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Adhesion of *Pediococcus acidilactici* Ch-2 Isolated from Chuli to Gastric Mucin and its Potential in Pathogen Exclusion

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ABSTRACT

To appraise probiotic attributes such as cell surface hydrophobicity, adhesion to mucus and inhibition of pathogens to gastrointestinal tract, *P. acidilactici* Ch-2 isolated from a traditional Apricot product of Himachal Pradesh–Chuli has been used. In this study, the hydrophobicity, adhering abilities to gastric mucin and potential of isolate to inhibit the pathogenic adherence was assessed. Hydrophobicity was determined by bacterial adherence to hydrocarbons, xylene, chloroform and ethyl acetate. Mammalian laryngeal cell line HEp-2C was used to investigate the ability of cell to adhere the epithelial cells. The percentage of hydrophobicity of the strain was found to be 86.0%. Competitive exclusion of pathogens was found to be exhibited by the isolate. Efficient adhesion of the tested strain to laryngeal epithelial cells was observed. Our findings indicated that *P. acidilactici* Ch-2 expressed high capability to adhere to the epithelial cells as shown by hydrophobicity, mucin and mammalian cells adhesion, indicating that this isolate can be a good candidate for probiotic use.

INTRODUCTION

Lactic acid bacteria are one of the most significant bacterial groups living in the large intestine and exert a range of biological activities related to host health. Probiotics which mostly include lactic acid bacteria are defined as "live microorganisms, which when administered in adequate amounts confer a health benefit to the host (1). They are generally members of the healthy indigenous microflora and their incorporation to food products as nutraceuticals or functional foods can assist in returning a disturbed microbiota to its normal beneficial composition (2).

Characteristics which are important for lactic acid bacteria (LAB) to be a successful probiotics include safety, processing and storage stability, antagonism against pathogenic bacteria, microorganisms capable of surviving in the gastrointestinal system and adherence potential to the intestinal epithelium of the host (3). Lactic acid bacteria possess some important activities such as significant adhesion to gastrointestinal mucus, inhibition of pathogenic attachment and stimulate immune system reactivity (4). The important prerequisite for colonization of probiotic strains in the gastrointestinal tract is their ability to adhere the lining of intestinal epithelial cells (5). Usually successful probiotic bacteria are able to colonize the intestine at least temporarily by adhering to the intestinal mucosa. Several studies also have suggested the ability of probiotic bacteria to prevent the attachment of pathogens such as coliform bacteria and clostridia and encourage their removal from the infected intestinal tract (6-8).

A necessary step in the infection process is the adherence of pathogens to the host tissue (9). Probiotic bacteria have been reported to interfere with the adhesion of other microbes. The capability of probiotic bacteria to bind to the intestinal lining is predicted to have enduring beneficial health effects some of which include the pathogenic exclusion, immunomodulation and the production of beneficial bacterial molecules. Binding of bacterial cells to intestinal cell surfaces is thus commonly considered to be an important property and in addition to survival it is the main feature often investigated in relation with the probiotic characteristics of bacteria (10). These considerations led to the present study, which was focused on an assessment of the cell surface hydrophobicity and adhesion properties of *Pediococcus acidilactici* Ch-2 and its ability to inhibit and displace food borne pathogens.

MATERIALS AND METHODS

Experimental Section

1. Bacterial strains and culture conditions

P. acidilactici Ch-2 (accession no KJ541886) investigated in the present study was isolated from Chuli-a traditional Apricot product of Himachal Pradesh. The isolate was cultured in MRS broth in anaerobic jars at 35^0C. Before the adhesion test, bacteria were counted by viable cell plate count method.

2. Cell culture

HEp-2C (human laryngeal carcinoma) cell line tested which was kindly provided by Dr. S S Kanwar (Department of Biotechnology, HPU, Shimla (HP) India). HEp-2C cells were routinely grown in Dulbecco-modified minimal essential medium (DMEM; HiMedia) supplemented with 10% inactivated fetal calf serum (FCS). Cells were cultured in 25 cm^2 flasks in an incubator with 5% CO_2 at 37^0C. For adhesion assay, monolayer of HEp-2C was prepared in Leighton tubes (1.5 ml) with 10^4 cells/ml.

