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EVALUATION OF *ENTEROCOCCUS FAECIUM* CH-1 ISOLATED FROM CHULI- A TRADITIONAL FERMENTED APRICOT PRODUCT OF TRANS HIMALAYAN REGION

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CONFLICTS OF INTEREST

There are no conflicts of interest for any of the authors.

ABSTRACT

Background:
Chuli is a naturally fermented apricot fruit product of Himachal Pradesh and is a rich source of polyphenols and other phytochemicals such as betacarotene and ascorbic acid**.** In the present research work potential lactic acid bacteria were isolated and explored for its novelty as potential probiotics.

Methods: Isolates were screened on the basis of broadest inhibitory spectrum against various food borne pathogens i.e , and hence was selected for further study i.e acid and bile tolerance, adherence to gastric mucin sensitivity towards different antibiotics. its relative safety as probiotic candidate was also assessed.

Results: Highest antagonism was show by *Enterococcus faecium* Ch-1 and was found to be tolerant to low pH and high bile concentrations, adherance to gastric mucin. *E. faecium* Ch-1 was found negative for gelatinase, DNase enzyme activity and haemolysis thus validating its relative safety as probiotic candidate.

Conclusion: *Enterococcus faecium* Ch-1 was found to be a good probiotic strain with cumulative probiotic score of 100% therefore, could be promising for the development as suitable isolate for use in functional foods.

Keywords: acid and bile tolerance, antagonism, antibiotics, chuli, DNase, functional foods, gastric mucin , gelatinase, haemolysis, probiotic.

INTRODUCTION

The Trans Himalayan region of Himachal Pradesh is an arid high altitude desert unlike any other part of the Indian subcontinent. This landscape is a panorama of high snow capped peaks and bare multi hued hills sculpted by the forces of nature. The high valleys range from 2,500 m to 4,500m. Approximately 10% of the world's population lives in mountain areas [1]. However indigenous people possess an immense traditional knowledge of food products. The fermented foods have been prepared and consumed for thousands of years, and are

strongly linked to culture and tradition of the Trans Himalayan regions. A naturally fermenting Apricot fruit product chuli of Himachal Pradesh and is consumed as dried fermented fruit by the local people of hilly areas of this regions especially Lahaul Spiti and Kinnaur.

Lactic acid bacteria are generally considered to be safe (GRAS status) and are present naturally in the fermented food products and human intestine, therefore they are preferentially exploited for the commercial use as probiotics [2,3]. Lactic acid bacteria viz. *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Enterococcus, Oenococcus* and *Weissella* spp. and some yeasts,[4,5] produce a number of vitamins and increase the nutritional value of the food products. *Enterococcus faecium* along with Bacillus spp. – and some yeast strains are among the popular commercially available probiotic products in animal nutrition in the European countries [4,5] . Though the *Enterococci* are ubiquitous microorganisms, some fermented food products and the gastrointestinal tract of humans and warm-blooded animals are some of their major habitats [6]. *Enterococci* have been involved as the predominant probiotic lactic acid bacteria (LAB) in the development of the typical organoleptic characteristics of a variety of fermented foods such as cheeses, fermented sausages and vegetables [7], various studies have reported the benefits of using *Enterococcus*, especially *Enterococcus faecium* strains, as adjunct cultures in fermented foods, due to the ability to inhibit the growth of food-borne pathogens commonly present in the food products [7] . The consumption of probiotics has beneficial effects such as balancing colonic microbiota, protection of the normal intestinal microbiota, prevention of gastrointestinal disorders, modulation of immune system function, antitumor, anti-inflammatory effects, alleviation of lactose intolerance, reduction of serum cholesterol, antagonism against food-borne pathogens and improvement in the nutritional value of foods [8-11].

Criteria for selection of probiotic strains formulated by the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) are gastric and bile acid resistance, competition with pathogens for adhesion sites, growth inhibition of potentially pathogenic bacteria and antibiotic susceptibility [3]. Antibiotic resistance, haemolytic activity and some enzymes such as Gelatinase and DNase production are considered as important virulence factors and good indicators in order to select potential probiotics strains. Probiotics are able to coaggregate with pathogens and will efficiently inhibit and kill pathogenic bacteria as antimicrobial compounds can move directly on pathogens [12].

Coaggregation with a pathogen is important as it may prevent the pathogen from binding to mucus or epithelial cells. The inhibitory activities of cell free supernatant are related to the production of various secondary metabolites by the probiotic candidates that exert a direct antibacterial action towards the pathogenic bacteria [13]. These include organic acids, bacteriocins, other low-molecular mass peptides and hydrogen peroxides [14] . The present study was aimed to evaluate *E. faecium* Ch-1 isolated from rare and novel traditional fermented apricot product- chuli for potential use as probiotic strain, concerning its safety assessment, survival during simulated gastrointestinal tract passage, autoaggregation and adhesion to gastric mucin and mammalian epithelial cells, antimicrobial activity and its use as nutraceutial or in functional food development.

2. Material and methods

All chemicals used in the present study were of analytical grade from HiMedia Laboratories, Mumbai, India.

