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Characterization of Potential Probiotic Lactic Acid Bacteria- *Pediococcus acidilactici* Ch-2 Isolated from Chuli- A Traditional Apricot Product of Himalayan Region for the Production of Novel Bioactive Compounds with Special Therapeutic Properties

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Abstract

Objectives: In the present study, *Pediococcus acidilactici* Ch-2 isolated from Chuli has been evaluated for its probiotic potential and its ability to produce bioactive compounds.

Methodology: Isolation was done on MRS agar followed by the safety assessment of isolates. Isolates were screened on the basis of their antagonistic potential against pathogenic bacteria. Probiotic attributes of screened isolate Ch-2 i.e. acid and bile tolerance, survival in simulated gastrointestinal conditions, bile salt tolerance, auto- and co-aggregation, antimicrobial potential and metabolic profiling have been evaluated.

Results: It was observed that *P. acidilactici* Ch-2 was resistance to low pH and bile salts (0.3%) and simulated gastric and intestinal conditions, was able to produce bacteriocin and lactic acid against a number of serious food borne and spoilage causing microorganisms. The susceptibility to selected eleven antibiotics, inability to produce gelatinase and DNase and non-hemolytic nature revealed its safe status for further use in food and nutraceutical industry. In the present study, Squalene – a rare and therapeutic anti-cancerous compound has been reported for the very first time from a probiotic bacteria isolated from fermented food product and recommended for its further investigation as a potential source of Squalene.

Conclusion: In conclusion, most of the results in the present work revealed *P. acidilactici* Ch-2 as a potential probiotic candidate with many functional properties and novel compounds.

Keywords: Chuli; *Pediococcus*; Probiotics; Himachal Pradesh; Squalene; Lactic acid bacteria

Introduction

Probiotics are defined as live microorganisms that contribute to the health and well-being of the hosts by maintaining the intestinal microbial balance [1]. Among these, lactic acid bacteria (LAB) are the potential probiotic bacteria which are already used in many fermented food products and occur naturally in several raw food materials viz. milk and milk products, meat and flour [2]. LAB is used as natural or selected starters in food fermentations in which they perform acidification due to the production of lactic and acetic acids. The protection of food from spoilage and pathogenic microorganisms by LAB is related to their antagonistic activity [3].

The interest in the microorganisms occurring in foods is primarily due to the biotechnological potential of new bacterial species and strains. Recently, use of safe and natural supplements instead of chemotherapeutics has been incorporated because of the advantageous and useful effects to the environment. The excessive use of antibiotics and rapid appearance of multiple-antibiotic resistant pathogenic bacteria has refocused clinical attention on the field of probiotics and their safe and effective use in the pharmaceuticals. The efficacy of lactic acid bacteria as probiotic supplementation has been studied in number of human diseases viz. irritable bowel syndrome, inflammatory bowel diseases, obesity and other allergic diseases. The bioactive compounds secreted by these bacteria with immunomodulation and therapeutic potential are being utilized more now- a -days due to their effectiveness and safe status [4].

Use of probiotics with broad antagonistic potential and natural antimicrobial substances is considered as an appropriate alternative to antibiotic treatment and a better pharmaceutical approach [5]. *Pediococcus acidilactici*, a gram positive coccus, cells arranged in tetrad, is a general inhabitant of fermented fruit and vegetable products and efficiently colonize the digestive tract of the host [6]. Even though the importance of probiotics has been principally coupled with the improvement of gastrointestinal health, recent reports have revealed that potential probiotics also have contributed in reducing other metabolic diseases leading to antihypertensive effects [7]. In the present study metabolic fingerprint of the isolate has been elucidated to find out the possibility of novel compounds with therapeutic potential and Squalene has been reported for the very first time from probiotic *Pediococcus acidilactici* (probiotic lactic acid bacteria) isolated from Chuli with antioxidant properties.

