PRODUCTION OF HEAT AND ALKALI STABLE BACTERIOCINS BY STRESS TOLERANT PROBIOTICS FROM FAECES

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Abstract: A total of six probiotic strains were isolated from faecal sample of infant and calf. These strains were analyzed and partially identified by morphological (Gram staining) and biochemical (catalase, indole production, methyl red, urease, citrate utilization and H$_2$S production) tests. All the isolates showed a broad inhibitory action against different pathogenic microorganisms including *Klebsiella pneumoniae* (zone of inhibition: 7-8mm), *Staphylococcus epidermidis* (7-8mm), *Pseudomonas aeruginosa* (7-8mm), *Escherichia coli* (8-9mm), *Enterococcus faecalis* (8-9mm) and *Serratia marcescens* (7-9mm) as exhibited by disc-diffusion method. The isolates were found to have great potential to resist a wide variety of commonly used antibiotics like amoxycillin/sulbactam, nalidixic acid, ciprofloxacin, penicillin G, norfloxacin, oxacillin, amoxycillin and ampicillin. The probiotic isolates were found to tolerate stresses like acid (pH 2.0, 3.0, 4.0, 5.0), bile salt (0.5%, 1.0%, 1.5%, 2.0% (w/v)), lysozyme (0.5 mg ml$^{-1}$), 0.4mM hydrogen peroxide (30% (w/v)) and NaCl (1.0%, 2.0%, 4.0%, 5.0% (w/v)), thus indicating their persistence under *in vivo* conditions. Almost negligible haemolytic activity indicated that these probiotics were safe for human health. Moreover, the bacteriocins produced by these probiotics exhibited prominent antimicrobial activity against all the test microorganisms. Some of these bacteriocins were also found to be highly stable as they retained their activity against *E. coli* even at high temperatures upto 121°C and under acidic as well as alkaline conditions (pH 1.5 to 9.0). Among all the probiotic strains examined, F3 and C1 and C2 possessed most of the desirable probiotic properties and can therefore claim their potential for applications in human health.

Keywords: Antagonistic, Bacteriocins, Haemolytic, Lysozyme, Probiotics

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INTRODUCTION

Probiotics are indigenous micro-flora of humans and colonize various parts of the body\(^1\text{--}^3\). The human intestinal microflora is complex with total counts of \(10^{11}\text{--}10^{12}\) bacteria per gram of stool\(^4,\)\(^5\). Most probiotics commercially available today belong to the genera *Lactobacillus* and *Bifidobacterium*\(^6\). Indeed, particular *Lactobacillus* strains have been found to be long-term residents of the intestinal tracts of some humans\(^7,\)\(^8\) and are usually spread throughout the gastrointestinal tract (GIT) of healthy humans\(^9\). This study emphasizes the characteristics and potential of probiotics isolated from faecal sample.

Probiotics are defined by the WHO/FAO group, as the live microorganisms which when administered in adequate amounts confer a health benefit on the host\(^10\). They are known to play an important role in the maintenance of human health especially against acute diarrhoea in childhood by stimulating the natural immunity, inhibiting the growth of harmful bacteria and increased resistance to infections. Probiotics thus contribute to the balance of microflora, mainly through competitive exclusion and antimicrobial activity against pathogenic bacteria\(^11,\)\(^12\). The acid and bile tolerance are two fundamental properties that indicate the ability of probiotic microorganisms to survive the passage through the gastrointestinal tract, resisting the acidic conditions of pH as low as 1.5 in the stomach and the bile acids at the beginning of the small intestine\(^13,\)\(^14\). Lysozyme is an enzyme found in saliva at a concentration of 180 µg ml\(^{-1}\) and is effective in killing gram positive bacteria by promoting cell wall disruption and subsequent cell lysis\(^15\). For survival in the GI tract, probiotic bacteria should be resistant to the enzymes, mainly lysozyme in the oral cavity\(^16\).

Among the probiotic bacteria, antibiotic resistance has been reported for strains isolated from animals as well as humans\(^17\). This is due to the use of the antimicrobial agents for therapy and prophylaxis of bacterial infections and in some cases to the abuse of antibiotics in general. Tolerance of probiotic bacteria to antibiotics is interesting due to their possible use to reconstitute the intestinal microflora of patients suffering from antibiotic-associated colitis\(^18\).

Antioxidative strains i.e., strains with capability to tolerate hydrogen peroxide (H\(_2\)O\(_2\)) have significantly increased resistance to harsh media compared to the non-antioxidative strain; sodium chloride concentration affects the growth of probiotics and is a physiochemical requirement for the production of bacteriocins\(^19\). The non-haemolytic nature of probiotics indicate that they would not be fatal for mammalian host if entered into the blood and food chain\(^20\).

