purified bacteriocin E-760 produced by E. faecium, upon treatment with various proteolytic enzymes.

Conclusion
The results in this experiment revealed that the bacteriocin isolated from Enterococcus faecium has great potential to inhibit pathogenic bacteria like Staphylococcus aureus.

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Conflict of interest
The authors declare that there is no conflict of interest

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