2.1 Isolation of lactic acid bacteria

A nutritionally enriched product chuli, being a rich source of naturally fermented microflora was explored for the very first time ever in the history to assess the profile of potential probiotic lactic acid bacteria (Fig.1) using De Man Rogosa and Sharpe (MRS) mediums [15]. The samples before isolation were aseptically collected, weighed (1 g) and enriched in MRS broth for 24 h. Sample dilutions were prepared followed by plating on MRS agar and incubated at 35°C for 24-48 h anaerobically. In total 6 isolates were obtained and were further tested for Gram reaction, catalase test, cell morphology and antimicrobial activity.

Figure 1: Traditional food product chuli explored for the isolation of lactic acid bacteria

2.2 Identification of isolates
The isolates were identified according to their phenotypic and molecular characterization.

2.3 Phenotypic characterization
The following biochemical tests were carried out using 24-48 h old cultures as per standard procedures: gram reaction, catalase reaction, citrate utilization, casein hydrolysis, urease test and indole production. Out of six isolates, *Enterococcus faecium* Ch-1 was selected for further study on the basis of its broadest antagonistic spectrum against spoilage and food borne pathogens by using Bit/disc method [16].

2.4 Molecular identification
Molecular identification of the screened isolate Ch-1 (on the basis of biochemical and antagonistic potential) was carried out on the basis of 16S rRNA gene sequence. The DNA of isolate was extracted and amplified using PCR gene amplification technique with the universal primers of expected product size (1500 bp) i.e. BITS-1 (5'AGAGTTTGATCCTGG) and BITS-4-(5'-TACCTTGTTACGACTT). All amplification reactions were carried out in a PCR thermocycler. The PCR analysis was carried out with a volume of 25 µl mixture and the procedure consisted of 35 cycles of 92^{o}C for 1 min, 55^{o}C for 1 min, 72^{o}C for 1 min. The PCR products were resolved by electrophoresis in a 1.8 (w/v) agarose gel (Sigma) and visualized by Ethidium Bromide staining. Alignment of the 16S-rRNA sequence was conducted by using the BLASTN program from NCBI web site (http://www.ncbi.nlm.nih.gov). Based on maximum identity score, the sequences were selected and aligned using multiple alignment software program Mega6.

2.4.1 Protocol for DNA extraction
250 ml of TAE buffer (1X) was taken in a 500 ml Erlenmeyer flask. 2.5 g of agarose was weighed and added into the TAE buffer (1X). The contents were heated on hot plate till agarose dissolves completely. Contents were cooled to 60^{o}C and 5 µl of ethidium bromide was mixed with pre-cooled agarose. Gel casting tray was fixed properly and kept in an electrophoretic tank. Comb was inserted in the gel casting tray. Agarose solution was poured into the casting tray and was allow to set for 30 min at room temperature. Comb was removed as gel polymerized completely. Electrophoresis buffer i.e. TAE (1 X) was added to the buffer tank. An aliquot of 5 µl DNA sample was mixed with 2µl gel loading dye (Genei, make) and samples were loaded in agarose gel slots. The lid of the electrophoresis tank was closed and leads were attached. A voltage of 1-5 V/cm was applied. Gel was run for 1.5 h. After 1.5 h, electric current was turned off and the gel was examined under transmitted UV light. Photograph was clicked by gel documentation system (Genei, make).

2.5 Screening of isolates on the basis of antagonistic pattern
2.5.1 Antimicrobial activity
All lactic acid bacterial isolates which showed catalase test -ve were tested against indicators by following methods:

Bit method [16]
Well diffusion method [17]

2.5.2 Test indicators
Serious food borne pathogens/food spoilage bacteria i.e. *Listeria monocytogenes* MTCC 839, *Leuconostoc mesenteroides* MTCC 107, *Enterococcus faecalis* MTCC 2729, *Bacillus cereus* CRI, *Clostridium perfringenes* MTCC 1739*, Pectobacterium carotovorum* MTCC 1428, *Escherichia coli* IGMC, *Pseudomonas syringae* IGMC and *Staphylococcus aureus* IGMC were used in screening of bacterial isolates on the basis of their antagonistic potential. The test strains were procured from Institute of Microbial Technology (IMTECH), Chandigarh, Central Research Institute, Kasualli and Indira Gandhi Medical College (IGMC), Shimla. All these test strains were revived twice for 24 h at 37^{o}C before performing the experiments, as all these indicators were preserved in 30% glycerol at -20^{o}C.

2.6 Safety assessment of *E. faecium* Ch-1
2.6.1 Antibiotic resistance (Thirabunyanon et al. 2009) [18]
Twenty four h old active culture of *E. faecium* Ch-1 was seeded on MRS agar plates using swab. Antibiotic impregnated discs (HiMedia, India) were placed on seeded plates and sensitivity of the isolate was detected. The antibiotic susceptibility was determined towards antibiotics such as Ampicillin (AMP) 10 (µg), Augmentin (AMC) 30 (µg) , Gentamicin (GEN) 10 (µg), Cephalothin (CEP) 30 (µg), Cloxacillin (COX) 1 (µg), Cefotaxime (CTX) 30 (µg), Cefoxitin 30 (µg) (CX), Lincomycin (L) 2 (µg), Tetracycline (TE) 30 (µg), Amoxyclav (AMC) 30 (µg), Co-trimoxazole (COT) 25 (µg) and Cefuroxime (CXM) 30 (µg).

