Antagonistic Potential of *Lactobacillus* Spp against Enteropathogenic Bacteria; Purification and Characterization of their Bacteriocins

Asha and D. Gayathri
Department of Microbiology, Davanagere University, Davanagere-577002, India

Abstract: In the present study, *Lactobacillus* (160) isolates were isolated from curd sample. The isolates were aimed to analyze the antibacterial potential against *Escherichia coli*, *Vibrio cholerae* sub sp., *ogawa*, *V. cholerae* sub sp., *inaba*, Klebsiella sp., Proteus sp. and *Shigella dysenteriae*. All the isolates were inhibiting the tested Enteropathogenic bacteria except *S. dysenteriae*. *Lactobacillus* isolates produced highest inhibition zone (30 to 37 mm) against *V. cholerae* sub sp., *inaba* and Klebsiella sp., of the 160 isolates only ten *Lactobacillus* isolates (L1-L10) were used for the production of bacteriocins, purified by ammonium sulphate precipitation and ion exchange (DEAE cellulose) chromatography. Maximum bacteriocin activity has been observed with Lf3 against *V. cholerae* spp *Inaba* at 30°C, pH 6.0, 1.5 to 2.0% Na Cl/18 h in addition to L8, L9 and L10 (MW 100 to 106 KDa) and Lf3 was found to be the most prominent potential isolate.

Keywords: Bacteriocins, enteropathogens, inhibition zone, *Lactobacillus fermentum*, *Lactobacillus plantarum*, SDS-PAGE

INTRODUCTION

Probiotics are the microorganisms which when administered in a required amount confer a health benefit on the host (FAO/WHO, 2002). Among the numerous intestinal bacteria that affect beneficially to the host intestine could be selected as probiotics. The major aerobic probiotics group includes *Lactobacillus* spp such as, *Lactobacillus acidophilus*, *L. casei*, *L. plantarum*, *L. reuteri*, *L. fermentum* and *L. rhamnosus* and others. Various studies have been indicated that the *Lactobacillus* spp have a positive influence on the intestinal flora of humans, alleviated lactose intolerance, have hypercholesteremic effects, stimulate immunity and ant colon cancer effects, prevent Crohn’s and candidacies infection in addition to diarrhea and infantile diarrhea (Pant et al., 1996) pseudo membranous colitis and ant allergic effects (Vanderhoof, 2001).

*Lactobacilli* with potential in inhibiting many zoonotic, fish, poultry enteroto and common porcine pathogens have been reported (Hacin et al., 2008). Antimicrobial activity of *L. crispatus* and *L. amylovorus* against *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* and *Clostridium perfringens* was due to the production of hydrogen peroxide and metabolites’ other than organic acids. Additionally, the spent culture supernatant of *L. fermentum* isolated from swine and poultry showed antagonistic activity against *E. coli*, *Salmonella* sp., *Shigella sonnei* and some enterotoxigenic *S. aureus* (Lin et al., 2007). Further, antimicrobial activity of *L. brevis*, *Enterobacter faecium*, *Padicoccus acidolactis* from urinary tract of healthy children against uropathogens such as, *E. coli*, *S. saprophyticus*, *E. cloacae*, *Pseudomonas aeruginosa* and *B. anthracis* was also observed (Lim et al., 2009). Further, *L. rhamnosus*, *L. gasseri*, *L. casei* and *L. plantarum* adhered to colon epithelial cell lines and inhibited the enter hemorrhagic *E. coli* in vitro (Hirano et al., 2003). In addition, *L. fermentum* inhibited the adhesion of enterotoxigenic *E. coli* releasing a compound with the molecular weight approximately 1700 kDa (Ouwehand and Conway, 2008). Although, Lactobacilli are known for antibacterial potential and their bacteriocins were evaluated, comparison of the antibacterial potential of lactobacilli bacteriocin with whole cell bacteria is scarce. In present study, the antibacterial of potential *Lactobacillus* isolates (isolated from malnad districts of Karnataka, India) against Enteropathogenic bacteria was evaluated with their bacteriocins.

MATERIALS AND METHODS

Samples:
*Curd and stool samples*: The curd/stool samples were collected from most remote regions of malnad districts (Shivamogga and Chikkamagalure) of Karnataka, India.

Isolation and identification of *Lactobacillus* spp: The samples were serially diluted and pour plated on MRS (de Man, Rogosa, Shrape) selective medium and...
Enteropathogenic bacteria: Intestinal pathogens viz., Escherichia coli, Proteus sp., Vibrio cholerae ssp ogava, Vibrio cholerae ssp inaba, Shigella dysenteriae and Klebsiella sp., were kindly supplied by the Department of Microbiology, JJM Medical College and Davangere, India.