The most important criteria to be considered for the survival and potential benefits on the host are ability to overcome gastric pH and

the toxic effects of bile salts, the advent in a viable physiological state at the site of action, should be capable of adhering effectively the intestinal mucosa and coaggregation ability to reduce the ill effects of pathogens [8]. Most commonly for the isolation of the probiotic bacteria dairy food products have been explored although recently new ideas of isolating new and potential probiotic bacteria from other fermented fruit as well as vegetable food products have been adopted worldwide. Chuli is a traditional fermented Apricot product of Himachal Pradesh with high nutritional profile and a potential source of probiotic lactic acid bacteria (LAB). Therefore, in the present study, *Pediococcus acidilactici* Ch-2 isolated from Chuli has been evaluated for its probiotic potential and its ability to produce bioactive compounds with therapeutic potential.

Materials and Methods

Isolation and identification of probiotic lactic acid bacteria

Lactic acid bacteria were isolated from Chuli- a traditional fermented Apricot product of Himachal Pradesh for the very first time for their potentiality as probiotic (Figure 1) using De Man Rogosa and Sharpe (MRS) medium (HiMedia, India) [9]. Chuli (5 g) was mixed with 45 ml of MRS broth and enriched in it for 24 h. Sample was serially diluted and plated on MRS agar plates at 35°C for 24-48 h anaerobically using anaerobic gas packs (HiMedia, India).

Figure 1: Chuli- A fermented Apricot product of Himachal Pradesh.

All the isolates obtained after anaerobic incubation were cultivated in MRS broth and were maintained in 30% glycerol at -20°C. In total 6 isolates were obtained and were further tested for Gram reaction [10] catalase test [11], cell morphology and antimicrobial activity [12]. Gram positive, catalase negative isolates were selected and identified to the genus level by their morphological, physiological and biochemical characteristics. Out of six isolates, Ch-2 was selected for further study on the basis of its broadest antagonistic spectrum against spoilage and food borne pathogens by using Bit/disc method [12] and was identified to species level by 16S rRNA sequence analysis.

Safety assessment

Some of the important criteria for the assessment of safe status of probiotic bacteria are antibiotic resistance [13], haemolytic activity [14] and some enzymes such as Gelatinase [15] and DNase production [16] and are considered as good indicators in order to select potential probiotics strains. So, antibiotic susceptibility, hemolytic activity, Gelatinase and DNase enzyme production were evaluated to ensure the safety of the isolate to be used as potential probiotic candidate in food and pharmaceutical industry.

Tolerance to low acid conditions and bile salts

Acid tolerance of Ch-2 was studied by the method of Liong and Shah [17] with modifications while tolerance to bile salts was determined by Giililand and Walker [18]. Acid tolerance was determined by comparing the final plate count after 3h with the initial plate count at 0h. Viability of cells in MRS broth supplemented with 0.3, 1 and 2% bile salts (HiMedia, India) upto 8h was observed by plating 100 µl of culture onto MRS agar plates and incubated at 35°C for 24h. Growth of bacteria was expressed in colony forming units per milliliter (log CFU/ml) and the percent survival of strain was then calculated.

Survival in simulated gastrointestinal conditions

Survival in simulated gastrointestinal conditions was assessed by following the method of Charteris et al. [19] with minor modifications. An aliquot of 0.1 ml for gastric and intestinal transit assay was removed after 0, 60 and 240 min. The tolerance was assayed by determining the viable count in simulated gastric juice after the incubation for different time intervals up to 4h. All the experiments were carried out in triplicates.

BSH activity

To evaluate bile salt hydrolase activity, isolate was cultivated in MRS agar containing 0.5 % (w/v) of the sodium salt of taurodeoxycholic acid (TDCA) (HiMedia, India). MRS agar plates were incubated at 35°C for 72 h. The plates were examined for white precipitates which were the sign of bile salt hydrolysis [20].

Autoaggregation

Autoaggregation assay was performed according to Del Re et al. [21] with minor modifications. 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)

Coaggregation

Coaggregation ability of the isolate was determined by following the method described by Del Re et al. [21] with minor modification. Absorbance at λ=600 nm was observed for mixture and each of individual strain. Co-aggregation % was calculated according to Handley's equation [22].