3. Adhesion assay

The cell monolayer was washed twice with phosphate buffered saline (PBS) before the adhesion test. Bacterial strains were grown for 48h at 35^0C in MRS broth, centrifuged and re-suspended in PBS at a concentration of 10^8 cells/ml. Aliquot of the bacterial suspension (1.5 ml) was added to the Leighton tubes and incubated for 2h at 37^0C. After incubation, monolayers were washed 5 times with PBS, fixed with methanol, Gram stained and counted under imaging microscope (Zeiss), using 20 mammalian cells in 10 randomized microscopic fields. Adhesion was measured as the number of bacterial cells adhering to monolayer. Adhesion score was expressed as: non adhesive for fewer than five bacteria adhered to 100 cells, adhesive with six to 40 bacteria adhering to 100 cells and strongly adhesive with more than 40 bacteria adhering to 100 cells (11).

4. Measurement of bacterial hydrophobicity

Microbial adhesion to hydrocarbons (MATH) was evaluated to measure the hydrophobicity. Bacterial cells after growth for 24h and Centrifugation, were washed twice in phosphate buffer and diluted in the same buffer to 0.5 OD units at 600 nm. One hundred ml Xylene (Merck) was

added to 3 ml of the bacteria. The tube was vortexed for 1 min. After 15 min, the OD of the aqueous phase was determined (12). The decrease in the absorbance of the aqueous phase was taken as the measure of the cell surface hydrophobicity (H%) which was calculated with the following formula:

$$\%H = [\{A0- At\}/A0)] \times 100$$

Where At represents the absorbance at time t=2h and A0 the absorbance at t =0h.

5. Mucin binding assay

Adherence ability of *P. acidilactici* Ch-2 was investigated with the mucin type III form porcine stomach (Sigma Aldrich). Gastric mucin (0.5 mg/ml in PBS) was immobilized passively into microtiter plate wells (Maxisorp; Nunc, Denmark) by overnight incubation at 4^0C. Bacterial cells were added as a volume of 150 µl into microtiter plate wells already coated with mucin and allowed to adhere at 37^0C for 1h and after that these were stained with crystal violet (150 µl/well; 0.1% solution. The absorbance values at 620 nm were determined using Microtiter plate reader (Maxisorp; Nunc, Denmark). Stained mucus without added cells was used as negative control and absorbance values of this control were subtracted from absorbance values of the sample (13).

6. Inhibition/Exclusion of pathogen adhesion to intestinal mucus

Potential of *P. acidilactici* Ch-2 to inhibit the adhesion of pathogens viz. *L. monocytogenes* MTCC 839, *C. perfringens* MTCC 1739 and *B. cereus* CRI was assessed by using the same procedure for bacterial adhesion to gastric mucin with minor modifications i.e. probiotic bacteria were adhered first followed by pathogen adhesion. The inhibition of pathogens was calculated as the difference between the adhesions of the pathogen in the absence and presence of probiotic bacteria (14).

7. Displacement of pathogen adhered to intestinal mucus

The displacement of already adhered pathogens by *P. acidilactici* Ch-2 was assessed by following the method used for microbial adhesion to mucin with minor modifications. Displacement of pathogens was calculated as the difference between the adhesions after the addition of the probiotic strains [14].

8. Competence between pathogen and *P. acidilactici* Ch-2 to adhere to intestinal mucus

Competitive exclusion of pathogens by probiotic isolates was determined by following the same procedure for microbial adhesion to gastric mucin with minor modifications i.e. probiotic bacteria and pathogens were immobilized simultaneously. Competitive exclusion was calculated as the percentage of pathogens bound after the combination with probiotic bacteria relative to pathogens bound in the absence of probiotic bacteria (14).

RESULTS AND DISCUSSION

Three different solvents were used to evaluate hydrophobic/hydrophilic cell surface properties. The results (Fig. 1) revealed that *P. acidilactici* Ch-2 showed very high affinity to xylene (86%) which indicates strong hydrophobic properties of cell surface to apolar solvents. Affinity to chloroform exhibited by isolates shows the electron donor character of cell surface and the basic character while the affinity to ethyl acetate exhibited by probiotic isolates shows the electron acceptor characters and acid character (15). *P. acidilactici* Ch-2 showed a higher affinity to chloroform (85%) as compared to ethyl acetate (82%) indicating that the cell surface of Ch-2 also act as electron donors and have basic character.