2.6.2 Hemolytic activity
Hemolytic activity of *E. faecium* Ch-1 was determined by spot inoculating fresh overnight bacterial culture on Blood agar plates (Hi Media) supplemented with 5% Sheep blood and incubated at 35°C for 24-48h. Hemolytic activity of the isolate was examined for signs of β-hemolysis (clear zones around colonies), α-hemolysis (green zones around colonies) or γ-hemolysis (no clear zones around colonies) on blood agar medium plates [19].

2.6.3 DNase production
DNase enzyme production of *E. faecium* Ch-1 was evaluated by following Gupta and Malik method (2007) [20]. Isolate was streaked on the DNase agar medium (HiMedia) plates and was incubated at 35°C for 24-48h. A clear pinkish zone around the colonies against dark blue background was considered as positive result for DNase enzyme production [20].

2.6.4 Gelatinase production
Gelatinase enzyme production of *E. faecium* Ch-1 was determined by streaking 24h old culture on plates containing MRS agar supplemented with 3% gelatin. The plates were incubated at 35°C for 24-48h and was then flooded with saturated ammonium sulphate solution (HiMedia). Development of clear zones around the colony against the opaque background indicated a positive reaction [21].

2.7 Assessment of probiotic attributes
2.7.1 Tolerance to low acid conditions
Acid tolerance of *E. faecium* Ch-1 was studied by the method of Liong and Shah (2004) [22] with slight modifications. In brief, culture was inoculated in MRS broth and incubated at 35°C for 24 h followed by centrifuged at 11,200 g for 10 minutes at 4°C. Pellet obtained after centrifugation was washed twice with sterilized phosphate buffer saline and the bacterial cells suspended in phosphate buffer saline were further diluted to 1/10 in buffers of different pH such as 1, 2, 3 and 6.5 followed by incubation for 3 hours. Acid tolerance was determined by comparing the final plate count on MRS agar after 3 hours with the initial plate count at 0 hour.
2.6.2 Effect of bile salts on the growth rate of *E. faecium* Ch-1

2.7.2 Tolerance to bile salt conditions
Effect of bile salt on the growth of *E. faecium* Ch-1 was studied by the method Gilliland and Walker (1990) [23] . MRS broth containing 0.3% (w/v) of ox bile (HiMedia Laboratories, Pvt., Ltd.) was inoculated with 10% (v/v) (approximately 10^8 CFU/ml) of the isolates and incubated at 35°C for 8 hours. The effect of bile salt on growth was measured on the basis of time required to increase the absorbance at 620mn by 0.3 units in MRS broth with and without 0.3% ox bile. The difference in time (h) for attaining desirable absorbance between both culture media was considered as the lag time (LT). Viability of cells in MRS broth supplemented with 0.3, 1 and 2% of bile salt for 8 hours was observed by plating 100µl of culture onto MRS agar plates and incubated at 35°C for 24 hours. Growth of bacteria was expressed in colony forming units per milliliter (log CFU/ml) and the percent survival of strain was then calculated.

2.7.3 Survival in simulated *in vitro* digestion
Simulated gastric and intestinal juices were prepared by dissolving pepsin (HiMedia) and pancreatin from porcine pancrease, (HiMedia) in sterile saline (0.85 % w/v) to a final concentration of 3g/L and 1g/L, respectively. The pH of simulated gastric juice was adjusted to 2.0 and 3.0 and the pH of simulated intestinal juice was adjusted to 8.0. *E. faecium* Ch-1 cells were incubated in MRS broth at 35°C for 18 hour and centrifuged at 11,200 g at 4°C for 10 min. The cell pallets obtained were washed three times in PBS buffer solution (pH 7.0). The cells were inoculated at 10^8 CFU/ml into simulated gastric (pH 2.0 and 3.0) and intestinal juice (pH 8.0). The mixture was then vortexed at maximum setting for 10s and incubated at 35°C. 0.1ml aliquot for gastric and intestinal transit assay was removed after 0, 60 and 240min. The pH in human stomach ranges from 1, during fasting, to 4.5 after a meal, and food ingestion can take up to 3 hour. Thus, the tolerance was assayed by determining the viable count in simulated gastric juice after the incubation for different time intervals up to 4 hour. All the experiments were carried out in triplicates [24] .

2.7.4 Autoaggregation
Autoaggregation assay was performed as described by Del Re et al. (2000) [25] with minor modifications. The active bacterial culture of *E. faecium* Ch-1 was grown in MRS broth at 35°C for 24 hour. The cell suspension was centrifuged at 11,200 g at 4°C for 10 min. Pellet was collected and washed twice in sterile phosphate buffer saline (PBS; 0.1 M Phosphate buffer, 0.85% NaCl, pH 7.0). Cells were re-suspended in PBS, mixed by gentle vortexing for 10s and optical density (OD) was set to 0.5 at 600 nm followed by incubation at 35°C for 5 hours.

