Research Article

In Vitro and In Vivo Survival and Colonic Adhesion of Pediococcus acidilactici MTCC5101 in Human Gut

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The present study aims to investigate the probiotic nature of Pediococcus acidilactici MTCC5101 by an in vitro assay of bacterial adherence to intestinal epithelial cells of human gastrointestinal (GI) tract using Caco-2 cell line. Further to assess the in vivo survival in the GI tract, oral feeding was carried out with the help of 10 healthy volunteers. The effect on wellness was assessed by studying blood biochemical parameters of the volunteers. The survival of the bacteria was assessed using PCR-based detection of P. acidilactici MTCC5101 in fecal samples. The probiotic nature of P. acidilactici MTCC5101 was strengthened by its adherence to the intestinal epithelial Caco-2 cell line in the in vitro SEM observations. Oral feeding study for assessing the survival of bacteria in GI tract of volunteers showed the strain to be established in the GI tract which survived for about 2 weeks after feeding.

1. Introduction

Human intestinal microbiota is a metabolically active microbial environment that is relatively stable in the guts of healthy individuals [1]. The commensal intestinal flora of humans and animals includes the genera Lactobacillus, Pediococcus, and Lactococcus [2, 3]. Strains of these genera are frequently used on large scale as starter cultures in food industries because of their generally recognized as safe (GRAS) and probiotic status [4–6].

Probiotics are known to exert protective influence in the human intestine through various mechanisms and represent promising applications in prophylaxis and therapy. It has also been acknowledged by WHO that probiotic microorganisms when administered in adequate amounts confer a health benefit to the host [7]. Improvement of health status by probiotics can be explained by the fact that these bacteria reduce production of toxic substances, aid in production and absorption of vital nutrients, stimulate gastrointestinal activity as well as immunity, and reduce colonization of anaerobic pathogens by competitive exclusion [8, 9].

For all the health giving effects, the probiotic bacteria should also be capable of surviving while passage through the GI tract. It is essential for them to overcome highly acidic environment of stomach, digestive enzymes, and bile salts of the small intestine, which thus become important selection criterion for probiotic strains. Apart from this, adherence of bacterial cells to intestinal epithelial cells and/or mucus is considered to be a desirable feature of probiotics, as it promotes enhanced gut residence time, pathogen exclusion, and interaction with host epithelial and immune cells [10, 11].

Pediococcus acidilactici MTCC5101 is an acid and bile tolerant probiotic strain that secretes a potent antibacterial bacteriocin designated as Pediocin CP2. Pediocin CP2 exhibits a wide range of antimicrobial activity against Gram-positive, Gram-negative bacteria as well as fungi [12]. Genes encoding production of Pediocin CP2 are localized to the ped operon present on the 8.9 kb plasmid pCP289 of P. acidilactici MTCC5101 [13–15]. These properties make this sp. of Pediococcus an attractive prophylactic and therapeutic agent against pathogenic bacteria in GI tract. However, the final confirmation of its probiotic nature can come only from human trials. It is these trials that provide evidence of the survival of the probiotic strain in vivo and provide the required basis for a credible claim.

The present study aims to investigate the probiotic nature of P. acidilactici MTCC5101 by an in vitro assay of bacterial adhesion to intestinal epithelial cells of human GI tract using...
Caco-2 cell line. Further, to assess the *in vivo* survival in the GI tract, oral feeding was carried out with the help of 10 healthy volunteers. The effect on wellness was assessed by studying blood biochemical parameters of the volunteers.

### 2. Materials and Methods

#### 2.1. Bacterial Strain Procurement and Maintenance

Pediacoccus acidilactici MTCC5101, characterized in the laboratory of Department of Biotechnology, Punjabi University, Patiala [12], was used in the present study. It was revived and maintained at 37°C for 18–24 h under microaerophilic conditions in de Man, Rogosa, and Sharpe (MRS) broth [16].