Probiotics are live microbial feed supplements that improve the health of man by its valuable secondary products\(^21\). They inhibit pathogenic bacteria by producing inhibitory substances such as antibiotics, bacteriocins or acids among others\(^22\). Bacteriocins are small, ribosomally synthesized, antimicrobial peptides or proteins\(^23\), produced by probiotics which play a role

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during *in vivo* interactions occurring in the human gastrointestinal tract, hence contributing to gut health \(^{24}\). Bacteriocins thus have considerable attention as natural bio-preservatives and as potential replacement of antibiotics \(^{19}\). Hence, bacteriocin producing strains have *in situ* application as part of or adjuncts to starter cultures for fermented/heated food products in order to improve safety and quality \(^{25,26}\).

The purpose of the present study was to isolate probiotic strains from faecal sample of humans and calf and their partial characterization. The antimicrobial potential against a range of microbes as well as their tolerance to other antibiotics and environmental stresses including acid, bile salts, lysozyme, hydrogen peroxide and sodium chloride was determined; anti-haemolytic property was also assessed to ascertain the safety of these probiotics *under in vivo* conditions. The probiotics were also assessed for the production of inhibitory compounds i.e., bacteriocins; their pH and temperature stability analyzed.

**MATERIALS AND METHODS**

**Isolation of microorganisms**

Procured strains were isolated from faecal sample of infant (aged below 2 years) and calf from Tangori village, Banur (Punjab), India. These samples were collected and stored in sterile boxes. Samples were serially diluted up to \(10^{-6}\) in sterile distilled water and 100µl was spread on MRS agar plates (de-Mann, Rogosa and Sharpe agar; Hi Media, Mumbai, India). These plates were then incubated at 37°C for 24 hours. After incubation, 6 well isolated typical colonies were picked and streaked on MRS agar plates, which were then incubated at 37°C for 48 hours for further use.

**Identification of microorganisms**

The isolated microorganisms were identified by morphological tests such as Gram staining \(^{27}\) and various biochemical tests including catalase, indole production, methyl red, urease, citrate utilization and \(\text{H}_2\text{S}\) production \(^{28}\).

**Detection of antagonistic activity**

The antagonistic properties of all the 6 isolated strains were determined by the disc diffusion method \(^{29}\) against the test pathogens, obtained from IMTech, Chandigarh, India. Autoclaved filter paper discs (6mm) were impregnated with 60µl of MRS broth containing 48 hours grown isolated cultures. These impregnated discs were dried and then placed on solidified nutrient agar plates seeded with freshly grown culture of test pathogens, like *Klebsiella pneumoniae* MTCC 9024, *Staphylococcus epidermidis* MTCC 6810, *Pseudomonas aeruginosa* MTCC 2297, *Escherichia coli* MTCC 443, *Enterococcus faecalis* MTCC 3159 and *Serratia marcescens* MTCC 482; *Lactobacillus plantarum* was used as a positive control. The plates were then incubated at
37°C for 24 hours and zones of inhibition were estimated by measuring the diameter of the clear area around the disc.

**Acid and bile salt tolerance**

Isolated strains were inoculated into MRS broth of varying pH, i.e. pH 2.0, 3.0, 4.0 and 5.0 adjusted using 0.1 M HCl; broth with pH 7.0 was used as control. Inoculation was also done in broth with varying concentrations of bile salts i.e., 0.5%, 1.0%, 1.5% and 2.0% (w/v) (Thomas Baker Chemicals) and the control was without any bile salt. Flasks containing these broths were incubated at 37°C for 48 hours. Hundred microlitre of inoculum from each MRS broth (of varying pH and from varying concentrations of bile salts) were taken, and transferred to individual flasks containing MRS agar (at 40°C-45°C) and plating was done. After 24 hours of incubation at 37°C, the growth of isolates were observed on MRS agar plates in order to designate isolates as acid and/or bile salt tolerant.

**Lysozyme tolerance**

Dilutions of all the isolated strains, from the MRS broth were prepared in peptone water and loop full of culture were streaked on MRS agar plates supplemented with 0.5 mg ml⁻¹ of lysozyme (Hi Media Laboratories, Mumbai, India). Plates were then incubated for 24 hours at 37°C and tolerance to lysozyme was ascertained by the presence of growth of desired strains.

