

Original Article

Preliminary characterization and inhibitory activity of bacteriocin like substances from *Lactobacillus casei* against multi-drug resistant bacteria

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Abstract Antimicrobial resistance is a mounting threat to the control of infectious diseases worldwide. Due to the increasing rate of drug resistance, there is continuous requirement for new and safe antimicrobial agents both for therapeutic purposes and food industries. The potential of bacteriocin has attracted much attention in recent years. We characterized bacteriocin like substances produced by *Lactobacillus casei*. This bacterium produces bacteriocin like substances that have the ability to inhibit multi-drug resistant pathogenic bacteria such as *Staphylococcus aureus*, *Klebsiella pneumoniae*, and *Proteus vulgaris*. To investigate the antimicrobial property of the bacteriocin like substances produced by *L. casei*, cell free supernatant (CFS) was prepared and antagonistic activity of cell free supernatant was determined by agar well diffusion method. Production of bacteriocin like substances was greatly affected by nutrient media, pH, temperature and incubation period. Highest inhibitory activity was observed when *L. casei* was grown in MRS broth (De Man, Rogosa and Sharpe broth) (pH 4.0) for 72 hours at 37°C. The active components of CFS responsible for antagonistic activity are proteinaceous in nature since they are susceptible to heat treatment. Partial purification of this CFS was done by organic solvent extraction method. Among the eight isolates tested, only *S. aureus*, *Proteus vulgaris* and *K. pneumoniae* showed more sensitivity to the partially purified crude bacteriocin like substances (CBLs) than CFS.

Keywords: Bacteriocin, *Lactobacillus casei*, multi-drug resistant bacteria, urinary catheter, urinary infection,

Introduction

Emergence of antibiotic resistance has become a serious threat to human and animal health. This is largely attributed to extensive and improper use of antibiotics, especially in developing countries, where regulations and drug policies are not stringently controlled. The Infectious Disease Society of America (IDSA) reported that approximately 70% pathogens will become resistant to at least one of the drugs used to treat infection caused by them¹. Urinary catheters pose a risk of recurring infections in patients suffering from urinary tract infections (UTIs) as they provide an adhering surface for potential urogenital pathogens to form biofilms. These infections are characterized by a poor response to antibiotic therapy and recurrence of infections. The problems associated in managements of these infections with drug resistant pathogens have prompted research focusing on finding alternative therapeutics. Antibiotic resistance in Gram-negative uropathogens is a major global concern having major implications for urological practice, particularly in relation to catheter-associated urinary tract infections (UTIs) and infectious complications following transrectal-ultrasonography-guided biopsy of the prostate or urological surgery².

Bacteriocins, anti-microbial peptides (AMPs) produced by bacteria, are produced by both Gram positive and Gram negative bacteria possessing antagonistic activity against other bacteria.

These AMPs can be potential candidates for alternative therapeutic agents against emerging multi-drug resistant infections including UTIs. Bacteriocins of Gram positive bacteria are classified into four groups on the basis of their size and mode of action³. Class 1 bacteriocins are called lantibiotics contain thermostable peptides having molecular weight below 5 kDa. Class 2 groups include small (<10 kDa), heat stable, non-lantibiotic containing bacteriocins which contain no lantionine. Class 3 bacteriocins include thermo labile bacteriocin having high molecular weight (>30 kDa). Class 4 bacteriocins consist of glycoprotein (lactocin 27) or lipoprotein (lactostrepcins) that require non-protein moiety for their activity⁴⁻⁷. Bacteriocins produced by Gram negative bacteria can be classified into two major groups, colicin and microcin, on the basis of their size⁸. Largest group of bacteriocin produced by Gram negative bacteria is Colicin (molecular weight 25–80 kDa). They are mainly synthesized by *E. coli* strains and also by *Yersinia pestis*, *Serratia marcescens*, *Shigella*, *Klebsiella* and *Pseudomonas*⁹. Microsin, another group of bacteriocin produced by Gram negative bacteria are characterized by low molecular weight (<10 kDa), thermostability and hydrophobicity¹⁰. Though hundreds of different types of bacteriocins have been discovered, only few have been allowed to be used as preservative or antibiotic due to concerns of toxicity and generation of hypersensitivity¹¹. Studies

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on animal model revealed that bacteriocins like nisin, mersacidin and lactacin 3147 can be used as potent therapeutic agents¹². Research on lantibiotics demonstrated that it has antagonistic activity against *Strep. pneumoniae* and MRSA in mice¹³, tooth diseases in dogs¹⁴ and bovine mastitis in dairy cows¹⁵. Besides, *Salmonella* infection in a mouse model has been drastically reduced by the bacteriocin microcin J2¹⁶.