The enterocyte-like Caco-2 cells were obtained from National Centre for Cell Science (NCCS), Pune. Cells were routinely grown in Dulbecco modified Eagle’s minimal essential medium containing 25 mM glucose, 10% fetal bovine serum, and 1% antibiotic solution containing penicillin and streptomycin (Himedia) [15].

The adherence of pediococci to Caco-2 cells was examined as described previously by Bernet et al. [17] with a few modifications. For the adhesion assay, monolayers of Caco-2 cells were prepared on glass coverslips placed in six-well tissue culture plates seeded at a concentration of 10^6 cells/mL. Experiments were carried out at 37°C in a 5% CO_2 atmosphere. The culture medium was changed every alternate day and monolayers at late postconfluence stage (after 15 days) were used for adhesion assay. To begin with the assay, monolayers were washed twice with phosphate-buffered saline (PBS, pH 7.2). 1 mL bacterial culture containing one million CFU/mL suspended in PBS was added to the monolayers on glass coverslips placed in tissue culture plates containing DMEM without antibiotic solution. The plates were incubated for 1 h in 5% CO_2 at 37°C. After 1 h, the monolayers were washed five times with sterile PBS and fixed for SEM observations. The adhesion assay was repeated thrice.

#### 2.2. In Vitro Adhesion Assay of Pediacoccus acidilactici MTCC5101

The overnight grown *P. acidilactici* MTCC5101 cells were harvested by centrifugation (6000 rpm, 10 min) and resuspended slowly in PBS (pH 8.0). Cells at a concentration of 10^8–10^10 cells/mL were added to fresh buttermilk as medium for oral feeding study (Verka Co. Ltd., Patiala, India). Cell counts were taken on a haemocytometer [19] and after thorough microbiological examination, cells at an average dosage level of 10^8–10^10 cells/mL/day were fed to 10 healthy subjects in 1 mL buttermilk base, administered once a day for 4 weeks continuously [20]. Equivalent amount of buttermilk without *P. acidilactici* MTCC5101 was fed to a group of 3 control volunteers which served as negative controls. The subjects were asked to abstain from taking other fermented milk products and supplements with probiotics during the trial period. They were explained how to collect, store, and deliver the fecal samples. Participants were asked about changes in their wellbeing and/or had taken any kind of medication during study period.

#### 2.4. In Vivo Study Design

The study was approved by the Institutional Clinical Ethics Committee vide clearance no. ICEC/3/2011. Experiments were strictly performed according to the guidelines of Indian Council of Medical Research for conducting research on human trials. Ten healthy volunteers were selected after taking their informed consent. It was a controlled study consisting of parallel 6 weeks trials, with 4 weeks of intervention and 2 weeks wash-out period.

#### 2.4.1. Preparation of Probiotic Buttermilk

The overnight grown *P. acidilactici* MTCC5101 cells were harvested by centrifugation (6000 rpm, 10 min) and resuspended slowly in PBS (pH 8.0). Cells at a concentration of 10^8–10^10 cells/mL were added to fresh buttermilk as medium for oral feeding study (Verka Co. Ltd., Patiala, India). Cell counts were taken on a haemocytometer [19] and after thorough microbiological examination, cells at an average dosage level of 10^8–10^10 cells/mL/day were fed to 10 healthy subjects in 1 mL buttermilk base, administered once a day for 4 weeks continuously [20]. Equivalent amount of buttermilk without *P. acidilactici* MTCC5101 was fed to a group of 3 control volunteers which served as negative controls. The subjects were asked to abstain from taking other fermented milk products and supplements with probiotics during the trial period. They were explained how to collect, store, and deliver the fecal samples. Participants were asked about changes in their wellbeing and/or had taken any kind of medication during study period.

