Brazilian Journal of Microbiology (2010) 41: 643-648
ISSN 1517-8382

BACTERIOCINS PRODUCED BY *L. FERMENTUM* AND *L .ACIDOPHILUS* CAN INHIBIT CEPHALOSPORIN RESISTANT *E .COLI.*

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Submitted: September 05, 2009; Approved: March 29, 2010.

ABSTRACT

Reemerging infections occur due to resistant bacteria. Such infections create restrictions for clinicians and microbiologists in drug selection. Such problems demand new strategies for solution. Use of bacteriocins for this purpose may be fruitful. In the present research work, the inhibitory effects of bactericins on cephalosporin resistant *Escherichia coli* are used as model system for the control of antibiotic resistant pathogenic bacteria. Cephalosporin resistant *Escherichia coli* strain was isolated from pus by using conventional methodology. For bacteriocin production, *Lactobacilli* strains were selected by using selective media. Out of seventy two strains isolated from yogurt, fecal materials of human, chick, parrot and cat, only two strains (strain 45 and strain 52) were found to produce bacteriocins having antimicrobial potential against cephalosporin resistant *Escherichia coli.* Biochemical characterization showed that strain 45 belonged to group of *Lactobacillus fermentum* and strain 52 to *Lactobacillus acidophilus*. Both strains showed maximum growth at 25°C and 35°C respectively. Suitable pH was 5.5 and 6.0 for *Lactobacillus fermentum* and *Lactobacillus acidophilus* respectively. Bacteriocins produced by both strains were found stable at 50, 75 and 100°C for 60min. Function of bacteriocin was also not disturbed due to change in pH. These findings suggest that bacteriocin produced by *Lactobacillus fermentum* and *Lactobacillus acidophilus* can be used for the infection control of cephalosporin resistant *Escherichia coli.*

Key words: Cephalosporin resistant *Escherichia coli,* Bacteriocin, Antibiotic resistance, *Lactobacillus fermentum, Lactobacillus acidophilus*

INTRODUCTION

Discovery of antibiotics revolutionized the world of medicine. Increased rate of morbidity and mortality due to epidemics came into control. As a result, average life span increased for human beings. With the passage of time, non judicious use of antibiotics became common practice resulting in the reemerging infections. For solution, the derivatives of antibiotics were discovered and applied but pathogenic bacteria developed the resistance against them also. Now it was the need of time to search for new strategies for infection control of resistant pathogenic bacteria (10). *Lactobacillus* group of bacteria is famous for its uses as probiotic and food preservative. It has won this prestige due to its production of inhibitory compounds such as organic acids, hydrogen peroxides, diacetyl and bacteriocins. Bacteriocins are the ribosomally produced cationic proteins inhibiting other bacteria living in the same ecological niche. So, lactobacilli use it as weapon for its survival (2, 15).

Bacteriocins have withdrawn special interest of

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microbiologists for the control of pathogenic bacteria (9). However, reports on the effects of *Lactobacilli* bacteriocins on antibiotic resistant pathogenic *Escherichia coli* have not yet appeared in the medical press. Pathogenic *Escherichia coli* is very common bacteria which causes infections like diarrhea and other extraintestinal infections (2).

In the present research project, lactobacilli strains producing bacteriocin effective against cephalosporin resistant *Escherichia coli* were isolated and characterized biochemically. Effects of pH and temperature for the promotion of growth of bacteria were also monitored. Bacteriocins were characterized in terms of sensitivity to pH and temperature also.

MATERIALS AND METHODS

Isolation and characterization of cephalosporin resistant *E. coli*

For the isolation of cephalosporin resistant bacterial strain, pus samples from 10 patients (Age group 35-50) were collected. Susceptility testing was performed against amikacin, augmentin, ampicillin, cephtazidime, ciprofloxacin, gentamycin, ceftriaxone, cefuroxime, imepenum, meropenem, cotrimoxazole and tazocin. Cephalosporin resistance potential was confirmed by double disc synergism test following Feizabadi and coworkers (7). Identification of the strain was completed following standard operating procedures (3, 11).