**Resistance to antibiotics**

The antibiotic resistance of the isolated strains was assessed using pre-impregnated antibiotic discs (Hi Media Laboratories, Mumbai, India). Two hundred microlitre of each of the freshly grown isolates were spread over the surface of individual MRS agar plates. The sterile antibiotic discs containing amoxycillin/sulbactam (30/15mcg/disc), nalidixic acid (30mcg/disc), ciprofloxacin (5mcg/disc), penicillin G (10 units/disc), norfloxacin (10 mcg/disc), oxacillin (1 mcg/disc), amoxyccillin (30 mcg/disc) and ampicillin (10 mcg/disc) were then placed on the MRS agar surface, spreaded with probiotic lawn and incubated for 24 hours at 37°C.

**H₂O₂ tolerance**

One millilitre of all the isolated strains cultivated in MRS broth were suspended in 1 ml of isotonic saline (0.85 gm NaCl suspended in 100 ml of distilled water) in test tubes and incubated with 1 ml of 0.4 mM of 30% (w/v) H₂O₂ solution (LOBA Chemie Ltd.). The results were recorded by measuring the optical density (OD) (Metertech, UV/VIS spectrophotometer) at 650 nm after a time interval of 1 hour, 2 hours, 3 hours, 4 hours and 24 hours for observing their maximum tolerance to H₂O₂.
**NaCl tolerance**

For the determination of NaCl (LOBA Chemie Ltd.) tolerance of isolated strains, test tubes containing MRS broth were adjusted to different concentrations of NaCl (1.0%, 2.0%, 4.0% and 5.0% (w/v)) and autoclaved. Each sterile tube was then individually inoculated with 1% (v/v) freshly grown culture of the 6 isolated strains (in MRS broth) and incubated at 37°C for 24 hours. After incubation, their growth was determined by observing their OD at 600 nm\(^{19}\).

**Anti-haemolytic activity**

The isolated strains were streaked on individual blood agar plates containing 5% human blood and incubated at 37°C for 24 hours. Haemolytic activity was checked by the presence of clear zones on blood agar plates around the bacterial colonies\(^{32}\).

**Bacteriocin/s production**

For bacteriocin/s production, MRS broth containing freshly grown culture of each of the 6 isolates were taken in centrifuge tubes and centrifuged using cooling microfuge (Remi Pvt. Limited) at 8000 rpm for 15 minutes at a temperature of 4°C. The pH of the cell free supernatant was adjusted to pH 6.5-7.0 with 1M NaOH to neutralize the acids in broth culture of probiotics\(^{29}\) and this supernatant was further used as the source of bacteriocin/s.

**Bacteriocin assay**

Autoclaved filter paper discs were impregnated with 60µl of the supernatant containing bacteriocin/s from the 6 probiotic isolates, dried and placed on MRS agar plates seeded with test pathogens (*K. pneumoniae* MTCC 9024, *S.epidermidis* MTCC 6810, *P. aeruginosa* MTCC 2297, *E. coli* MTCC 443, *E. faecalis* MTCC 3159 and *S. marcescens* MTCC 482). The antagonistic activity of the bacteriocin/s was determined by measuring the diameter of zones of inhibition around the discs; supernatant from *L. plantarum* was used as a positive control for bacteriocin activity.

**Heat sensitivity of bacteriocin/s**

To test the heat sensitivity, culture supernatant containing bacteriocin/s was heated for 15 minutes at 60°C, 80°C, 100°C and 121°C and the activity was tested against *E. coli*, as the test pathogen (by disc-diffusion method), using culture supernatant at room temperature (37°C) as the control.

**Acid/alkaline sensitivity of bacteriocin/s**

Sensitivity of bacteriocin/s to different pH was tested by adjusting the pH of culture supernatant (containing bacteriocin/s) of the probiotic isolates to 1.5, 3.0 and 5.0 using 0.1M HCl and to pH 9.0 using 1 M NaOH. The antibacterial activity of the bacteriocin/s was detected against *E. coli* using culture supernatant with pH 7.0 as a control.
RESULTS

Isolation of microorganisms

A total of 6 microorganisms were isolated from faecal sample of infant and calf. Four isolates from faecal sample of infant were named as F1, F2, F3 and F4; and 2 isolates from faecal sample of calf were named as C1 and C2.

Morphological and biochemical identification

All the probiotic isolates F1, F2, F3, F4, C1 and C2 were identified to be Gram positive and their biochemical characteristics have been shown in Table 1, Figure 1(a) and (b).