#### 2.4.2. Collection and Analysis of Fecal Samples

The fecal swab samples were collected using ear buds in 2 mL of sterile normal saline (0.85% NaCl) after feeding trials of 5, 10, 20, and 30 days [38]. The fecal suspension was cultured in MRS broth for bacterial enumeration for 18 h at 37°C. Cell pellets were harvested by centrifugation and were stored at −20°C for plasmid DNA isolation.

#### 2.4.3. Molecular Identification of *P. acidilactici* MTCC5101 in Fecal Samples

A metagenomic approach was followed for the isolation of plasmid DNA from fecal samples [39]. Isolated DNA was stored at −20°C until further use and its quality, quantity, and purity were determined using agarose gel electrophoresis and absorbance measurements on spectrophotometer (UV mini-1240, UV-Vis spectrophotometer, Shimadzu Corporation). PCR was performed in PROGENE (Techne) using forward primer 5’-CTCGGTTGATAGGCCAGGT-3’ and reverse primer 5’-ACCTTGATGTCACCAGTACG-3’ which are specific for 323 bp fragment of pedA gene located on the cryptic plasmid pCP289 of *P. acidilactici* MTCC5101 [13]. The amplification program consisted of 1 cycle of 94°C for 3 min and 30 cycles of amplification (94°C for 1 min, 63°C for 1 min, and 72°C for 1 min).

#### 2.4.4. Bacteriocin Activity Assay

Well diffusion assay was performed as per standard methodology of Sarkar and Banerjee [40]. MRS hard agar (1% w/v) was overlayed with MRS soft agar (0.75% w/v) preseeded with approximately one million cells of indicator *Enterococcus faecalis*. Wells were filled with heat inactivated supernatant of MRS grown fecal
3. Results

3.1. Adhesion of P. acidilactici MTCC5101 to Caco-2 Cells. Figures 1(a) and 1(b) represent SEM micrographs of untreated Caco-2 cells and P. acidilactici MTCC5101 with their typical tetrad arrangement. After examining the SEM images of cocultured P. acidilactici MTCC5101 with Caco-2 cells, it is clearly illustrated that the selected probiotic has a very high tendency to adhere to intestinal epithelium or Caco-2 cells at the mucosal surface. Adhesion of selected probiotic strain onto monolayers of Caco-2 cells was evaluated on different microscopic fields per coverslip and at an average 152 ± 33 cells adhered per 100 Caco-2 cells. A biofilm of adherent bacteria constituted of pediococci was observed. The bacteria were also found to adhere to each other (Figures 1(c) and 1(d)).

3.2. Total LAB Count in Fecal Samples. In Table 1 the total cell counts of P. acidilactici MTCC5101 in buttermilk base are recorded. All the subjects have received oral doses of 10^8 – 10^10 cells/mL/day of P. acidilactici in buttermilk base. The fecal swab samples cultured in MRS medium showed growth of mixed LAB with cell counts of 9.27 ± 1.01 cells/mL at the baseline (day 0) and 9.31 ± 0.98 cells/mL after 4 weeks of intervention. Similarly, fecal swab samples from control group showed cell counts of 10.17 ± 1.06 cells/mL at the baseline and 9.76 ± 0.33 cells/mL after 4 weeks (Table 2).

3.2.1. Molecular Identification of P. acidilactici MTCC5101 in Fecal Samples. Results of the PCR analysis coincide with count data that indicates detectable levels of P. acidilactici MTCC5101 in all the volunteers after a 4-week intervention. Figures 2(a) and 2(b) depict agarose gel analysis of 323 bp fragment of partial pedA gene amplified from isolated whole LAB plasmids of 5th and 10th day’s fecal samples. Intensity of the PCR amplicon is lesser in the 5th day’s samples which show that the establishment of the probiotic strain requires a little longer time to persist and colonize in the human gut. The fragment of the expected size was obtained in nearly all 10th day’s samples and the bands in agarose gels were more prominent as compared to 5th day which confirms that the pediococci have started gut colonization. A similar pattern is observed in the 20th and 30th day’s fecal samples on 2% agarose (Figures 2(c) and 2(d)).