Isolation of *Lactobacillus* bacteria

Lactobacillus strains were isolated from the dilutions of 10^3 samples collected from yogurt, fecal material of chick, fecal material of parrot, fecal material of cat and fecal material of male healthy human. MRS agar plates were prepared and 50µl of each sample was inoculated on each plate following conventional method (5). These plates were incubated in anaerobic condition at 37°C for 48h to obtain isolates. Anaerobic condition was created by using anaerobic sachet (Sigma-Aldrich Chemie GmbH) commercially available. Lactobacillus strain was confirmed by using standard operating procedures (3, 11).

Screening of bacteriocin producer and its characterization

Bacteriocin producing bacteria were screened as previously described (12). The culture of bacteriocin producers was identified according to their morphological, and biochemical properties. The used tests were: Gram reaction; growth at 15, 25 and 45°C; production of catalase, cytochrome oxidase; acid and gas production from glucose; acid production from carbohydrates (1% w/v) lactose, sucrose, mannitol, ribose, sorbitol and mannose in MRS broth devoid of glucose and beef extract with phenol red as indicator.

Determination of optimum growth conditions

Bacterial strains showing most significant inhibitory zone against cephalosporin bacterial strain were selected for determination of optimum growth conditions. For determination of optimum temperature, 5 sets each of 3 test tubes were used for this study. In each tube 5ml of MRS medium was inoculated with 50µl of log phase growing bacterial cells and incubated at 15, 25, 35, 45 and 55°C for overnight. The bacterial growth was assessed by measuring absorbance at 600nm.

For ascertaining optimum pH, MRS broth was adjusted at various pH viz 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 in 3 sets of test tubes. The cultures were inoculated with log phase growing bacterial cells (50µl) and incubated at 37°C for overnight. Absorbance was noted at 600nm.

Determination of growth curve

For preparation of growth curves, 10 test tubes for each bacteriocin producing strain were prepared with MRS broth medium and inoculated with 50µl of log phase bacterial culture. The tubes were incubated in shaking incubator at 37°C and 150rpm. After every two hours the tubes were taken out and optical density (O.D) was noted at 600nm. Growth curves were prepared by plotting graph between time of incubation and O.D.

Effects of pH and temperature on bacteriocin

Bacteriocins were characterized by noting the effects of

heat and pH. Effects of heat were monitored by incubating the cell free supernatant containing bacteriocin at 50, 75 and 100°C in incubator for five minutes. Agar well diffusion test was performed to detect residual activity (8). Resistant culture supernatant was incubated at 100°C for 15, 30 and 60 min (9). pH effects on bacteriocin activity was analyzed by using HCl and NaOH for maintaining the pH of 3.0, 5.0, 7.0, 9.0 and 11.0. Agar well diffusion test was performed following Alpay and coworkers (1).

RESULTS

Cephalosporin resistant bacterial strain was isolated from pus culture of a 45 years old female. Gram staining, colonial morphology on Macconkeys, and positive results for indol, EMB agar, TSI and other biochemical test confirmed that the resistant strain is *E .coli* (3). Out of 72 gram +ve, catalase and oxidase -ve, bacilli isolates, only two strains i-e strain 45 and strain 52 were found to produce effective bacteriocin against cephalosporin resistant *E. coli.* Strain 45 was isolated from fecal material of chick and strain 52 was isolated from fecal material of cat. Biochemical characterization showed that strain 45 belonged to *Lactobacillus fermentum* and strain 52 belonged to *Lactobacillus acidophilus.* There was prominent difference between the sizes of zones of inhibitions produced by *L. fermentum* and *L. acidophilus.* The zone of inhibition due to bacteriocin produced by *L. fermentum* was larger as compared to that of *L .acidophilus* (Figure 1). The growth of *L. fermentum* was maximum at 25°C and pH value equal to 5.5 (Figure 2 and 3). For *L. acidophilus,* optimum temperature for growth was 35°C and pH was 6.0 (Figure 2 and 3). Figure 4 indicates the growth curves of both strains. Bacteriocins of both strains were found stable on all temperature and pH ranges under observation (Table 1).