Table 1: Morphological and biochemical identification tests of isolates

<table>
<thead>
<tr>
<th>Source</th>
<th>Isolated strain</th>
<th>Gram staining</th>
<th>Shape</th>
<th>Catalase test</th>
<th>Indole production</th>
<th>Methyl red</th>
<th>Urease test</th>
<th>H₂S production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infant</td>
<td>F1</td>
<td>+*</td>
<td>Cocco-bacilli</td>
<td>-*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>F2</td>
<td>+</td>
<td>Cocci</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>F3</td>
<td>+</td>
<td>Cocco-bacilli</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>F4</td>
<td>+</td>
<td>Bacilli</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Calf</td>
<td>C1</td>
<td>+</td>
<td>Cocci</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>C2</td>
<td>+</td>
<td>Cocco-bacilli</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Note: + indicates isolates are test positive; - indicates isolates are test negative.

Figure 1: Biochemical analysis of isolates (a) H₂S production test and (b) Urease test
Antagonistic activity of isolates

All the 6 probiotic isolates showed the antagonistic activity against pathogenic microorganisms (Table 2). Probiotic isolate F1 showed the zone of inhibition in the range of 7-8mm against all the test pathogens with maximum zone of inhibition (8mm) against *E. coli*, *E. faecalis* and *S. marcescens*; isolate F2 showed the zone of inhibition in the range of 7-9mm with maximum zone of inhibition (9mm) against *E. coli* and *S. marcescens*. The isolate F3 exhibited inhibition zone between 7-9mm size range with maximum activity (9mm) against *E. coli*, while isolate F4 showed the inhibition range to be 7-8mm and *E. coli*, *S. epidermidis*, *K. pneumoniae* and *E. faecalis* were the most susceptible with zone size of 8mm. Isolate C1 showed inhibition within a zone size range of 7-9mm, exhibiting maximum activity (9mm) against *E. coli*, *E. faecalis*, *S. marcescens* and isolate C2 also showed inhibition (range 7-8mm), with the maximum activity against *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. marcescens* (8mm).

Hence, it could be seen that out of all the test pathogens, *E. coli* was the most sensitive against all the probiotic isolates. Moreover, isolates F2, F3 and C1 showed quite appreciable zone of inhibitions against most of the test microorganisms, comparable to that obtained using the positive control *L. plantarum*.

Table 2: Antagonistic activity of isolates against bacterial pathogens

<table>
<thead>
<tr>
<th>Isolates</th>
<th><em>E. coli</em> MTCC 443</th>
<th><em>S. epidermidis</em> MTCC 6810</th>
<th><em>P. aeruginosa</em> MTCC 2297</th>
<th><em>K. pneumoniae</em> MTCC 9024</th>
<th><em>E. faecalis</em> MTCC 3159</th>
<th><em>S. marcescens</em> MTCC 482</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>8mm*</td>
<td>7mm</td>
<td>7mm</td>
<td>7mm</td>
<td>8mm</td>
<td>8 mm</td>
</tr>
<tr>
<td>F2</td>
<td>9mm</td>
<td>7mm</td>
<td>7mm</td>
<td>7mm</td>
<td>8mm</td>
<td>9mm</td>
</tr>
<tr>
<td>F3</td>
<td>9mm</td>
<td>8mm</td>
<td>7mm</td>
<td>8mm</td>
<td>8mm</td>
<td>8mm</td>
</tr>
<tr>
<td>F4</td>
<td>8mm</td>
<td>8mm</td>
<td>7mm</td>
<td>8mm</td>
<td>8mm</td>
<td>7mm</td>
</tr>
<tr>
<td>C1</td>
<td>9mm</td>
<td>7mm</td>
<td>7mm</td>
<td>7mm</td>
<td>9mm</td>
<td>9mm</td>
</tr>
<tr>
<td>C2</td>
<td>8mm</td>
<td>7mm</td>
<td>8mm</td>
<td>7mm</td>
<td>8mm</td>
<td>8mm</td>
</tr>
<tr>
<td><em>L. plant- arum</em></td>
<td>10mm</td>
<td>10mm</td>
<td>8mm</td>
<td>8mm</td>
<td>9mm</td>
<td>9mm</td>
</tr>
</tbody>
</table>

*Note: mm denotes the diameter of zone of inhibition in millimetre; *L. plantarum* was taken as control

Acid and bile salt tolerance

All the 6 isolates were able to tolerate the acidic media (pH-2.0, 3.0, 4.0, 5.0) as well as bile salts (0.5%, 1.0%, 1.5%, 2.0 % (w/v)) upto 24 hours of incubation, as indicated by the presence of
growth under these extremely acidic pH conditions and high bile salt concentrations [Table 3, Figure 2(a) and (b)].