Absorbance of upper suspension was measured after each hour. 0.1ml of upper suspension was taken and 3.9ml of PBS was added to it. Autoaggregation % was measured as 1- (At/A0) × 100, where At represents the absorbance at time t=1, 2, 3, 4, 5 h and A0 the absorbance at t = 0 h (i.e. 0.5)

2.7.5 Coaggregation

Coaggregation ability of *E. faecium* Ch-1 was determined by following the method described by Del Re et al. (2000) [25] with minor modification. *E. faecium* Ch-1 was inoculated into MRS broth and the indicators such as *Listeria monocytogenes*, *Clostridium perfringens* and *Bacillus cereus* were inoculated in nutrient broth followed by incubation at 35°C for 24 h. Bacterial suspension of *E. faecium* Ch-1 was diluted to OD = 1.0 (λ = 600 nm) and indicators were diluted to OD = 0.6 (λ = 600 nm). Mixtures were then made for the isolate with each indicator strain at 1:1 ratio. *E. faecium* Ch-1 bacterial cells and indicator bacteria were kept as control and were incubated at 35°C for 4 h. Absorbance at λ = 600 nm was observed for mixture and each of individual strain. Coaggregation % was calculated according to Handley's equation [26].

2.7.6 Bacterial adhesion to solvents: Hydrophobicity

The test of adhesion to hydrocarbons (xylene) was adopted to evaluate *E. faecium* Ch-1 for its cell surface hydrophobicity. Microbial adhesion to hydrocarbons (MATH) in terms of the cell surface hydrophobicity (%H), was determined according to the method of Rosenberg et al. (1980) [27] with slight modifications. 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:

$\%H = [\{A_0 - A_t\}/A_0)] \times 100$

Where A_t represents the absorbance at time t=2 h and A_0 the absorbance at t =0 h.

2.7.7 Mucin binding assay

Ability of *E. faecium* Ch-1 to adhere mucin type III from porcine (pig) stomach (Sigma Aldrich) was investigated. An aliquot of 18-24 h old bacterial cells were harvested by centrifugation at 11,200 g for 10 min at 4°C and washed twice with phosphate buffer saline (PBS) (pH 7.3). The optical density of bacterial suspension at 600nm was adjusted to 1.0, giving approximately 10^8 CFU/ml of the bacterial cells. Gastric mucin (0.5mg/ml in PBS) was immobilized passively into microtiter plate wells (Maxisorp; Nunc, Denmark) by overnight incubation at 4°C. Bacterial cells were added as a volume of 150µl into microtiter plate wells already coated with mucin and allowed to adhere at 37°C for 1 h. After 1 h, wells were washed 3 times with 200 µl of PBST to remove non-adhered bacteria cells. Adherent bacteria were fixed at 65°C for 45 min and stained with crystal violet (150µl/well; 0.1% solution) [28] . Wells were subsequently washed five times with PBST to remove excess stain. The stain bound to bacterial cells was released by adding 150µl of Citrate buffer (50mM, pH 4.3). The absorbance values at 620nm were determined using Microtiter plate reader. Stained mucus without added cells was used as negative control and absorbance values of this control were subtracted from absorbance values of the sample. All observations were obtained in triplicate.

2.7.8 Inhibition/ exclusion of pathogen adhesion to intestinal mucus

The ability of *E. faecium* Ch-1 to inhibit the adhesion of pathogens was assessed by using the same procedure for bacterial adhesion to gastric mucin with minor modifications. *E. faecium* Ch-1 was inoculated first followed by adherence of serious food borne and spoilage causing pathogenic bacteria such as *L. monocytogenes, C. perfringens* and *B. cereus.* The inhibition of pathogens was calculated as the difference between the adhesions of the pathogen in the absence and presence of probiotic bacteria [29]

2.7.9 Displacement of pathogen adhered to intestinal mucus

The ability of *E. faecium* Ch-1 to displace already adhered pathogens was assessed by following the method used for microbial adhesion to mucin with minor modifications. Pathogenic bacteria were inoculated first to the mucin adhered to the microtiter wells followed by adherence of *E. faecium* Ch-1 cells to the wells. Displacement of pathogens was calculated as the difference between the adhesion after the addition of *E. faecium* Ch-1 [29].

2.7.10 Competence between pathogen and *E. faecium* Ch-1 to adhere to intestinal mucus

Competitive exclusion of pathogens by *E. faecium* Ch-1 was determined by following the same procedure for microbial adhesion to gastric mucin with minor modifications. In case of competence measurement, *E. faecium* Ch-1 and pathogenic bacterial cells were adhered simultaneously in the ratio of 1:1. 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 [29].

2.7.11 Production of bacteriocin

An overnight culture of *E. faecium* Ch-1 was inoculated (OD_{600} = 1, 10%) into 100ml of MRS broth and cultivated at 37°C for 24 h. Samples were taken every 2 hours for 24 h and bacteriocin activity was measured. To quantify the bacteriocin activity, the agar well diffusion method was used. Neutralized cell free culture supernatant (NCFS) was serially diluted twofold in sterile distilled water and 150 µl of each dilution was added into the wells. Activity units of bacteriocin production were estimated as AU/ml, where AU/ml is defined as the reciprocal of the highest dilution that resulted in inhibition of the indicator strains. *L. monocytogenes* was used as a bacteriocin sensitive indicator strain to determine bacteriocin activity levels [16].