3.2.2. Bacteriocin Activity Assay. To check for pediocin CP2 profile in the mixed fecal cell cultures of 30th day, standard well diffusion assay was carried out using E. faecalis as the indicator strain. After 24 h, definite zone of inhibition was seen in the plates that also confirms our previous findings on successful establishment of P. acidilactici MTCC5101 in GI tract of volunteers (Figure 3).
**Figure 1:** SEM micrographs of (a) untreated Caco-2 cells (magnification level 4,000x) (b) *P. acidilactici* MTCC 5101 (magnification level 9,000x), and (c) and (d) adherent *P. acidilactici* MTCC 5101 on Caco-2 cells (magnification level 2,000x and 6,500x).

**Table 4:** Adhesion studies based on Probiotics.

| Organism(s)                        | Cells/cell line(s) | Adhesion index* | Reference |
|------------------------------------|--------------------|-----------------|-----------|
| *Bifidobacterium breve* (4, 5, 25) |                    |                 | [17]      |
| *B. longum* (4, 16, 18, 22)       | Caco-2             |                 |           |
| *B. bifidum* (7, 8)               | HT29-MTX           |                 |           |
| *B. infantis* (1)                 |                    |                 |           |
| *Lactobacillus rhamnosus* GG      |                    |                 |           |
| *L. casei* strain Shirota          |                    |                 |           |
| *L. johnsonii* Lal1               |                    |                 |           |
| *L. rhamnosus* LC 705             | Mucus from feces   |                 |           |
| *B. lactis* Bb12                  |                    |                 |           |
| *L. rhamnosus* GG                 |                    |                 | [21]      |
| *B. animalis* subsp. *lactis* Bb12| Caco-2             |                 |           |
| *B. animalis* IATA-A2             | HT29-MTX           |                 |           |
| *B. bifidum* IATA-ES2             |                    |                 |           |
| *L. plantarum* (9, 72, 75, 77, 90, 91) |                |                 | [22]      |
| *L. delbrueckii* subsp. *bulgaricus* CH4 |            |                 |           |
| *L. plantarum* CSCC5276           |                    |                 |           |
| 163 *Lactobacillaceae* sp.         | HT-29              |                 | [23]      |
|                                    | Caco-2             |                 |           |
| *P. acidilactici* MTCC 5101       |                    |                 |           |
|                                    | Caco-2             |                 |           |
| *P. acidilactici*: 152 ± 33       |                    |                 | Present study |

* Adhesion is indexed as % adhesion or mean ± SD of the number of bacterial cells adhered per 100 cells of cell line used.
Figure 2: 2% agarose gel showing 323 bp DNA fragment amplified using plasmids from fecal samples (a) M: marker pUC 19/Msp Digest, C(+): positive control, Lane 1–10: 5th day samples, C(−): negative control. (b) M: marker pUC 19/Msp digest, C(+): positive control, lane 1–10: 10th day’s samples, C(−): Negative control. (c) M: Marker pUC 19/Msp Digest, C(+): Positive control, Lane 1–10: 20th day’s samples, C(−): negative control. (d) M: marker pUC 19/Msp Digest, C(+): positive control, lane 1–10: 30th day’s samples, C(−): negative control.

Figure 3: Well diffusion assay of cultured fecal samples of 30th day’s against E. faecalis. C: P. acidilactici MTCC 5101; C1–C3: Fecal swab samples of volunteer controls; 1–10: Fecal swab samples of volunteer subjects.

3.3. Wellness Parameters Performed. Routine blood tests were performed to estimate the effect of probiotics on wellness parameters of volunteers. The tests include WBC and RBC counts, levels of Hb in blood, bleeding time, and clotting time (Table 3). Haematological survey was carried out before as well as after feeding trial to estimate the effect of probiotics on some of the wellness parameters of test subjects. Results indicated a small yet insignificant increase in the values of RBC counts and Hb levels of subjects. The findings confirm the safe oral consumption and health improvement capacity of probiotic strain.