Due to the alarming surge of antibiotic resistance in uropathogens, development of novel antimicrobial agent against uropathogens has become an urgent need. Lactic acid bacteria (LAB) and their byproducts are considered to be safe by the U.S. Food and Drug Administration (FDA)¹⁷. Lactobacilli can produce various metabolic byproducts having inhibitory activity against pathogens including hydrogen peroxide, lactic acid and bacteriocins¹⁸. Being active in nanomolar range and nontoxic in nature, bacteriocins produced by lactic acid bacteria have attracted attention from many industries¹⁹. Lactobacilli have been found to be effective against catheter associated pathogens as they can displace the pathogens from catheter materials such as silicone rubber and can block binding of the pathogens to epithelial cells of urinary tract¹⁸. This study was designed to investigate antibacterial activity of bacteriocin like substances from *Lactobacillus casei* against multi-drug resistant bacteria isolated from urinary catheters. Our study indicates that the bacteriocin like substances used in our study can inhibit multi-drug resistant *Shigella sp.*, *Salmonella sp.*, *Pseudomonas sp.*, *E. coli* and *B. cereus*.

Materials and Methods

Preparation of cell free supernatant (CFS) from Lactobacillus casei culture

One loopful of *L. casei* culture, isolated in a previous study in our laboratory²⁰, was grown overnight in 10 ml MRS broth at 37°C following the method as described by Al-Mathkhury *et. al.*²¹. Then 1 ml of actively growing culture was inoculated into 500 ml fresh MRS broth medium and incubated at 37°C for 24 hours under stationary condition. The cultured broth was centrifuged at 8,000 x g for 20 minutes at 4°C (KUBOTA 6930, Japan). The supernatant was filtered through Whatman No.1 filter paper (pore size 0.11 µm) to remove residual cells and stored at 4°C until use²².

Determination of Inhibitory activity of CFS on multi-drug resistant pathogenic bacteria

The inhibitory activity was determined by agar-well diffusion method using sterilized and solidified Mueller-Hinton agar (MHA) (HiMedia, Mumbai, India) plates. The suspension of pathogen was prepared by inoculating 1 loopful of test organism (*Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *E. coli*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Shigella sp.*, *Salmonella sp.*) into 10 ml sterile distilled water and turbidity was adjusted with 0.5 McFarland Standard¹⁸⁻¹⁹. Suspension of the individual test organism was seeded homogeneously on agar plates. Wells were made on the surface of solidified media with

the help of sterile cork borer. One drop of melted agar was poured into each well and allowed to solidify to make a base layer. Then the wells were filled with 100 µl of CFS. The plates were kept at 4°C for 1 hour to allow maximum diffusion, followed by incubation at 37°C for 24 hours.

Characterization of the inhibitory substance present in CFS

The pH of CFS (pH 6) was checked. 100 µl of CFS was added into one well in seeded Mueller Hinton plate. pH of remaining CFS was adjusted to pH 7.0 with 1M NaOH in order to rule out possible inhibition effects due to organic acids and filtered. A total of 100 µl of NaOH treated CFS was added into another well. 4 ml of neutralized CFS was then treated with 1 mg/ml of catalase (Merck, Germany) at 25°C for 30 min to eliminate the possible inhibitory action of H₂O₂ and filtered. Then 100 µl of catalase treated CFS was added to a different well. Remaining catalase treated CFS was heated 121°C for 15 minutes and 100 µl of heat treated CFS was added to another well²⁵. Zone of inhibition of observed after incubation at 37°C for 24 hours²⁵.