3. Results :

3.1 Isolation of lactic acid bacteria

Six lactic acid bacterial isolates were obtained from chuli and 2 out of 6 were confirmed as rods while other four were confirmed as coccus by microscopic examination. All the isolates were gram positive, catalase negative, unable to utilize citrate, unable to hydrolyze casein and no urease and indole production were observed by any of the isolate. Fig. 1 and Table 1-2

S. No.	Isolate	Food Source	Color	Form	Margin	Elevation	Texture
1.	Ch1	Chulli	Dirty white	Circular	Entire	Raised	Smooth
2.	Ch2	Chulli	Cream	Circular	Entire	Flat	Smooth
3.	Ch3	Chulli	Cream	Circular	Entire	Raised	Smooth
4.	Ch6	Chulli	White	Circular	Entire	Raised	Smooth
5.	Ch7	Chulli	Cream	Circular	Entire	Raised	Smooth
6.	Ch9	Chulli	Translucent	Punctiform	Entire	Flat	Smooth

Table 1: **Morphological characteristics of Lactic acid bacteria (LAB) isolated from Chulli- a traditional fermented product of Himachal Pradesh**

S. No.	Isolate	Gram's reaction	Catalase test	Shape	Mode of growth	Tentative Identification
1.	Ch1	+ ve	-ve	Coccus	Facultative anaerobe	*Lactococcus*
2.	Ch2	+ ve	-ve	Rod	Anaerobe	*Lactococcus*
3.	Ch3	+ ve	-ve	Coccus	Anaerobe	*Lactococcus*
4.	Ch6	+ ve	- ve	Rod	Facultative anaerobe	*Lactobacillus*
5.	Ch7	+ ve	-ve	Coccus	Anaerobe	*Lactococcus*
6.	Ch9	+ ve	-ve	Coccus	Anaerobe	*Lactococcus*

Table 2: Biochemical characterization and tentative identification of Chulli- a traditional fermented product of Himachal Pradesh

3.2 Genotyping:

These data can only guide to the genus level identification. On the basis of morphological and biochemical characteristics, isolates were tentatively identified at genus level as *Lactobacillus* and *Lactococcus/Enterococcus*. (Fig. 2), analysis of the 16S rRNA sequences revealed that the LAB isolated from chuli displayed 99% homology with *Enterococcus faecium* NR042054 and was named as *Enterococcus faecium* Ch-1. The 16S rRNA gene sequences were deposited in gene bank under accession no. KJ541885. Number of nodes in neighbor- joining phylogenetic tree are levels of bootstrap support (%) from 1000 resample database. This isolate has been reported for the very first time from chuli with an exceptionally high probiotic potential.

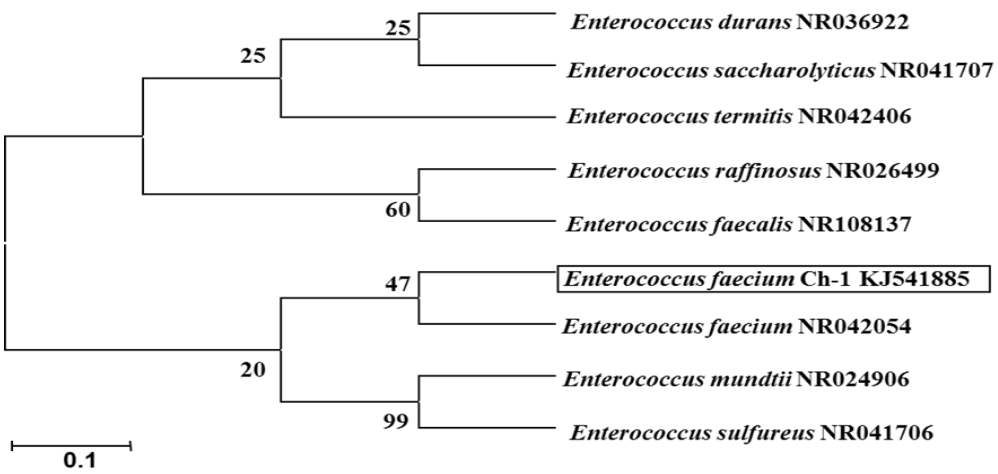
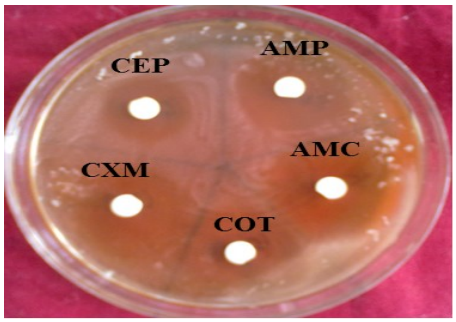


Figure 2: Neighbor-joining tree with 1000 bootstrap values in MEGA 6.0 showing phylogenetic relationship of *Enterococcus faecium* Ch-1 based on a distance matrix analysis of 16S rRNA sequences