4. Discussion

Bacterial adhesion to epithelial cells in gut is initially based on nonspecific physical interactions between the two surfaces [41, 42]. After primary attachment to epithelial surface, secondary interactions between bacterial adhesins and complementary epithelial receptors play a key role in adhesion of bacterial cells to intestinal mucin and enterocytes [22, 43–47]. Since there is difficulty in studying bacterial adhesion in vivo, intestinal cell lines are widely used as in vitro models for assessment [48]. We have used a well-characterized cultured colon carcinoma Caco-2 cell line displaying typical features of enterocytic differentiation in the form of villi to study adhesion of P. acidilactici MTCC5101. A strong adherence of probiotic strains to intestinal epithelial cells has been reported previously in a number of studies (Table 4). In a recent study by Jensen et al. [49], it has been reported that the adhesion capacity of probiotics varies from species to species as a variation from 1% to 25% has been observed in case of 18 known probiotic lactobacilli and pediococci.
A comparative analysis of such of probiotic bacteria and prevention of diseased condition. Colonization studies prove successful intestinal colonization in intolerance, and inflammatory bowel diseases [27, 29, 34, 50–55], induced diarrheal disease, viral and bacterial diarrhea, lactose intolerance, and inflammatory bowel diseases such as antibiotic-induced diarrheal disease, viral and bacterial diarrhea, lactose intolerance, and inflammatory bowel diseases [27, 29, 34, 50–55].

Persistence of probiotic strains in GI tract is also demonstrated by their bile and acid resistance properties, as shown in earlier studies carried out on the present strain [12, 61–64].

The current study provides clear evidence that P. acidilactici MTCC5101 adheres strongly to villi of Caco-2 cells. These results further strengthened the claim of this strain for selection as a probiotic for human use.

Oral consumption of probiotics has been advocated with prophylactic and curative properties that have been observed in case of intestinal disorders such as antibiotic-induced diarrheal disease, viral and bacterial diarrhea, lactose intolerance, and inflammatory bowel diseases [27, 29, 34, 50–55]. Recently, there has been accumulation of evidence from rigorous clinical studies on well-characterized probiotics having real health-promoting properties [56, 57].

Although minimum effective dose is not known exactly, usually an oral dose of $10^8$ CFU/day or more than this has been followed in most studies (Table 5). Previous clinical and colonization studies prove successful intestinal colonization of probiotic bacteria and prevention of decreased condition. A comparative analysis of such in vivo clinical studies demonstrates that the persistence time of probiotic bacteria in gut varies from strain to strain (Table 5). The relative strain-specific persistence in vivo correlates accurately and significantly with in vitro outcomes as evident from a recent study on Lactobacillus plantarum [58]. Studies on probiotic Lactobacillus casei strain DN-114 001 and L. casei strain Shirota have proven the capacity of these strains to survive and colonize human gut [59, 60]. Persistence of probiotic strains in GI tract should lead to shedding of live cells in fecal samples which can be detected using quantitative methods like PCR [37, 70]. In the present parallel, controlled human intervention study, in vivo persistence and colonization of P. acidilactici MTCC5101 in GI tract provides a clear evidence for intimate interactions between the selected probiotic bacteria and intestinal mucosal surface. These interactions allow probiotic strain to persist in gut for a considerable time period, regardless of the dietary and physiological differences among individuals selected in the study. Furthermore, results indicate that buttermilk is a suitable carrier medium for P. acidilactici MTCC5101 strengthening the use of buttermilk as a probiotic product.

