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

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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* ssp *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. renetri*, *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 hypercholesteromic 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 entero 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

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characterized (Asha *et al*., 2012; Kandler and Weiss, 1986). For comparison *L. fermentum* (NCIM 2166) and *L. bulgaricus* (NCIM 2671) were obtained from NCIM, Pune, India.

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.

Antimicrobial activity: Antimicrobial activity of *Lactobacillus* sp., against intestinal bacteria (*Escherichia coli*, *Vibrio cholerae* sub sp., *ogawa*, *V. cholerae* sub sp., *inaba*, *Klebsiella* sp. and *Proteus* sp., and *Shigella dysenteriae*) was performed by agar disc diffusion technique (Jin *et al*., 1996) with modifications.

Agar disc diffusion technique:

Preparation of *Lactobacillus* suspension: *Lactobacillus* isolates were sub cultured on MRS broth and incubated overnight at 37°C for 24 h, resuspended in MRS broth and 0.1 µL (10^6/mL) of the *Lactobacillus* suspension was used to examine the antimicrobial activity against test Enteropathogenic bacteria.

Preparation of enteropathogen suspension: *Klebsiella* sp. and *S. dysenteriae* was sub cultured on nutrient agar, *E. coli* on EMB agar media, *V. cholerae* ssp *ogava*, *V. cholerae* ssp *Inaba* on TCBS slants and further incubated in nutrient broth at 37°C/24 h (10^6/mL).

Antimicrobial activity assay: (0.1 µL) of overnight suspension of the test organisms (enteropathogens) was swabbed on nutrient agar plates. (0.1 µL) of *Lactobacillus* suspension was placed onto the filter paper disc (6 mm) and placed at the centre of lawn culture of enter bacteria and incubated at 37°C for 24 h.

Production of crude bacteriocin: Out of 160 isolates only ten *Lactobacillus* isolates (L1 to L10) were chosen for bacteriocin production based on high antimicrobial activity and inoculated to MRS broth, maintained an aerobically, centrifuged (10,000 g/15 min/4°C). The cell free supernatant was adjusted to pH 6.0 using 1N Na OH and it was used as crude bacteriocin (Rajaram *et al*., 2010).

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* (Lf3) 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*.

Crude bacteriocin was produced from ten *Lactobacillus* isolates and was optimized at various temperature, pH and sodium chloride concentration and

Table 1: Antibiogram of enteric bacteria against *Lactobacillus* sp., isolated from curd sample

Lactobacillus sp. isolated from curd sample	*E. coli* (%)	*V. cholerae* ssp *Inaba* (%)	*V. cholerae* ssp *ogava* (%)	*Proteus* sp. (%)	*Klebsiella* sp. (%)	*Shigella dysenteriae* (%)
Low inhibition (5-10 mm)	58	72	72	39	77	43
Average inhibition (10-25 mm)	18	9	14	55	28	Nil
Large inhibition (25-37 mm)	1.25	1.6	Nil	1.6	1.6	Nil

Nil: No inhibition zone

Table 2: Antibiogram of enteric bacteria against *Lactobacillus* sp., isolated from fecal sample

Lactobacillus sp. isolated from curd sample	*E. coli* (%)	*V. cholerae* ssp *Inaba* (%)	*V. cholerae* ssp *ogava* (%)	*Proteus* sp. (%)	*Klebsiella* sp. (%)	*Shigella dysenteriae* (%)
Low inhibition (5-10 mm)	40	50	20	30	70	20
Average inhibition (10-25 mm)	10	10	10	40	10	Nil
Large inhibition (25-37 mm)	Nil	Nil	Nil	Nil	Nil	Nil

Nil: No inhibition zone

Fig. 1: Highest inhibition produced by the *Lactobacillus* sp., *Klebsiella* sp

Fig. 2: Inhibition zone produced by L3 bacteriocin against *E. coli*

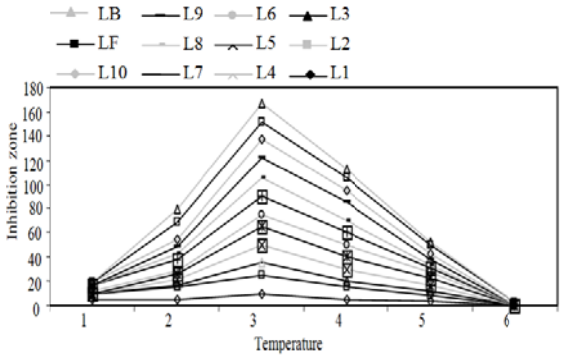

Fig. 3: Effect of temperature on bacteriocin activity
1: 20°C; 2: 25°C; 3: 30°C; 4: 35°C; 5: 40°C; 6: 45°C; L1: Lf1; L2: Lf2; L3: Lf3; L4: Lp1; L5: Lp2; LF: *L. fermentum*; LB: *L. bulgaricus*