Optimization of culture conditions: The Lactobacillus isolates were subjected to different culture conditions to derive the optimum conditions for maximum bacteriocin production. Growth and bacteriocin production were estimated at various temperatures (20, 25, 30, 35, 40 and 45°C, respectively), pH (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0, respectively), sodium chloride (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0%, respectively) and incubation period (6, 12, 18, 24, 30, 36, 42 and 48 h, respectively). Samples were collected after 48 h (except for incubation time effect) and examined for bacteriocin production (µg/mL).

Purification of bacteriocin: The crude bacteriocin was precipitated with 80% ammonium sulfate saturation. The precipitate was dialyzed against 20 mm potassium phosphate buffer (pH 7.0) for 12 h at 4°C. Further, purification was carried out in ion exchange chromatography (DEAE-Cellulose). The dialyzed protein was applied to a DEAE-Cellulose A-50 column (20×60 mm), pre-equilibrated with 20 mM potassium phosphate buffer (pH 7.0). Columns were washed with 3 vol. of equilibrated buffer. Bound proteins were eluted stepwise using phosphate buffers of increasing molarity and decreased pH values at room temperature. The flow was adjusted to 24 mL/h and fractions (1 mL each) were collected. OD of elutes were measured using UV spectrometer (280 nm). Protein concentration of bacteriocin in the supernatant was determined (Lowry et al., 1951).

Antibacterial activity of bacteriocin: Bacteriocin (0.1 µL) disc was prepared using standard methods as described above and placed at the centre of lawn culture plate of test Enteropathogenic bacteria.

SDS-PAGE: The molecular weight of the bacteriocin (L1-L10) was determined using standard protein markers (18-215 KDa) (Genei, Bangalore, India) using 15% gel in a mini gel electrophoresis unit by SDS-PAGE.

RESULTS

A total of 160 Lactobacillus isolates (150 species from curd and 10 species isolated stool) were used for the present study. An antibiogram pattern against potential intestinal pathogens using Lactobacillus sp., was determined (Table 1 and Fig. 1). Ten isolates (L1-L10) were chosen based on maximum inhibition zone production against Escherichia coli (Fig. 2), Proteus sp., Vibrio cholerae ssp ogava, Vibrio cholerae ssp inaba, Shigella dysenteriae and Klebsiella sp., for further study. Among the tested isolates 1.6% of Lactobacillus isolates (isolated from curd sample) showed highest inhibition (37 mm) against Vibrio cholerae ssp inaba and Klebsiella sp., while, none of the Lactobacillus isolates isolated from stool samples produced high zone of inhibition (Table 2). Inhibition zone produced by L. fermentum (L6) was maximum of 15 mm against S. dysenteriae and L. bulgaricus (NCIM) could able to produce maximum of 15 mm of zone inhibition against V. cholera inaba.

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incubation period. All the isolates produced highest bacteriocin activity at 30°C, pH 6, 1.5-2% NaCl and incubation period of about 18 h (Fig 3, 4, 5 and 6) and their inhibition patterns against enteric bacteria were determined. All ten isolates showed an inhibition zone ranged between 12-15 mm except Lf3, which showed 25 mm diameter inhibition zone (Fig. 1). At low (4.0) or high pH (9.0), at high temperature (45°C), at low sodium chloride concentration (0.5%) or high sodium chloride concentration (3.0%), in less incubation period (6 h) and more incubation period (48 h), no inhibition was observed (Fig. 3, 4, 5 and 6). The concentration of bacteriocin ranged between 230-440 µg/mL (Table 3).

**SDS-PAGE:** Molecular weight of the bacteriocin was determined using SDS-PAGE. Ten bacteriocins purified from potential Lactobacillus isolates showed almost similar banding pattern ranged between 100-110 KDa(100, 104, 106 and 110 KDa). A distinct variation in the banding pattern was observed in Lf3 isolate.
DISCUSSION