Antimicrobial activity

Antagonistic potential of isolate Ch-2 was evaluated against serious food borne as well as spoilage causing microorganisms. Overall antimicrobial activity of non-neutralized cell free supernatant was checked against *Listeria monocytogenes* MTCC 839, *Clostridium perfringens* MTCC 1739, *Bacillus cereus* CRI, *Staphylococcus aureus* IGMC, *Escherichia coli* IGMC, *Enterococcus faecalis* MTCC 2729, *Leuconostoc mesenteroides* MTCC 107, *Pectobacterium carotovorum* MTCC 1428 and *Pseudomonas syringae* IGMC using well diffusion method [23]. The wells were poured with 150 µL of 24 h old culture

supernatant and the plates were incubated at 35°C for 24 h. The antibacterial activity was determined and zones of inhibition were measured in millimeter (mm).

HPLC- determination of lactic acid

LAB with industrial potential are mostly homofermentative (*Pediococcus*) which produce only lactic acid as major end product of their metabolism. Lactic acid, mainly responsible for the antagonistic potential of isolate Ch-2, was detected by using HPLC (Novapak C-18) column. Mobile phase used was Methanol: Water (double distilled) (95:5) (Sigma Aldrich). Standard organic acid solution i.e. 5% standard solution of lactic acid (Sigma Aldrich) was prepared in double distilled water. Twenty four hour old culture grown in MRS broth was centrifuged to get the culture supernatant. Sample was filtered using a 0.22 µm Millipore filter membrane before using for analysis. HPLC analysis was firstly performed with standard organic solution followed by the samples. The monitoring was done at 210nm.

Production of bacteriocin

An overnight culture of Ch-2 was inoculated (OD600 = 1, 10%) into 100ml of MRS broth and cultivated at 37°C for 24 h. Samples were taken every 2 hours for 48 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 150µl 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 [24].

H_2O_2 production

Quantitaive estimation of Hydrogen Peroxide (H_2O_2) was done by following the method given in AOAC [25]. Suspension was titrated against 0.1 N $KMnO_4$ (Sigma Aldrich) until the suspension becomes colorless. Each ml of 0.1 N $KMnO_4$ is equivalent to 1.070 mg of H_2O_2.

Metabolic fingerprinting

Metabolites were extracted by following the method given by Coucheney et al. [26] with minor modifications using GC-MS. Microbial suspensions (20 ml) of isolate (grown in MRS broth for 24 h at 35°C) were disrupted using pulsed, high frequency sound waves (>20kHz) for five cycles (30 sec. run; one min break) to extract intracellular metabolites. Suspensions obtained after sonication were centrifuged for 10 min at 10,000 rpm in order to separate the extra as well as intracellular solution from the cells. The supernatant was removed and the metabolites were extracted with a methanol:water:chloroform mixture (2:0.8:1):2.5 ml of cold chloroform and 5 ml of cold methanol (-20°C) were added to and the phases were allowed to separate. Metabolic fingerprints were measured in the aqueous phase after freeze drying while in the organic phase; the dried chemical extracts obtained after complete evaporation of the solvent was used for metabolic profiling.

Results

Isolation and identification

Samples of Chuli taken from various parts of Himachal Pradesh were used as isolation source. Lactic acid bacteria were isolated on MRS agar at 37°C under anaerobic conditions. Six lactic acid bacterial isolates were obtained from Chuli and 4 out of 6 were confirmed as rods while two 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 was observed by any of the isolates. On the basis of morphological and biochemical characteristics, isolates were tentatively identified at genus level as *Lactobacillus* and *Pediococcus* (tetrad). Out of six isolates, Ch-2 gave clear zones around the lawn of indicator baterial strains using bit/disc method with broadest antimicrobial spectrum and was selected for further study. The largest diameter of inhibition (up to 26.6mm) was obtained against *L. monocytogenes*, *C. perfringens*, *B. cereus* and *P. syringae* (Figure 2).