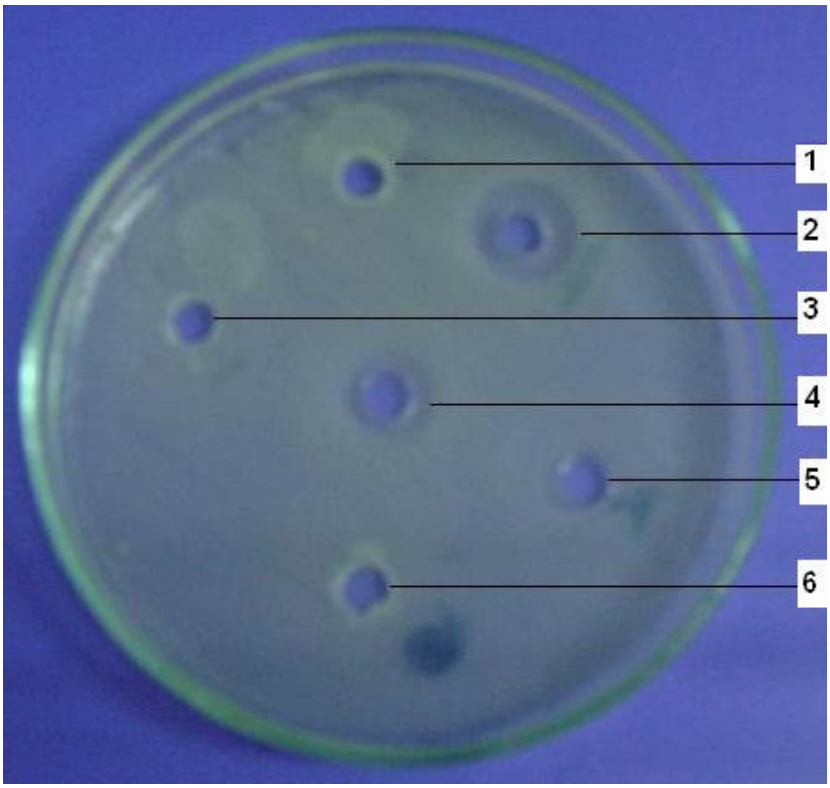

Figure 1: Well no. 1, 3, 5 and 6 indicates that cell free supernatant of strains have no antimicrobial activity against cephalosporin resistant *E. coli.* Cell free supernatants in well 2 and well 4 produce zone of inhibitions against test strain. Well 2 carries cell free supernatant of strain 45 and well 4 have cell free supernatant of strain 52. Well 1, 3, 5 and 6 keep cell free supernatants of other *Lactobacilli strains.*