Effect of culture media on production of bacteriocin or bacteriocin like substances

To determine effect of culture media on production of bacteriocin or bacteriocin like substances three culture media (MRS broth, LB broth and Nutrient Broth) were used to make CFS by following the same method described in the previous sections. Antimicrobial activity of CFS of *L. casei* isolate were observed against test organisms following the same agar well diffusion method using CFS prepared from three different media.

Effect of incubation temperature on production of bacteriocin or bacteriocin like substances

CFS was prepared from *L. casei* by culturing in MRS broth at four different incubation temperatures (27°C, 33°C, 37°C and 40°C) for 24 hours by following same method described in the previous section. Agar well diffusion method was used to investigate effect of temperature on the production of bacteriocin like substances by *L. casei* using same test organisms as mentioned in the previous section.

Effect of pH on production of bacteriocin or bacteriocin like substances

MRS broth was prepared, sterilized and the initial pH was measured which gave a reading of pH 6. To reduce the pH to 4 and 5, 1M HCl was added to MRS broth separately and to increase the pH 1M NaOH was used. pH meter (HANNAH pH 201, Wisconsin, RI, USA) was used to adjust the pH. Agar well diffusion method was used to check the antimicrobial activity of the CFS prepared from media with different pH values against the test organisms.

Effect of incubation period on production of bacteriocin or bacteriocin like substances

Three separate conical flasks containing 50 ml sterilized MRS broth was inoculated with 100 µl suspended fresh culture of

L. casei followed by incubation at 37°C for 24, 48 and 72 hours, respectively. Effect of incubation period on the antagonistic activity of CFS was observed against test organisms following agar well diffusion method.

Results

Inhibitory activity of CFS against multi-drug resistant pathogenic bacteria

In our study CFS from *L. casei* grown in MRS broth media was used to examine antimicrobial activity by agar well diffusion method against eight multi drug resistant pathogenic bacteria (*S. aureus*, *K. pneumoniae*, *P. vulgaris*, *Shigella sp.*, *Salmonella sp.*, *Pseudomonas sp.*, *E. coli* and *B. cereus*) isolated in a previous study conducted in our laboratory³¹. These organisms showed multidrug resistance and biofilm forming capabilities in catheters of urinary tract infected patients. CFS was found to exhibit inhibitory activity against all the test organisms as shown in Table 1. Among the isolates, *S. aureus* and *Pseudomonas sp.* were found to show higher susceptibility to CFS as compared to other test organisms. Both Gram negative and Gram positive pathogens were found to produce zone of inhibition around the wells containing CFS. The results indicated that *L. casei* used in our study produces antibacterial substances.

Table 1: Inhibitory activity of CFS from *Lactobacillus casei* against pathogenic bacteria determined by agar well diffusion method. The experiment was performed at least three times maintaining triplicates for each isolate. Zone of inhibition was measured and analyzed to determine mean and standard deviation (SD).

Isolates	Zone of inhibition (diameter in mm, Mean ± SD)
<i>S. aureus</i>	21± 1.5
<i>K. pneumoniae</i>	19± 2.5
<i>P. vulgaris</i>	19 ± 1.0
<i>Salmonella sp.</i>	17± 1.5
<i>Shigella sp.</i>	18± 1.5
<i>Pseudomonas sp.</i>	21 ± 1.0
<i>B. cereus</i>	15 ± 1.0
<i>E. coli</i>	17± 0.6

Characterization of the inhibitory substance(s) present in CFS

Lactic acid bacteria are known to produce organic acids and hydrogen peroxide to inhibit other competing microorganisms²⁶. Both organic acids and hydrogen peroxide have antibacterial property and therefore these metabolites were neutralized to exclude the possibility that the antibacterial activity exhibited by CFS is not due to these antibacterial metabolites present in the CFS. CFS was treated first with NaOH to neutralize organic acids, followed by treatment with both catalase and NaOH to neutralize possible combined inhibitory actions by organic acids and hydrogen peroxide. No zone of inhibition was found around the well

containing CFS neutralized with NaOH and catalase in case of *Shigella sp.*, *Salmonella sp.*, *Pseudomonas sp.*, *E. coli*, and *B. cereus* (Figure 1). Out of eight isolates, only three isolates (*P. vulgaris*, *S. aureus*, *K. pneumoniae*) were found to be sensitive to CFS after treating with NaOH, but the activity of CFS significantly decreased after NaOH treatment. Further reduction of antagonistic activity was observed after treatment with catalase. Finally, proteins in the CFS were denatured by heating at 121°C for 15 min at 15 psi. The