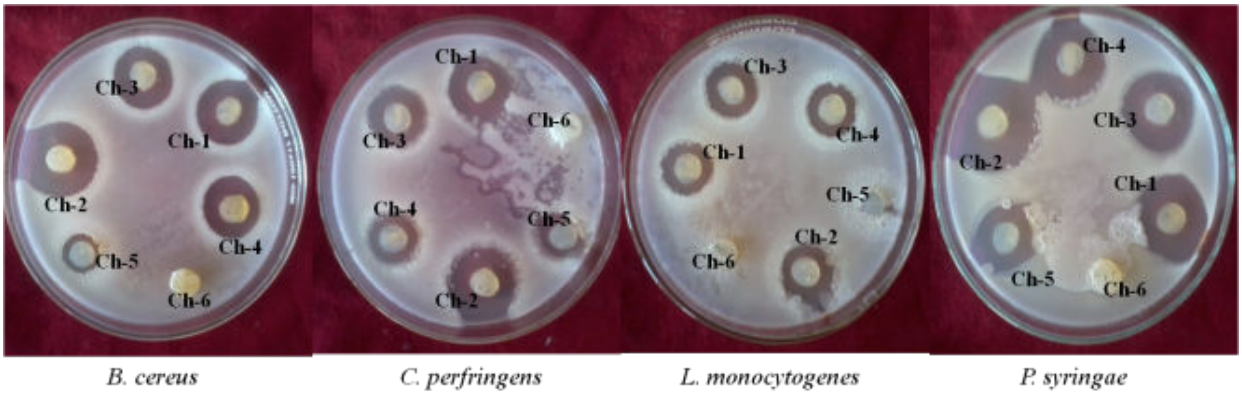

Figure 2: Antagonistic spectrum of lactic acid bacteria isolated from Chuli by Bit/disc method against bacterial indicators.

After screening, DNA of the isolate was subjected to PCR for amplification of small subunit of 16S rRNA genes using universal primers (BITS-1(5'AGAGTTTGATCCTGG) and BITS-4-(5'-TACCTTGTTACGACTT) having expected product size of 1500bp. The PCR products obtained after amplification were visualized using ethidium bromide (Thermo Fisher Scientific) on 1.5% agarose gel (Sigma-Aldrich). These after purification had got sequenced by Xceleris, India Pvt. Ltd. to identify the isolate. Sequence similarity

search for the isolate Ch-2 (BLAST, NCBI) showed 99% similarity with the available sequence of *Pediococcus acidilactici* and was assigned accession no KJ541886 by NCBI. Phylogenetic tree of *Pediococcus acidilactici* Ch-2 with respect to other lactic acid bacteria along with the boot strap values as inferred by the neighbor joining method (Mega 6) has been presented in Figure 3.

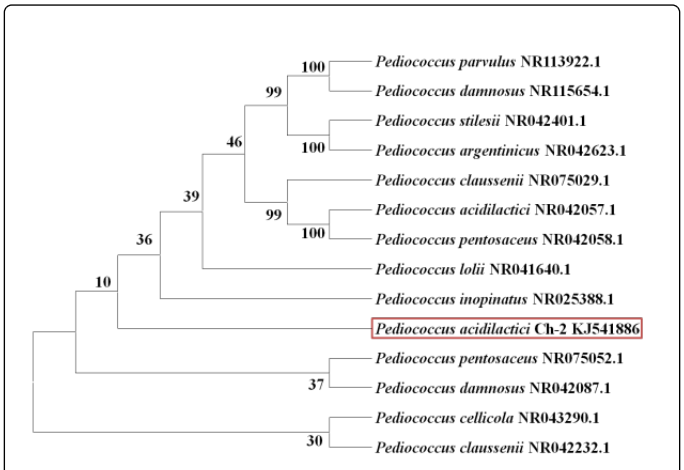

Figure 3: Neighbour-joining phylogenetic dendrogram of strain Ch-2 and related lactic acid bacterial species based on 16S rRNA gene sequence similarity. Numbers at branch points are levels of bootstrap support (%) based on analysis of 1000 re-sampled datasets.

Safety assessment of *P. acidilactici* Ch-2

P. acidilactici Ch-2 was found to be sensitive to 11 out of 12 antibiotic (with 91.66% sensitivity) used in the study (Table 1), thereby advocating its inability to grow in the presence of antibiotics and thus its safe status. Isolate showed a negative response in the production of DNase and gelatinase enzymes as pathogenicity factors. No clear zones around colonies were observed on blood agar medium (Figure 4). Haemolytic activity would break down the epithelial layer while the gelatinase activity would damage the mucoid lining. Absence of haemoloytic and gelatinase activity is a selection criterion for probiotic strains, indicating that this bacterium is non-virulent [27].