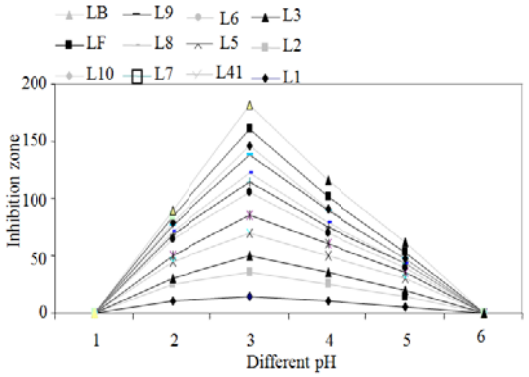

Fig. 4: Effect of pH on bacteriocin activity
1: pH 4.0; 2: pH 5.0; 3: pH 6.0; 4: pH 7.0; 5: pH 8.0; 6: pH 9.0; L1: Lf1; L2: Lf2; L3: Lf3; L4: Lp1; L5: Lp2; LF: *L. fermentum*; LB: *L. bulgaricus*

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.

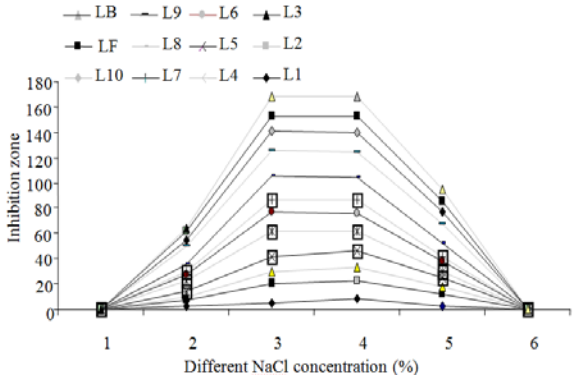

Fig. 5: Effect of sodium chloride on bacteriocin activity
1: 0.5; 2: 1.0; 3: 1.5; 4: 2.0; 5: 2.5; 6: 3.0; L1: Lf1; L2: Lf2; L3: Lf3; L4: Lp1; L5: Lp2; LF: *L. fermentum*; LB: *L. bulgaricus*

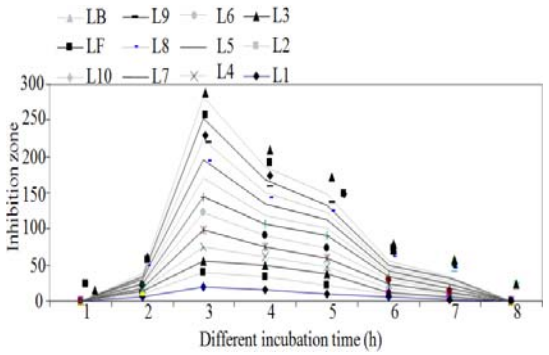

Fig. 6: Effect of incubation period on bacteriocin activity
1: 6; 2: 12; 3: 18; 4: 24; 5: 30; 6: 36; 7: 42; 8: 48; L1: Lf1; L2: Lf2; L3: Lf3; L4: Lp1; L5: Lp2; LF: *L. fermentum*; LB: *L. bulgaricus*

Table 3: A detailed representation of optimum temperature, pH, sodium chloride concentration and incubation period for high bacteriocin activity against enteropathogens

L1- *Lactobacillus fermentum* 1, L2- *Lactobacillus fermentum* 2,
L3- *Lactobacillus fermentum* 3, L4- *Lactobacillus plantarum*1,
L5- *Lactobacillus plantarum*2, L6- *Lactobacillus* sp6,
L7- *Lactobacillus* sp7, L8- *Lactobacillus* sp8, L9- *Lactobacillus* sp9,
L10- *Lactobacillus* sp10, Lf- *Lactobacillus fermentum*(NCIM),
LB- *Lactobacillus bulgaricus* (NCIM)

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^{o}C/pH6/1.5-2% NaCl/18h. Similarly, supernatants of *L. brevis*, *E. faecium*, *Pedicoccus acidilactici* had produced inhibition zone ranged between 18 to 24 mm against urotoxigenic *E. coli*, *S. saprophyticus*, *Citrobacter freudii*, *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. substilis*, *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/mLof 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* based on plasmid curing experiment. While in the present study 100, 104, 106 and 110 KDa were obtained, which may indicate that the involvement of multiple molecular factors as bacteriocins. Combinations of these proteins perhaps play a vital role against enteropathogens consequently reflecting the better gastrointestinal system. However, the antibacterial action of bacteriocins were lesser (25 mm) when compared to whole cells (37 mm) indicating that the whole cells were better in inhibiting entero pathogens. Lesser inhibitory activity of the bacteriocins may be due to; few of the antibacterial factors may not be present in the bacteriocin suspension. Therefore, in the present isolate (Lf3) was a potential isolate and could be supplemented orally as food/nutrient adjust. It has been found that the intimate association of *Lactobacillus* with the intestine in the healthy individuals would indicate that these bacteria fill the role as important to human health as does many nutrients rather than medicines.

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