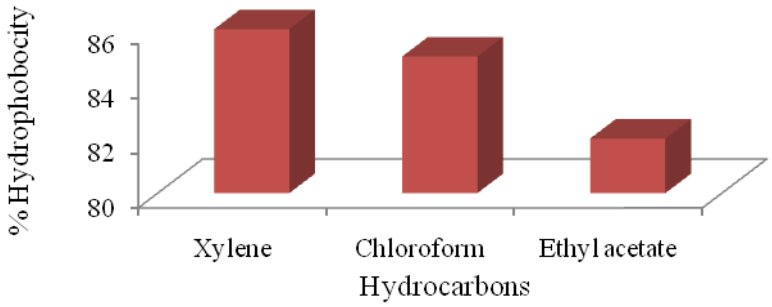

Figure 1. Cell surface hydrophobicity of *P. acidilactici* Ch-2

Adherence to the gastrointestinal mucosa is considered an essential requirement for successful colonization and plays an important role in immune modulation of the host by the Probiotics. Therefore, the adherence ability of *P. acidilactici* Ch-2 was investigated with the mucin type III form porcine stomach (Sigma Aldrich) and was observed to exhibit 49.5% adherence towards gastric mucin. Potential of *P. acidilactici* Ch-2 to inhibit, displace and compete with the pathogens viz. *L. monocytogenes* MTCC 839, *C. perfringens* MTCC 1739 and *B. cereus* CRI was assessed by using the same procedure for bacterial adhesion to gastric mucin with minor

modifications. Maximum exclusion of pathogenic bacteria was observed through competition for adhesion site against pathogens i.e. 66.26%. Maximum competition was observed against *C. perfringens* (87.58%) followed by *B. cereus* (78.70%) and *L. monocytogenes* (32.50%). *P. acidilactici* Ch-2 exhibited 66.0% inhibition of binding of pathogenic bacteria to gastric mucin. Maximum inhibition was observed against *B. cereus* (77.13%), followed by *C. perfringens* (71.85%) and *L. monocytogenes* (49.02%). Ability of *P. acidilactici* Ch-2 to displace pathogenic bacteria was a bit less (51.31%) where isolate exhibited 82.11, 66.49 and 5.33% against *C. perfringens*, *B. cereus* and *L. monocytogenes*, respectively.

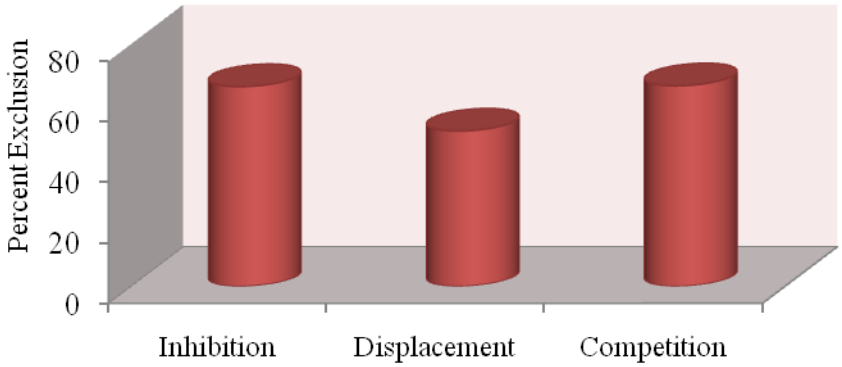

Figure 2. Exclusion of pathogen adhesion to intestinal mucus

The results of bacterial adhesion to HEp-2C cells are shown in Fig. 3. The adhesion of bacterial cells per 20 mammalian cells was observed and 400 bacterial cells were found to adhere showing strong adhesive potential to the mammalian cells.