A total of 160 Lactobacillus isolates (150 from curd and 10 from stool sample) were used in the present study. All the isolates were subjected to antibiogram assay by disc diffusion method. Curd and stool sample have been collected from remote regions of malnad where, the curd sample were prepared by traditional method and maintained since a very long time. The people of the region were the regular consumers of such curd and they generally showed disease endurance particularly to gastrointestinal diseases and longevity. All the Lactobacillus isolates showed inhibition against tested enteropathogens. Lactobacillus sp., isolated from curd sample showed largest inhibition (37 mm) against enteropathogens when compared to the Lactobacillus sp. isolated from the stool sample (20 mm). Lactobacillus acidophilus, L. bulgaricus, L. plantarum, L. lactis and L. rhamnosus isolated from milk samples of buffalo, cow and goat showed antagonistic activity against E. coli, Enterobacter aerogenes, Klebsiella pneumoniae, Proteus vulgaris and Salmonella typhi by disc diffusion method and the inhibition produced varied between 15 to 24 mm (Tambekar et al., 2009). Lactobacillus sp., isolated from chicken intestine demonstrated inhibitory activity ranged from 12.5 to 18 mm against S. enteritidis, S. pullorum, S. typhimurium, S. blockley and three serotypes of E. coli and it was suggested that some organic compounds may be responsible for antagonistic activity (Jin et al., 1996). However, in the present study, a maximum inhibition zone of 37 mm has been produced by Lf3 against V. Cholerae ssp inaba indicating that the present isolate is more potential against tested intestinal pathogens perhaps having novel properties. Further, the result correlates the disease resistance among regular consumers of such curd of the region. In addition, Lactobacillus isolates obtained from stool samples of curd consumers showed average inhibition zone (10-25 mm) against tested Enteropathogenic bacteria than curd Lactobacillus isolates. This may indicate that the stool isolates perhaps have lost some of the molecular factors in the intestine which are required for antibacterial activity as defense mechanism. Therefore, it may be suggested that regular supplement of these Lactobacillus isolates are required to maintain better gastrointestinal system.

Further, Lactobacillus isolates (L1-L10) showing highest zone of inhibition against Enteropathogenic bacteria was chosen for bacteriocin production. The bacteriocin production was optimized by various physiochemical conditions like temperature, pH, NaCl concentration and incubation period. Among the various bacteriocins isolated of Lactobacillus sp., Lf3 showed a maximum inhibition zone of 25 mm against V. Cholerae ssp inaba at 30°C/pH6/1.5-2% NaCl/18h. Similarly, supernatants of L. brevis, E. faecium, Pediococcus acidilactici had produced inhibition zone ranged between 18 to 24 mm against urotoxigenic E. coli, S. saprophyticus, Citrobacter freundii, P. vulgaris, E. cloacae, P. aeruginosa and B. anthracis (Lim et al., 2009). Further, a mixture of supernatant of L. casei and L. acidophilus showed antimicrobial activity ranged between 8 to 18 mm against S. sonnei. Bacteriocins from Lactobacillus lactis ssp cremoris isolated from kefir grains which inhibited food spoilage bacteria such as, E. coli, Pseudomonas sp., S. aureus, Bacillus cereus, K. pneumoniae, Proteus sp., Clostridium botulinum, fecal Streptococci and Salmonella sp. and the inhibition zone varied from 11-36 mm (Raja et al., 2009). In addition, bacteriocins of L. lactis showed inhibitory effect against B. subtilis, B. megaterium, B. cereus, S. aureus, Enterococcus faecalis, E. coli, P. aeruginosa, S. shiga, S. dysenteriae and S. boydii (Rajaram et al., 2010). Although, their group obtained a maximum inhibition zone of 23 mm,
in the present study Lf3 showed higher inhibition zone of 25 mm size with 440 μg/mL of protein indicating that the isolate (Lf3) could be a potential candidate to be a probiotic. However, LB, against VCI produced an inhibition zone 15 mm, with equal abundance of 440 μg/mL of protein as Lf3, their potential was lesser compared to Lf3. Furthermore, L8, L9 and L10 also showed a notable inhibitory zone. L8 showed 15 mm of an inhibition zone against E. coli, VCO and Proteus while L9 and L10 against Klebsiella. This may indicate that, in addition to Lf3, L8, L9 and L10 are also potential.

Although bacteriocins were purified by subjecting the crude bacteriocin to get precipitated by ammonium sulfate saturation, dialyzed and subjected to ion exchange chromatography (DEAE-Cellulose) and stepwise protein elution on SDS-PAGE, more than one protein band was obtained on 15% separating gel from L1 to L10 and LF, LB. SDS-PAGE resolved the 3-4 protein bands in all ten isolates; however, their intensity was varied. Furthermore, Lf3 produced high intensity protein band of 104 KDa when compared to the others indicating that the Lf3 bacteriocin may quantitatively affected the intestinal pathogens. Rajaram et al. (2010) obtained 94 KDa protein band of bacteriocin on SDS-PAGE. Another research group (Karthikeyan and Santosh, 2009) obtained bacteriocin of 2.5 KDa from L. plantarum. Pant, A.R., S.M. Graham, S.J. Allen, S. Harikul, A. Sabehareon, L. Cuevasand and C.A. Hart, 1996. Lactobacillus GG and acute diarrhoea in young children in the tropics. J. Trop. Pediatri., 42: 162-165.

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