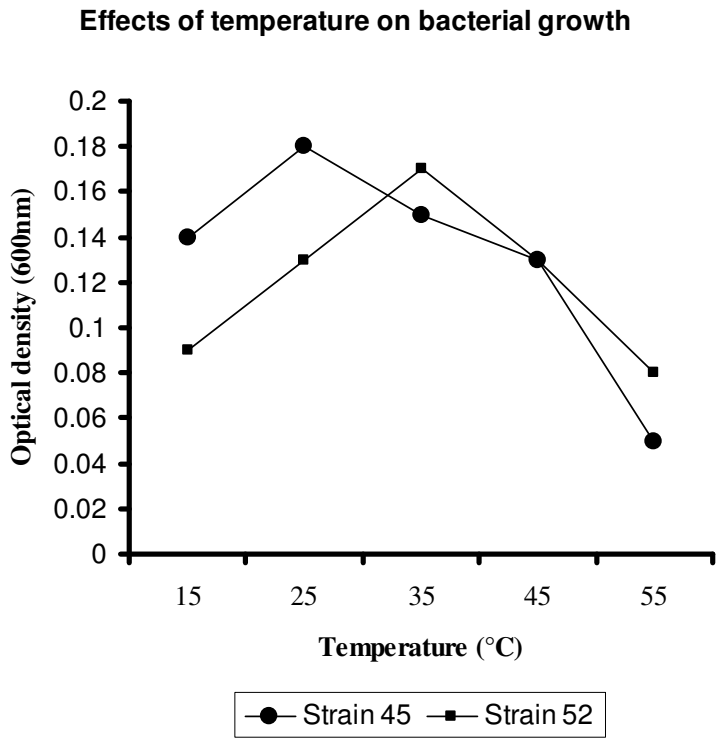

Figure 2. Effect of temperature on growth of strains 45 and 52.

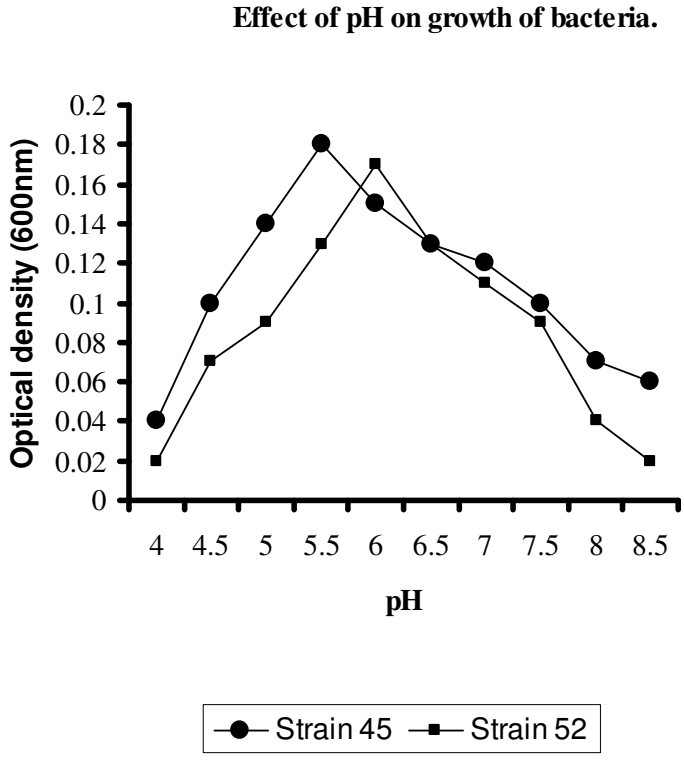

Figure 3. Effect of pH on growth of strains 45 and 52.

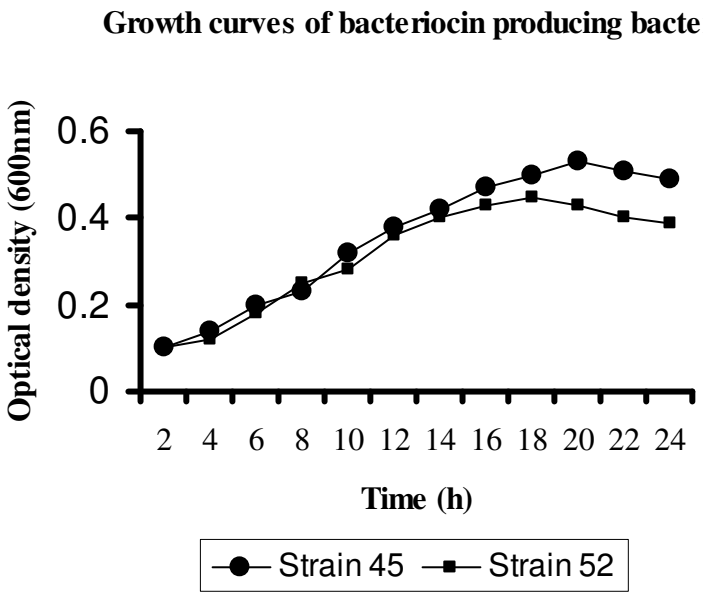

Figure 4. Growth curves of strains 45 and 52.