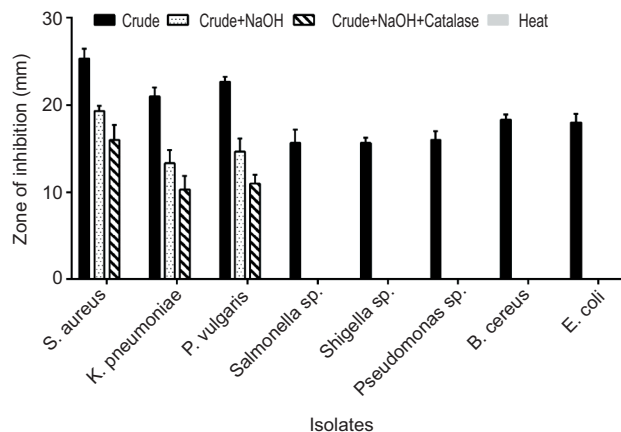


Figure 1. Zone of inhibition (in mm) by crude CFS, NaOH treated, both NaOH and Catalase treated and heat treated CFS against test organisms observed after incubation at 37°C for 24 hrs. in seeded Mueller Hinton agar plates (100% CFS in each agar well). The experiment was done in triplicate for each isolate to obtain mean and standard deviation (SD).

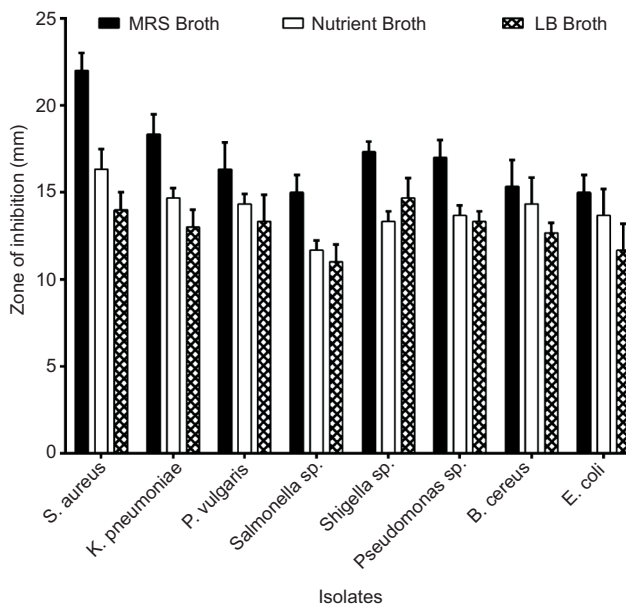


Figure 2. Comparison between the antibacterial activities (Zone of inhibition in mm) of CFS from *L. casei* cultured in different media observed after incubation at 37°C for 24 hrs. in seeded Mueller Hinton agar plates (100% CFS in each agar well). The experiment was done three times for each isolate to obtain mean and standard deviation (SD).

antagonistic activity was completely destroyed against all eight isolates after heat treatment indicating that the inhibitory substances in the CFS are proteinaceous in nature (Figures 1). Based on these results we conclude that the inhibitory substances present in the CFS are proteinaceous in nature.

Effect of culture media, incubation temperature, incubation period and pH on antibacterial activity of the bacteriocin like substances