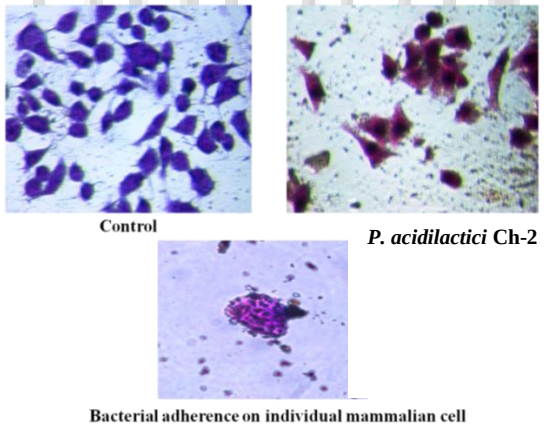

Figure 3. Adhesion of *P. acidilactici* Ch-2 to HEp-2C cells

Now-a-days screening of lactic acid bacteria from rare and least explored food sources for potential probiotic applications has been carried out. So, the focus of our study was to explore Chuli to isolate potential probiotic isolates with good adhesion properties. Bacterial cells with high degree of hydrophobicity generally form strong interactions with mucosal cells. Cell surface hydrophobicity and mucosal adherence demonstrate a direct correlation with each other (16). In the present study, isolate showing a high hydrophobicity reveals its potential to adhere to the gastrointestinal mucus efficiently, thus proving its strong probiotic character. Similar results were obtained (17, 18). Adherence to the gastrointestinal mucosa is considered an essential requirement for successful colonization and plays an important role in immune modulation of the host by the Probiotics.

In vitro adherence to gastrointestinal mucus mimics the *in vivo* conditions and strong ability of bacterial isolate to bind to the gastric mucin reveals its strong potential to adhere to the gastrointestinal epithelial cells, revealing its potential probiotic character. Similar study was investigated by Ouwehand et al. (19) where the adherence ability of lactic acid bacteria isolated of dairy origin to intestinal mucus was studied and significant variation in adhesion between the strains ranging from 3% (*Lactobacillus casei* 01) to 43% (*L. rhamnosus* GG) adhesion was found.

The competitive exclusion of pathogens by probiotic bacteria is another important characteristic for their successful survival and inhibition of pathogenic bacteria. In the present study, isolate Ch2 exhibited good exclusion and competition against pathogenic bacteria while the displacement percent was comparatively less, advocating that the probiotic bacteria Ch-2 inhibited the pathogenic bacteria through completion for adhesion site and exclusion from the respective site. Our results showed resemblance with report of Gueimonde et al. (20) who studied the competitive exclusion of enteropathogens from human intestinal mucus by probiotic *Bifidobacterium* strains and found that four *Bifidobacterium* strains tested were able to inhibit the adhesion of the five pathogens and also displaced them efficiently. Nuraida et al. (21) also evaluated the adhesion competition between *Lactocbacilli* and Enteropathogenic *E. coli* (EPEC) and found that lactobacilli isolates were able to displace indigenous *E. coli* while were able to compete with EPEC for adhesion. Since bacterial adhesion to intestinal epithelial cells is considered one of the most crucial selection criteria for probiotic strains (22), we observed the

adherence capacity of the *P. acidilactici* strain on HEp-2C cells. Adhesion of *P. acidilactici* Ch-2 to epithelial cells would allow colonization of the intestinal epithelial mucosa, therefore could limit the overgrowth of pathogens. Previous studies indicated that lactic acid bacteria are able to adhere to the surface of intestinal epithelial cells in tissue culture (23-25).

P. acidilactici Ch-2 isolated from Chuli has been found to fulfill all the adhesion criteria for successful establishment in the GI tract of human being viz. hydrophobicity, adhesion to gastric mucin, competitive exclusion of pathogenic bacteria and finally *in vitro* adherence to HEp-2C epithelial cell lines. Thus, the isolate proved its potential as a good probiotic candidate and can be further recommended for its use as nutraceutical agent and in functional foods and pharmaceutical preparations.

CONCLUSION

In conclusion, this study indicates that *P. acidilactici* Ch-2 isolated from Chuli exhibited effective adherence properties along with the ability to displace pathogenic bacteria from the host surface. This study has provided valuable information on the *in vitro* adherence characteristics of isolate to solvents, gastric mucin as well as mammalian epithelial cell lines and the competitive exclusion of pathogenic bacteria which has helped to prove the antagonistic potential of probiotic candidate that can be used further in foods, complementary and alternate medicines for development as potential probiotics.

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Author Contributions

Anupama Gupta conducted laboratory work and drafted this paper as a Ph.D. student. Nivedita Sharma, principal supervisor, finalized article write-up and is responsible for manuscript preparation.

Conflicts of Interest

The authors declare no conflict of interest.

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