Table 3: Acid and bile salt tolerance of isolates

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Acid tolerance (pH-2.0)</th>
<th>Bile salt tolerance (2% w/v)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>+*</td>
<td>+*</td>
</tr>
<tr>
<td>F2</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F3</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F4</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>C2</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Note: + indicates presence of growth; pH 2.0 was taken as minimum and 2% w/v bile salt was taken as maximum

Figure 2: Positive growth of isolates in media with (a) pH 2.0 and (b) Bile salt conc. 2% (w/v)

Antibiotic sensitivity test

The probiotic isolates exhibited different degree of resistance against various antibiotics. The isolates F4 and C2 showed resistance against all the eight antibiotics tested amoxycillin/sulbactam (AMS), nalidixic acid (NA), ciprofloxacin (CF), penicillin G (P), norfloxacin (NX), oxacillin (OX), amoxicillin (AM) and ampicillin (AMP) [Figure 3(a) and (b)]. Isolates F1 and F2 showed resistance against 5 antibiotics, whereas F3 and C1 showed resistance against 4 of the antibiotics tested (Table 4).
Table 4: Antibiotic resistance test of isolates

<table>
<thead>
<tr>
<th>Isolates</th>
<th>CF</th>
<th>AMS</th>
<th>AM</th>
<th>P</th>
<th>OX</th>
<th>AMP</th>
<th>NX</th>
<th>NA</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>R*</td>
<td>S*</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>F2</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>F3</td>
<td>R</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>F4</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>C1</td>
<td>S</td>
<td>S</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>S</td>
<td>S</td>
<td>R</td>
</tr>
<tr>
<td>C2</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>

*Note: R indicates resistance to antibiotics; S indicates sensitivity to antibiotics; Amoxycillin/sulbactam (AMS), nalidixic acid (NA), ciprofloxacin(CF), penicillin G (P), norfloxacin (NX), oxacillin (OX), amoxicillin (AM), ampicillin (AMP).

Figure 3: Resistance against all 8 test antibiotics exhibited by isolates (a) F4 and (b) C2

Lysozyme tolerance

All the 6 probiotic strains showed tolerance to lysozyme, as indicated by the presence of growth in a media supplemented with lysozyme at 0.5 mg ml⁻¹ concentration (Figure 4).
**H₂O₂ tolerance**

All the probiotic isolates showed tolerance to 0.4 mM H₂O₂ as indicated by persistence of growth up to 24 hours of incubation. F4 followed by F1 showed appreciable H₂O₂ tolerance, since they exhibited higher OD values at 650 nm even after 24 hours of incubation (Figure 5).

![Figure 5: H₂O₂ tolerance (0.4mM) of isolates (measured as growth in OD) after different time intervals](image-url)
Tolerance to NaCl

Among all the probiotics, isolates F3 and C1 showed tolerance to NaCl up to a concentration of 5% with maximum growth at 1% (O.D; 0.665 and 0.692, respectively) and minimum at 5% (0.292 and 0.252, respectively) NaCl concentration. Isolate F2 showed tolerance up to 4% NaCl with maximum tolerance at 1% (0.763), isolate F4 and C2 showed the tolerance to NaCl up to 2%, with maximum growth (0.150 and 0.249 respectively) at the same concentration while isolate F1 showed tolerance only at 1% (0.106) NaCl concentration, as indicated in Figure 6.

![Figure 6: Tolerance of isolates (measured as growth in OD) to different NaCl concentrations (1-5%)](image)

Anti-haemolytic activity

Anti-haemolytic activity was exhibited by most of the probiotic isolates, since no zone of clearance could be seen around most of the isolates grown on blood agar plates. Presence of only a small clearance zone around F3 and C2, indicated extremely weak haemolytic activity (Table 5, Figure 7).
Table 5: Anti-haemolytic activity of the isolates

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Haemolytic activity (Positives/weak/negative)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>Negative*</td>
</tr>
<tr>
<td>F2</td>
<td>Negative</td>
</tr>
<tr>
<td>F3</td>
<td>Weak*</td>
</tr>
<tr>
<td>F4</td>
<td>Negative</td>
</tr>
<tr>
<td>C1</td>
<td>Negative</td>
</tr>
<tr>
<td>C2</td>
<td>Weak</td>
</tr>
</tbody>
</table>

*Note: Weak activity indicates very few clear zones around bacterial colonies on blood agar plates; negative indicates absence of clear zones around bacterial colonies.