The CFS was further investigated to determine if culture media, incubation temperature, incubation period, and pH influence its antibacterial activity. It was found that CFS from MRS broth exhibited maximum inhibitory activity among the three media used in this study (Figure 2). CFS obtained from various temperature conditions were used against the selected organisms. CFS from the culture of *L. casei* incubated at 37°C exhibited the highest inhibitory activity among the temperatures examined in this study (Figure 3). The zone of inhibition gradually increased with the decrease of pH and highest antibacterial activity was observed at pH 4.0 among the pH values examined in this study (Figure 4). We observed significant increase of inhibitory activity at pH 4.0 as compared to that of pH 6.0 against *S. aureus*, *K. pneumoniae*, *P. vulgaris*, *Salmonella sp.* and *Shigella sp.* Incubation period also affected the inhibitory activity. Optimum inhibitory activity of the crude supernatant was observed after 72 hours as judged by the zones of inhibition against the MDR bacteria (Figure 5). Significant increase of inhibitory activity was observed against *S. aureus*, *K. pneumoniae* and *P. vulgaris* at 72 hours as compared to those at 24 hours incubation (Figure 5). The results suggest that the inhibitory activity of bacteriocin like substances varies significantly with different cultural conditions like media, incubation temperature, pH and incubation time.

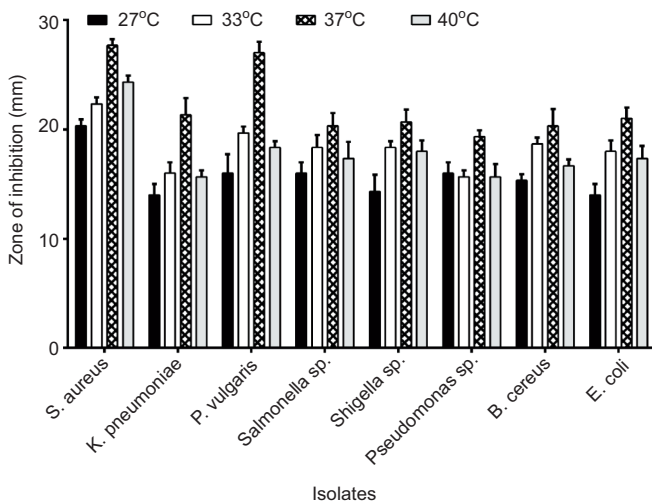


Figure 3. Comparison between the antibacterial activities (Zone of inhibition in mm) of CFS from *L. casei* cultured at different incubation temperature. Zones of inhibition were observed following incubation at 37°C for 24 hrs. in seeded Mueller Hinton agar plates (100% CFS in each agar well). This experiment was done three times for each isolate to obtain mean and standard deviation (SD).

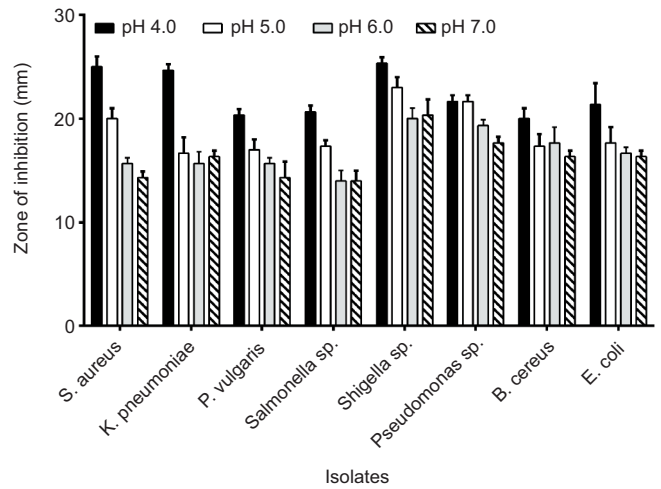


Figure 4. Comparison between the antibacterial activities of CFS (Zone of inhibition in mm) from *L. casei* cultured at different pH against eight isolates. Zones of inhibition were observed following incubation at 37°C for 24 hrs. in seeded Mueller Hinton agar plates (100% CFS in each agar well). The experiment was done in triplicate for each isolate to obtain mean and standard deviation (SD).