3.3 Safety assessment of *E. faecium* Ch-1

3.3.1 Antibiotic resistance

Selected strains LAB's were tested for antibiotic susceptibility/resistibility with antibiotic discs (HiMedia make). Different antibiotic discs were used viz. Ampicillin (AMP), Augmentin (AMC), Gentamicin (GEN), Cephalothin (CEP), Cloxacillin (COX), Cefotaxime (CTX), Cefoxitin (CX), Lincomycin (L), Tetracycline (TE), Amoxyclav (AMC), Co-trimoxazole (COT) and Cefuroxime (CXM). *E. faecium* Ch-1 exhibited 100% sensitivity towards the antibiotics. Mostly, lactic acid bacteria are generally sensitive to inhibitors of protein synthesis such as Tetracycline, Chloramphenicol, Erythromycin and Clindamycin and resistant to glycopeptides (Gentamycin, Kanamycin, Streptomycin, etc.)[24, 30, 31] . But in the present study, all the six screened isolates were found sensitive to protein inhibitors. The antibiotic susceptibility of all these isolates turns them safe and thus suggests their successful use as potential probiotics (Fig.3). According to world health organization (WHO), 2001 and European Safety Authority-EFSA, 2008, bacteria used as probiotics for human and animal use should not carry any transferable antimicrobial/antibiotic resistance gene.



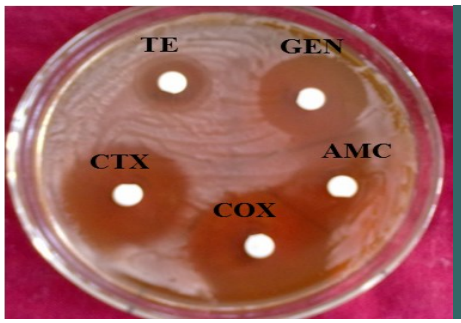
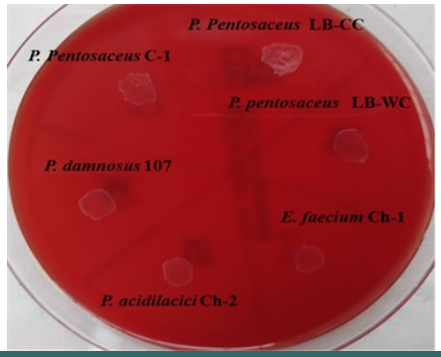


Figure 3: Antibiotic susceptibility profile of *E. faecium* Ch-1 against various tested antibiotics

3.3.2 Haemolytic, Gelatinase and DNase activity

Safety assessment with regard to hemolytic potential is an essential phase in the selection of Enterococci as potential probiotics (Fig.4). *E. faecium* Ch-1 showed no positive hemolysis, DNase and gelatinase enzyme activities, thereby revealing its safe status and its use as potential probiotic candidate.



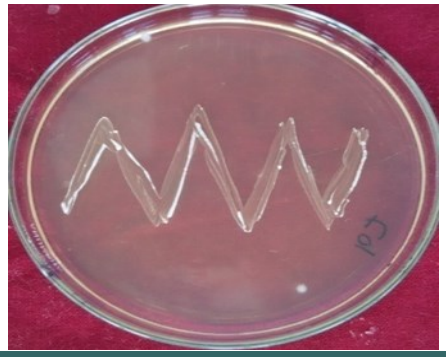


Figure 3: Haemolytic, Gelatinase and DNase activity of *Enterococcus faecium* Ch-1

3.3.3 Tolerance to low acid conditions

In this study this isolate has shown capability to resist as low pH as 1 (during fasting) (Table 3) for about one hour. The isolate tested for survival in acidic environment at varied pH levels showed the ability to grow well even at the minimum tested pH of 1.0 for 60 and 120 minutes of incubation.

pH	Incubation time (min)								
	Cell survival (log CFU/ml)*					**% Cell Survival			
	0	60	120	180	Mean	60	120	180	Mean
1.0	10.00	7.60	0.00	0.000	**4.42**	74.72 (59.79)#	0.00 (0.00)	0.00 (0.00)	**24.90 (19.93)**
2.0	10.04	7.90	7.80	7.50	**8.31**	77.67 (61.77)	76.52 (60.99)	73.02 (58.68)	**75.73 (60.48)**
3.0	10.07	10.12	10.04	9.90	**10.03**	99.50 (86.70)	98.52 (82.98)	96.39 (79.03)	**98.13 (82.90)**
Con-trol	10.14	10.17	10.19	10.27	**10.19**	100 (89.96)	100 (89.96)	100 (89.96)	**100 (89.96)**
Mean	**10.06**	**8.94**	**7.00**	**6.91**		**87.97 (74.55)**	**68.76 (58.48)**	**67.35 (56.91)**	
$CD_{0.05}$	Treatment (0.085) Incubation Time (0.085)					Treatment (0.834) Incubation Time (0.722)			

*log CFU/ml: Mean of results from three separate experiments
**% Survivability = (log cfu pH $_{1.2.3/}$ log cfu $pH_{.65}$) × 100
# Transformed values (Arc sign transformation)

Table 3: Acidity tolerance of screened *Enterococcus faecium* Ch-1

3.3.4 Tolerance to bile salt conditions

The culture was able to grow in the medium supplemented with bile salts upto 2%. Lag time for *E. faecium* Ch-1 was 4 hours. The culture showed good survival in the presence of 0.3, 1.0 and 2.0 % bile salts with survival rate of 95.04, 91.61 and 91.33 %, respectively on 8th h of incubation (Fig. 3). The survival rate of Ch-1 at pH 2.0, 3.0 containing pepsin (depicting stomach conditions) and pH 8.0 containing pancreatin (depicting intestinal conditions) was observed for 4 h. *E. faecium* Ch-1 exhibited good survival at pH 3 (5.39 log CFU/ml) upto 4 h and retained a moderate survival rate at pH 2.0 (4.4 log CFU/ml) after 1 h of incubation (Table 4).