Figure 7: Anti-haemolytic activity of the isolates (F1, F2, F3, F4, C1, C2)

Bacteriocin assay

Activity of the bacteriocin/s present in the supernatant was observed against different pathogenic microorganisms, by measuring zones of inhibition. The supernatant containing bacteriocin/s obtained from the 6 isolates showed activity against all test pathogenic strains including *E. coli*, *S. marcescens*, *P. aeruginosa*, *S. epidermidis*, *K. pneumoniae* and *E. faecalis*. Among the probiotic isolates, bacteriocins produced by F3 exhibited large inhibition zone sizes. *E. coli* was found to be the most sensitive pathogen to the bacteriocin/s produced by isolates
F1, F2, F3, C1 and C2 as exhibited by the zone of inhibition (12mm, 13mm, 20mm, 10mm and 10mm respectively). Bacteriocin/s produced by F4 isolate showed maximum inhibition against \( K. \) *pneumoniae* (zone size-10mm); bacteriocin/s from C2 along with exhibiting maximum inhibition against *E. coli*, also inhibited *K. pneumoniae* to an equal extent (zone size10mm); along with inhibiting *E. coli*, bacteriocins from isolate C1 also showed maximum activity against *P. aeruginosa* (zone size-10mm) (Table 6, Figure 8).

**Table 6: Antibacterial activity of bacteriocin/s of probiotic isolates against pathogens**

<table>
<thead>
<tr>
<th>Isolates</th>
<th><em>E. coli</em> MTCC 443</th>
<th><em>S. epidermidis</em> MTCC 6810</th>
<th><em>P. aeruginosa</em> MTCC 2297</th>
<th><em>K. pneumoniae</em> MTCC 9024</th>
<th><em>E. faecalis</em> MTCC 3159</th>
<th><em>S. marcescens</em> MTCC 482</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>12mm</td>
<td>7mm</td>
<td>8mm</td>
<td>7mm</td>
<td>7mm</td>
<td>10mm</td>
</tr>
<tr>
<td>F2</td>
<td>13mm</td>
<td>9mm</td>
<td>9mm</td>
<td>10mm</td>
<td>9mm</td>
<td>8mm</td>
</tr>
<tr>
<td>F3</td>
<td>20mm</td>
<td>9mm</td>
<td>12mm</td>
<td>10mm</td>
<td>11mm</td>
<td>13mm</td>
</tr>
<tr>
<td>F4</td>
<td>7mm</td>
<td>8mm</td>
<td>7mm</td>
<td>7mm</td>
<td>10mm</td>
<td>9mm</td>
</tr>
<tr>
<td>C1</td>
<td>10mm</td>
<td>8mm</td>
<td>10mm</td>
<td>9mm</td>
<td>7mm</td>
<td>9mm</td>
</tr>
<tr>
<td>C2</td>
<td>10mm</td>
<td>7mm</td>
<td>8mm</td>
<td>8mm</td>
<td>10mm</td>
<td>7mm</td>
</tr>
<tr>
<td><em>L. plantarum</em> (control)</td>
<td>9mm</td>
<td>8mm</td>
<td>10mm</td>
<td>9mm</td>
<td>10mm</td>
<td>8mm</td>
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</tbody>
</table>

**Figure 8: Bacteriocin assay of probiotic isolates (F1, F2, F3, F4, C1, C2)**
Heat sensitivity of bacteriocin/s

Bacteriocin/s produced by probiotic strain C1 exhibited maximum heat tolerance at even high temperatures of up to 121°C when exposed for 15 minutes, as indicated by the presence of their inhibitory activity (zone size of 9mm) against *E. coli* as test pathogen (Table 7); bacteriocin/s from C1 also exhibited tolerance at boiling temperature (at 100°C) with zone size of 9mm. Probiotic strains F2 and F3 produced bacteriocin/s which could also withstand boiling (for 15 minutes) as exhibited by the presence of zone of inhibition (8mm and 7mm, respectively). Bacteriocins from isolate F1 exhibited high temperature tolerance of up to 80°C with a zone size of 8mm; while those from strains F4 and C2 retained activity only up to 60°C (8mm each).