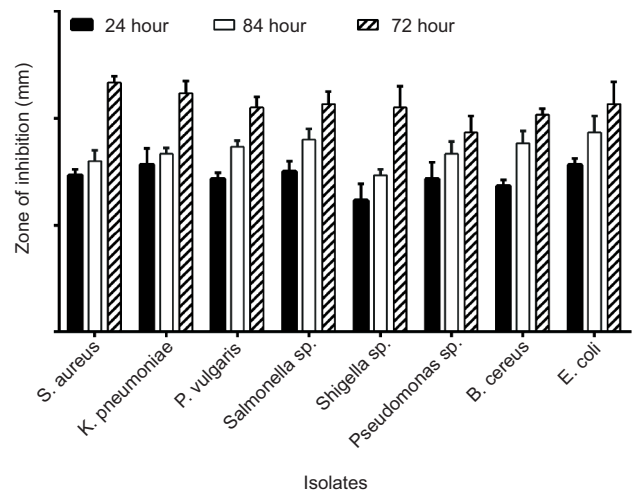


Figure 5. Comparison between the antibacterial activities of CFS (Zone of inhibition in mm) from *L. casei* cultured at different incubation period against eight isolates. Zones of inhibition were observed following incubation at 37°C for 24 hrs. in seeded Mueller Hinton agar plates (100% CFS in each agar well). This experiment was done in triplicate for each isolate to obtain mean and standard deviation (SD).

Discussion

We report here that bacteriocin like substances from *L. casei* can inhibit multidrug resistant bacteria isolated from urinary catheters. In addition to bacteriocin like substances, *L. casei* also produces other metabolites such as organic acids, and H₂O₂, which are inhibitory to other bacteria. Neutralization of these metabolites in the CFS led to reduced spectrum of inhibitory activity. After neutralization, five out of eight bacterial isolates (*Shigella sp.*,

Salmonella sp., *Pseudomonas sp.*, *E. coli* and *B. cereus*) exhibited antibacterial activity. Three bacterial strains (*P. vulgaris*, *S. aureus*, and *K. pneumoniae*) exhibited no sensitivity to the neutralized CFS. Complete absence of antibacterial activity after heat treatment indicates that the antibacterial activity was due to proteinaceous substances, which were denatured or degraded after heat treatment. As bacteriocins are protein in nature, antibacterial activity of CFS can be attributed to the activity of bacteriocin or bacteriocin like substances^{27, 28}.

Optimum temperature for bacteriocin production from *Lactobacillus sp.*, *L. lactis*, *L. fermentum*, *L. casei* and *L. plantarum* was reported to be 30°C²². It was also reported that zone of inhibition decreases with the increase of temperature above 30°C. However, Scolari *et al.* reported that *L. casei* and *Lactobacillus salivarius* CRL 1328 produce bacteriocin optimally at 37°C^{29,30}, which is consistent with our results. The discrepancy between our results and that of others is due to differences in strain background used. pH of the media was found to be important for antibacterial activity. For example, we observed decreased inhibitory activity with the increase of pH, and the lowest activity was observed at pH 7.0, which is consistent with the study of Tarelli *et al.* in which they reported that maximum bacteriocin production was found at pH 4.0 to 5.0³¹. This pH tolerance is an extremely important feature since the isolates have the ability to survive, grow and produce bacteriocins under acidic and alkaline conditions. Optimum antibacterial activity of the CFS was observed after incubation of the *L. casei* for 72 hours. This result is consistent with the report of Onwuakor *et al.* and Karaoglu *et al.* which showed that incubation time affects bacteriocin production^{22, 32}.

We observed that five out of eight bacterial isolates were sensitive to bacteriocin like substances. We also observed that the bacteriocin like substance can inhibit both Gram positive (*S. aureus*) and Gram negative bacteria (*P. vulgaris* and *K. pneumoniae*). This indicates that the target of this bacteriocin like substances are specific. Further investigation will provide the mode of action and the spectrum of bacterial species that can be inhibited.

Conclusion

Our results indicate that multi-drug resistant pathogenic bacteria can be inhibited by antibacterial substances from *L. casei*. The results also indicate that the antimicrobial substances responsible for inhibiting the bacterial pathogens are bacteriocin like substances (BLS). We found that different cultural conditions like types of media, incubation temperature, pH and incubation period had significant effects on the degree of inhibitory activity shown by bacteriocin like substances produced by *L. casei*. This is the first report showing inhibitory activity of bacteriocin like substances from *Lactobacillus casei* against multidrug resistant pathogens isolated from urinary catheters. Further investigation of these bacteriocin like substances might lead to an alternative of antibiotics against multi-drug resistant pathogenic bacteria.

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