Bile salt concentration	Incubation Time (h)						
	Cell survival (log CFU/ml)*				Cell survival (%)**		
	0	4	8	Mean	4	8	Mean
0.3	10.00	10.04	10.11	**10.05**	99.11 (10.00)#	99.70 (10.03)	**99.40 (10.02)**

1.0	9.92	9.94	10.04	**9.96**	98.12 (9.98)	99.01 (10.00)	**98.56(9.99)**
2.0	9.79	9.85	9.96	**9.86**	97.23 (9.91)	98.22 (9.96)	**97.72(9.93)**
Control	10.13	10.13	10.14	**10.13**	100 (10.05)	100 (10.05)	**100 (10.05)**
Mean	**9.96**	**9.99**	**10.06**		**98.78 (9.98)**	**99.23 (10.05)**	
$CD_{0.05}$	Treatment (N/A) Incubation Time (N/A) TxI (N/A)				Treatment (0.013) Incubation Time (0.009) TxI (0.018)		

*Log cfu/ml: Mean of results from three separate experiments
**% Survivability = (log cfu/ml 0.3,1,2%bile salt/ log cfu /ml0%bile salt) × 100
#Transformed values (Square root transformation)

Table 4: Bile salt tolerance of *Enterococcus faecium* Ch-1 during gastrointestinal transit

3.3.5 Autoaggregation and Coaggregation

Autoaggregation was investigated on the basis of sedimentation characteristics. The sedimentation rate of isolates was measured over a period of 5h. Results showed that the strain exhibited strong autoaggregating ability (97%). *E. faecium* Ch-1 exhibited co-aggregative properties with all the pathogenic strains tested after 4 h incubation at 35°C. In the present study, isolate *E. faecium* Ch-1 has been found to possess a very strong affinity (hydrophobicity) for organic solvent xylene after 2 h of incubation (91.0%) thus validating the isolate to be a potential probiotic strain. In the present investigation, the ability of *E. faecium* Ch-1 to adhere to gastrointestinal mucus, which mimics the GI conditions, was evaluated. The cells of *E. faecium* Ch-1 adhered significantly to the gastric mucin with adherence percentage of 57.19 %.

The competition, inhibition and displacement abilities of probiotics against pathogenic bacteria are strain dependent. *E. faecium* Ch-1 was able to inhibit the adhesion of the tested pathogens such as *L. monocytogenes, C. perfringenes* and *B. cereus* with 44.17, 60.59 and 73.29%, respectively. *E. faecium* Ch-1 was able to displace *C. perfringens* and *B. cereus* (65.72 and 68.76%, respectively) while *L. monocytogenes* remained adhered to the wells coated with mucin (-24.7%). Competition for adhesive site between *E. faecium* Ch-1 and pathogens was was found that *E. faecium* Ch-1 was able to compete for mucus site with *C. perfringens* and *B. cereus* (72.68 and 68.93%, respectively) while *L. monocytogenes* exhibited more competition for mucus sites (-10.19%). The data clearly demonstrated that *E. faecium* Ch-1 could only displace *C. perfringens* and *B. cereus*. *E. faecium* Ch-1 has been able to compete with *C. perfringens* and *B. cereus* for adherence, revealing that the mechanisms of displacement and competition of pathogenic bacteria by *E. faecium* might be similar.

The bacteriocin activity was evaluated by assaying serial twofold dilutions of neutralized culture filtrate supernatant (CFS) against *L. monocytogenes* and maximum bacetriocin production was observed at 18h of growth cycle with 666 AU/ml activity units rendering its potential to be used as a safe and efficient biopreservative as compared to harmful chemical preservative in food products.

4. Discussion

E. faecium Ch-1 isolated from Chuli reported for the first time was evaluated for potential probiotic attributes and has been found to exhibit all the properties required by a strain to be selected as a probiotic. Isolate showed a broad spectrum of antagonism against serious food borne and spoilage causing organisms i.e *Listeria monocytogenes*, *Leuconostoc mesenteroides* , *Enterococcus faecalis*, *Bacillus cereus*, *Clostridium perfringenes, Pectobacterium carotovorum* , *Escherichia coli* , *Pseudomonas syringae* and *Staphylococcus aureus* and hence proved its antimicrobial potential.