Table 7: Effect of heat treatment on bacteriocin/s from probiotic isolates

<table>
<thead>
<tr>
<th>Isolated strains</th>
<th>Temperature (°C)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>60°C</td>
</tr>
<tr>
<td>F1</td>
<td>*8mm</td>
</tr>
<tr>
<td>F2</td>
<td>12mm</td>
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<tr>
<td>F3</td>
<td>11mm</td>
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<tr>
<td>F4</td>
<td>8mm</td>
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<tr>
<td>C1</td>
<td>10mm</td>
</tr>
<tr>
<td>C2</td>
<td>8mm</td>
</tr>
</tbody>
</table>

Note: *E. coli* was taken as test organism

pH sensitivity of bacteriocin/s

Bacteriocin/s produced by the probiotic strain C1 were stable in the entire pH range (pH-1.5-9.0), as exhibited by their activity of 7-14mm zone size against *E. coli*, under extremely acidic as well as alkaline conditions (Figure 9). Bacteriocin/s produced by probiotic strains F2 and F3 also showed marked acidic and alkaline stability over a major part of the pH range i.e., 3.0-9.0 (zone size: 10-15mm for F2; and 9-15mm for F3). Bacteriocin/s produced by isolate C2 were stable more in the acidic side of pH range (1.5-7.0), as exhibited by their persistent activity of 8-11mm zone size. Probiotic strains F1 and F4 produced bacteriocin/s stable in 3.0-7.0 pH range (zone size: 10-12mm for F1; and 10-13 mm for F4).
Figure 9: pH sensitivity of bacteriocin/s from probiotic isolates against *E. coli*

DISCUSSION

The aim of the present research was to isolate the probiotics along with the inhibitory compounds produced by them (bacteriocins) and to evaluate their persistence under extremely stressful conditions, as encountered under *in vivo* conditions. This is one of only a few studies focusing on the isolation of the probiotics from both human and calf faeces. Some of the earlier studies have been done on either the faecal flora of child or that on the veal calves.

*Lactobacilli* and *Bifidobacteria* are the most commonly detected microorganisms in faecal samples. In our study, all the isolated probiotics were identified to be Gram positive. The biochemical tests revealed these isolates to be catalase negative, rod, cocci or cocco bacilli, which might fall into the category of *Bifidobacteria* or lactic acid bacteria. The antimicrobial activity of six isolates as seen against different pathogenic strains like *E. faecalis*, *E. coli*, *S. epidermidis*, *K. pneumoniae*, *P. aeruginosa* and *S. marcescens*, revealed that isolates F1, F3 and C1 were the most effective and exhibited large inhibition zones (7-9mm). Among the test pathogens, *E. coli* was found to be the most susceptible to majority of the isolated probiotics. Earlier studies on probiotics also demonstrated maximum antagonistic potential of probiotics against *E. coli* as exhibited by large diameter of inhibition zones.

Acid tolerant strains have an advantage for survival in stomach (upto extreme pH 2.0), to be able to resist the digestion process in the stomach, where hydrochloric and gastric juices are secreted. In our study, all the six isolates showed tolerance to extremely acidic medium with pH values 2.0-5.0, as exhibited by the growth of all the strains. Majority of the earlier studies have shown tolerance of probiotic isolates to acidic medium of pH upto 3.0; one of the only few reports suggested the survival of *Lactobacillus* strains, isolated from Moroccan traditional dairy products under extremely acidic gastric conditions (pH 2.0 and pH 3.0). The next barrier after gastric juice in the stomach is bile, which is present in the upper part of small intestine.
and for a bacteria to be effective as a probiotic, they should have the ability to survive the passage through the upper digestive tract to the large intestine, where its beneficial action is expected\textsuperscript{22,39-41}. Most of the earlier studies, report tolerance of probiotics like \textit{Leuconostoc mesenteroides} and \textit{Lactobacillus} species to bile salt concentration upto 1.0\% (w/v) after 24 hours of incubation\textsuperscript{35,42}. In our study, all of the 6 probiotic isolates showed tolerance to 0.5\% (w/v), 1.0\% (w/v) bile salts as well as to higher concentrations of 1.5\% (w/v) and 2.0 \% (w/v) after 24 hours of incubation. It was notable that, in this study, 100\% of the isolates were shown to be capable of growth at low pH and high bile salts’ concentrations, which is a mandatory requirement for a probiotic culture\textsuperscript{43}.

Antibiotic resistance can occur in two ways in a bacterial population; mutation of an endogenous gene or acquisition of a resistance gene from an exogenous source\textsuperscript{44,45}. Thus, intestinal bacteria can acquire resistance to antibiotics used for therapy, either by mutation or by horizontal transfer of resistance genes from another intestinal species or any species that passes through the colon\textsuperscript{46,47}. Some probiotic strains with intrinsic antibiotic resistance could be useful for restoring the gut microbiota after antibiotic treatment\textsuperscript{48}. In our study 2 isolates (F4 and C2) showed resistance against all the eight antibiotics tested; three isolates F1, F2 and C1 showed resistance against 5 antibiotics, whereas F3 showed resistance against 4 of the test antibiotics. This is in accordance with the previous studies\textsuperscript{49,33}, emphasizing the “selective antibiotic resistance” of probiotics isolated from human faeces. Bacteria used as probiotic adjuncts are commonly delivered in a food system and, therefore, begin their journey to the lower intestinal tract via the mouth and hence, probiotic bacteria should be resistant to the enzymes such as lysozyme present in the oral cavity\textsuperscript{50}. Like some previous studies\textsuperscript{31}, all our 6 isolated strains showed lysozyme tolerance, since they exhibited the growth in the presence of 0.5 mg ml\textsuperscript{-1} lysozyme.