| Organism(s) | Subjects | Dose levels (CFU/mL/day) | Response/outcomes | Reference |
|-------------|----------|--------------------------|-------------------|-----------|
| Lactobacillus paracasei subsp. paracasei (CRL-431) | Children and adults with diarrhea | $10^7$-$10^8$ | Three 15 day trial periods; reduction in incidence of intestinal disorders | [25] |
| L. acidophilus | Healthy volunteers | $10^8$-$10^{10}$ | 1-week trial; effective gut colonization | [26] |
| Bifidobacterium lactis BB-12 | Children with atopic eczema | $10^8$-$10^9$ | 2-month trial; controlled allergic reactions | [27] |
| Lactobacillus GG | Healthy volunteers | $10^9$ | 40-day trial; survival in the gut | [28] |
| B. longum SBT2928 | Children with acute infectious diarrhea | $10^5$ | Prophylactic; reduction in duration of diarrhea | [29] |
| Lactobacillus GG | Healthy volunteers | $10^8$-$10^{12}$ | 1-week study; successful colonization of gut | [30] |
| L. casei subsp. rhamnosus Lcr35 | Healthy volunteers | $10^8$-$10^{10}$ | 28-day trial; gut colonization; immune modulation | [31] |
| L. reuteri ATCC 55730 | Healthy volunteers | $10^8$ | Reduction in incidence of acute diarrhea and rotavirus shedding | [32] |
| B. animalis subsp. lactis BB-12 | Children with rotavirus diarrhea | $10^8$ | Prophylactic against acute diarrhea | [33] |
| Streptococcus thermophilus | Healthy breastfed infants | $10^6$ | 12-week trial; fewer and shorter episodes of diarrhea | [34] |
| B. animalis subsp. lactis BB-12 | Children with acute diarrhea | $10^7$ | 12-day trial; effective gut colonization | [35] |
| L. reuteri ATCC 55730 | Healthy volunteers | $10^7$-$10^{10}$ | 12-week trial; fecal recovery increases with increase in dose | [36] |
| L. delbrueckii subsp. bulgaricus | Healthy volunteers | $10^8$-$10^{10}$ (L. delbrueckii) | Reduction in incidence of intestinal disorders | [37] |
| S. thermophilus | Healthy volunteers | $10^9$-$10^{10}$ (S. thermophilus) | Three 15 day trial periods; reduction in incidence of intestinal disorders | [38] |
| B. animalis subsp. lactis BB-12 | Children with acute diarrhea | $10^8$-$10^{10}$ | 7-week study; fecal recovery increases with increase in dose | [39] |
| L. paracasei subsp. paracasei CRL-431 | Healthy volunteers | $10^8$-$10^{10}$ | 3-week trial; increase in fecal recovery of viable lactobacilli | [37] |
| L. reuteri DSM 17938 | Healthy volunteers | $10^8$-$10^{10}$ | 4-week trial; colonization and fecal recovery increases with time | Present study |
Abnormal blood biochemical parameters are an indicator of a number of clinical disorders. Oral consumption of probiotics has not been linked to any adverse subclinical effects on blood biochemistry so far [71, this study]. Probiotics have been reported to enhance absorption of essential vitamins and minerals from the diet into the body [72, 73]. The enhanced absorption of vitamins and minerals has led to improved haematological environment and gut health. A slight increase in RBC counts and Hb levels of volunteer subjects after oral feeding with probiotics strain was observed. Both in vitro models and in vivo studies have suggested the successful establishment of \textit{P. acidilactici} MTCC5101 in human gut that is being proposed herein with the possibility of providing beneficial health effects to the host.

5. Conclusions

In conclusion, \textit{P. acidilactici} MTCC5101 can survive passage through the human GI tract when administered orally in a buttermilk food base. Overall, results indicate that \textit{P. acidilactici} MTCC5101 is a safe and potent probiotic strain with strong adhesive and health-improving characteristics. The findings suggest an opportunity for successful use of \textit{P. acidilactici} MTCC5101 in functional food applications in future.

Conflict of Interests

The authors declare that they have no conflict of interests.

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