In general *E. faecium* strains are known to contain antibiotic resistance genes, but the absence of antibiotic resistance of *E. faecium* Ch-1against many antibiotics used in the present study depicts its safety and its further potential use in food and fermentation industry. Similar results were recorded for *E. faecium* 139 which when tested against antibiotics was found to be strongly susceptible to Chloramphenicol, Erythromycin, Penicillin G,

Streptomycin, Tetracycline and Vancomycin while *E. faecium* CE5-1 was susceptible to only Erythromycin, Penicillin G and Vancomycin [32]. Growth of *E. mundtii* ST4SA was also inhibited by Ampicillin, Bacitracin, Cephazolin, Chloramphenicol, Erythromycin, Novobiocin, Oflaxacin, Oxacillan, Rifampicin and Tetracycline, β -lactam penicillins (Promoxil and Cipadur) and acrolides [33] revealing its safe status. Absence of hemolysis, DNase and Gelatinase enzyme production establishing a possibility of *E. faecium* Ch-1 to be considered as safe and potential probiotic strain in food industry. Bile salts are the surface-active, amphipathic molecules with a potent antimicrobial activity and they act as detergents that disrupt biological membranes [13] The physiological concentration of bile salts in the small intestine is between 0.2- 2.0% [34] and the concentration of bile salt is the key factor which affects the viability of LAB. In this study, bile salt concentrations of 0.3, 1.0 and 2.0% were used and their effect on growth rate of isolate was studies. The results indicate that *E. faecium* Ch-1 can resist the effects of pepsin and pancreatin during the gastrointestinal (GI) transit, therefore could be a potential source for probiotic formulations with effective delivery in GI tract.Better growth of the bacteria on MRS broth than on MRS agar could be the reason for slightly better autoaggregation of cells grown on MRS broth. Similarly, Abdhul et al. (2014) [36] measured the autoaggregation ability of *E. faecium* BDU7 and observed the strain exhibited a strong autoagrregation of 72.7%. Also, the autoaggregation ability of *E. mundtii* ST4V was studied by Todorov et al. (2009) [37] where the observed autoaggregation was 41.34 %. The ability of bacterial isolates to aggregate could be associated to cell surface component, because it was not lost after washing and suspending of the cells in PBS [38] . As the autoaggregation ability is related to the cells' adherence properties of the isolate, the increased autoaggregation capacity might plays an important role in the adhesion of the strain to intestinal epithelium.It has been suggested that probiotic microorganisms that have the ability to coaggregate with pathogens may be better able to kill undesirable bacteria because they could produce antimicrobial substances in a very close proximity to them. Similar studies on coaggregation of lactic acid bacteria with pathogenic strains have been reported by various workers. The results of present study were in close agreement with the finding of Tareb et al. (2013) [39] that the *L. rhamnosus* 3698 and *L. farciminis* 3699 exhibited coaggregation ability with pathogenic strain *C. jejuni* i.e. 21.2% and 23.3%, respectively. The adherence of probiotics to the gastric and intestinal epithelial tissues is an important prerequisite that depends on the hydrophobicity of the bacterial cell surface [40] which helps the probiotic to colonize and modulate host immune system. Adhesion to hydrocarbons like xylene, toluene and n-hexadecane is considered as a biochemical marker for adherence to the gut epithelial cells. The adhesion to xylene (apolar solvent) demonstrates the hydrophobic surface characteristic of bacteria while the affinities to chloroform (polar acidic solvent) and ethyl acetate (polar basic solvent) describe the electron donor and electron acceptor properties of the bacterial cell surface, respectively. Bacterial cells with high hydrophobic properties usually form strong interactions with mucosal cells. *E. faecium* Ch-1 showed a high hydrophobic character and thus showing its potential to adhere to the GI tract efficiently. Similarly, Abdhul et al. (2014) [36] studied the adhesion ability of *E. faecium* BDU7 which was found to be 54.8%. On contrary, Todorov et al. (2009) [37] found *E. mundtii* ST4V to possess a low hydrophobic value i.e. 5.57%. In the strains with probiotic functions, adhesion is an important feature that favors the colonization and establishment of beneficial microbiota in the intestinal tract [41]. The substances responsible for adherence are adhesins. The mucin binding ability exhibited by isolate *E. faecium* Ch-1 contributes to its adhesion property and provides resistance to peristaltic elimination by providing competitive advantage in gut ecosystem. These results suggested the strain being potential probiotic for its use as good probiotic candidate in food as well as in pharmaceutical industry. Similar studies of probiotic adhesion to mucin were reported by many workers. Ability of *Lactobacillus acidophilus* LAB20 to bind mucus isolated from duodenum, jejunum, ileum, cecum and colon of canine intestine was studies and it was found that LAB20 exhibited statistically significantly higher adhesion to canine colonic mucus (1.6%) compared to adhesion to porcine (0.7%) mucus [42]. In the present study, the inhibition of pathogens' growth by bacteriocin production suggests that *E. faecium* Ch-1 isolate may have potential applications in preservation, safety and enhancement of shelf stability of food products. Taking into account that isolate Ch-1 survived in gastrointestinal tract passage, showed antimicrobial activity against important pathogenic and spoilage causing bacteria through lactic acid and bacteriocin production, proficient adherence to gastric mucin and mammalian epithelial cells and fulfilled the important safety criteria as the absence of antibiotic resistance, hemolysis, DNase and gelatinase enzyme production, the isolate *E. faecium* Ch-1 could be recommended as potential probiotic candidate and can be used for the preparation of fermented food products as well as in nutraceutical preparations.

5. Conclusion

Some crucial characteristics such as absence of antibiotic resistance, absence of hemolysis, DNase and gelatinase enzyme production, the ability to survive through gastrointestinal tract passage, to inhibit the growth of food borne pathogens and the ability to adhere to the gastric mucin , make the *E. faecium* Ch-1 isolate studied in this work a potential candidate for further investigations concerning its use as a potential probiotic culture.

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