Table 1. Sensitivity of bacteriocins to heat and different pH values

Bacterial Strains	Resistance to heating (5min) Temperature (°C)			Resistance to boiling (min)			Sensitivity to different pH values				
	50	75	100	15	30	60	3	5	7	9	11
L. fermentum	R*	R	R	R	R	R	R	R	R	R	R
L. acidophilus	R	R	R	R	R	R	R	R	R	R	R

*R: Resistant

DISCUSSION

The present article deals with the application of bacteriocins against cephalosporin resistant *E. coli*. The rationale behind is the production of this vital antimicrobial agent by *Lactobacilli* strains against different pathogenic strains of *E. coli* (6). Antimicrobial activity of bacteriocins was thought to be broad spectrum. But Ogunbanwo et al. proved that bacteriocins produced by a group are not effective against its own group member (13). So, it is important to screen effective bactericin against specific pathogenic bacteria. Two different strains i-e strain 45 and strain 52 were obtained to produce effective bacteriocin against cephalosporin resistant *E. coli.* Members of both groups have already been reported to produce bacteriocins effective against pathogenic bacteria. For instance, in a different research project, we obtained bacteriocin of *L. fermentum* having antimicrobial potential against methicillin resistant *Staphylococcus aureus* (12). In the same way, some other researchers observed inhibition of pathogenic *Salmonella* and *Escherichia* by the bacteriocin of *L. acidophilus* (4, 14). The sensitivity of gram negative bacteria to bacteriocins produced by lactic acid bacteria is not common. Previous finding of positive results for inhibitory action of bacteriocin produced by *Lactobacilli* on gram negative bacterium is consistent with the present observation. The noteworthy finding of the present project was the difference in the sizes produced by these bacteriocins. Inhibitory zone produced by *L. fermentum* was larger as compared to that of *L.*

acidophilus. Different reactions of the cephalosporin resistant *E. coli* to different bacteriocin indicate the different mode of actions of bacteriocins. This difference points out the presence of weak neutralization mechanism in cephalosoporin resistant *E. coli* for bacteriocin of *L. acidophilus*. For bacteriocin of *L.fermentum*, such neutralization mechanism can not be predicted due to presence of clear zone of inhibition.

Experiments related to the optimization show that both strains can be grown easily in lab and industry level due to survival in broad range of temperatures and pH. Different bacteriocins behave in different manner on exposure of different temperatures and pH values. For example, bacteriocins of vaginal *Lactobacilli* strains are stable between pH range of 4.5 to 7.0 but sensitive to pH 9 (1). Similarly, bacterocins of *L. acidophillus* and *L. bulgaricus* are stable at pH range of 3-10 (9). Conflictingly, bacteriocin produced by *L. helveticus* is stable at pH range of 3-9 but sensitive to pH 10 (9). In our experiment, bacteriocins from both strains were found stable at all pH ranges. Sensitivity of bacteriocins to different temperature ranges is also helpful in its characterization. For example, bacteriocins of *L. acidophillus, L. bulgaricus* and *L. helveticus* remain stable at 50, 70 and 80°C. But at 100°C, only bacteriocins of *L. acidophillus* and *L. bulgaricus* remain effective (9). In our experiments, both the bacteriocins remained stable at all temperature ranges. It can be concluded from the study that bacteriocins of *L. fermentum* and *L. acidophilus* can be tested for the treatment of infections caused by cephalosporin resistant *E. coli.* This was a preliminary but directional study which leads towards this strategy as therapy option. On the basis of its findings, advance research project can be initiated for the control of antibiotic resistant infections.

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