Haemolytic property of probiotics is a preliminary approach to address whether they are safe to mammalian system or not and is an important consideration as far as the food safety is concerned\textsuperscript{20}. Similar to some previous studies\textsuperscript{20,32,51}, our probiotics exhibited negative haemolysis, with only 2 strains showing negligible haemolytic activity. The anti-haemolytic activity indicated that our probiotics could be safely administered to humans. Antioxidative potential of probiotics is a desirable characteristic, being able to scavenge any peroxides. In the present study, all 6 isolated probiotics could tolerate 0.4 mM H\textsubscript{2}O\textsubscript{2}. Since our isolates could tolerate hydrogen peroxide for long duration upto 24 hours, hence our study is a step forward to earlier studies, emphasizing tolerance (0.4 mM concentration of H\textsubscript{2}O\textsubscript{2}) upto 4 hours and 6 hours\textsuperscript{19}.

Sodium chloride has been found to be a requirement by the probiotics for the production of inhibitory compounds including bacteriocins. The effect of NaCl on growth of 2 probiotic isolates in a medium was earlier seen, wherein, both the organisms tolerated 1\% (w/v) NaCl,
but less growth was found at 5% (w/v) NaCl. In our study, 2 isolates (F3 and C1) showed the maximum tolerance of NaCl up to 5% (w/v) concentration with persistent growth (0.292 and 0.252, respectively).

Bacteriocins, the peptides produced by probiotics contain bactericidal activity and enhance the survival of these species in a complex ecological system, thus focusing on prevention of growth of harmful bacteria in the intestinal tract and gut. Various previous works have emphasized the antagonistic potential of bacteriocins produced by probiotics against many pathogenic microorganisms. In our study, the supernatant containing bacteriocins showed the inhibitory effect against a broad spectrum of test pathogens such as *E. coli*, *P. aeruginosa*, *E. faecalis*, *S. epidermidis*, *K. pneumoniae* and *S. marcescens*. Highly significant activity was exhibited by the bacteriocins produced by the most effective isolate F3 against *E. coli* and *S. marcescens* (zone of inhibition-20mm and 13mm respectively).

Heat and pH tolerance by bacteriocin/s have been seen as a desirable characteristic. In the present study, the bacteriocins obtained from 6 probiotic isolates showed varied stability at different temperatures (60°C, 80°C, 100°C and 121°C) for 15 minutes, when tested against *E. coli* as the test pathogen. The bacteriocin/s obtained from the isolated strain C1 showed tolerance up to 121°C, as exhibited by the zone of inhibition (9mm), while bacteriocin/s obtained from probiotic isolates F2 and F3 showed stability up to 100°C. Our study is in accordance with some previous studies emphasizing production of heat stable bacteriocin/s (121°C up to 10 minutes and 15 minutes respectively). In our study, bacteriocin/s isolated from isolate C1 were stable in the entire pH range of 1.5-9.0 and bacteriocin/s from isolate C2 were stable in the pH range of 1.5-7.0, while bacteriocin/s from F2 and F3 exhibited stability in the range of 3.0 to 9.0. This property of pH tolerance by the metabolites produced by our probiotic isolates, ascertains its effectiveness in the highly acidic conditions (pH- 1.5-4.0) of the GIT.

**CONCLUSION**

Probiotics are of great interest and are generally recognized as safe organisms for human use. The usual selection procedure needs to be revised, since passage through the stomach is the first 'hurdle' that potentially probiotic bacteria face on their way to the intestines and acid tolerance is regarded as a very important pre-requisite in the selection of cultures. All our isolates exhibited remarkable tolerance to the extreme conditions, as could be encountered under *in vivo* conditions in the GIT. It is also desirable to continue to expand our understanding of the influences that environmental factors have on the survival of bacteriocin producing strains and the activity of their bacteriocins. The bacteriocins of the probiotic strains isolated in the present study showed excellent activity, along with possessing tolerance to extreme physical stresses. These properties are crucial in order to qualitatively estimate their efficacy for
future applications in food model systems and in the clinics and also, to establish adequate means of application of these bio-preservatives.

REFERENCES

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