Tolerance to low pH and bile salts

In the present investigation, Ch-2 was subjected to as low pH as 1 (during fasting) and was found to survive it for about 2 h with 74.95% survival. Under acidic conditions of pH 2 and 3, isolate Ch-2 showed resistance upto 3 h with 72.69 and 98.62% survival, respectively (Figure 5). Probiotic bacteria require to survive gastric passage, where the pH can be as low as 1.5 to 2.0 [28], and stay alive for 4 h or more [29] before they move to the intestinal tract. As the isolate in the present investigation was able to survive gastric pH (even as low as 1) upto 3h, therefore it can be considered as a good probiotic and can be recommended in the food preparations for its effective delivery in the system.

Isolate Ch-2 was able to survive the required concentration of 0.3% bile salts with 86.7% survival when evaluated through optical density method. Cell viability of isolate Ch-2 upto 8 h was reported to be 98.34%, 96.40% and 95.42% survival for 0.3, 1.0 and 2.0% bile salt concentration, respectively (Figure 6).

S. No	Antibiotics	Concentration (µg)	*S/R
1.	Ampicillin (AMP)	30	S
2.	Augmentin (AMC)	30	S
3.	Gentamicin (GEN)	10	S
4.	Cephalothin (CEP)	30	S
5.	Cloxacillin (COX)	1	S
6.	Cefotaxime (CTX)	30	S
7.	Cefoxitin (CX)	30	S
8.	Lincomycin (L)	2	S
9.	Tetracycline (TE)	30	S
10.	Amoxyclav (AMC)	30	S
11.	Co-trimoxazole (COT)	25	R
12.	Cefuroxime (CXM)	30	S
% Sensitivity			91.66

Table 1: Antibiotic sensitivity of *P. acidilactici* Ch-2. *Sensitive, Resistant.

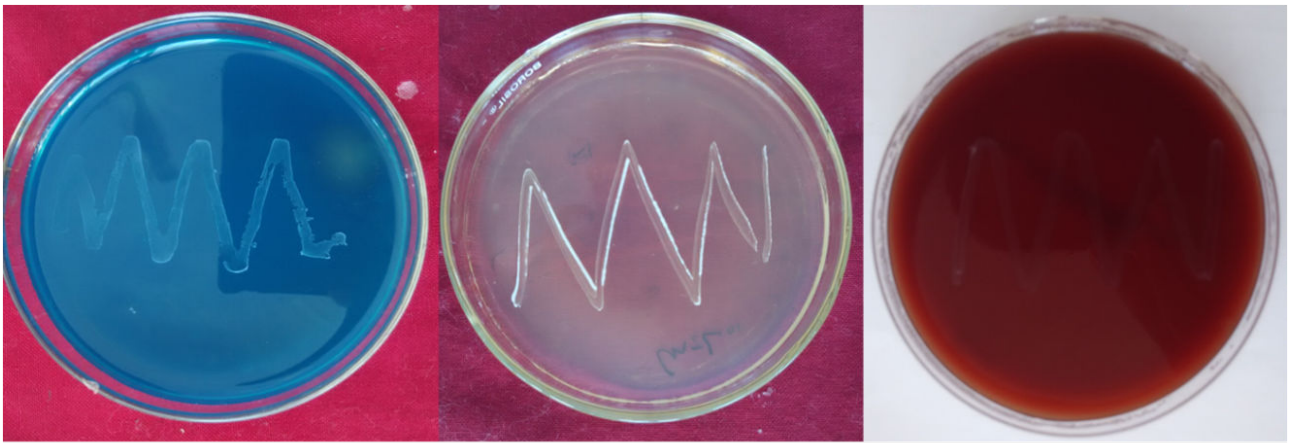

Figure 4: DNase, gelatinase and hemolytic activities of *P. acidilactici* Ch-2.

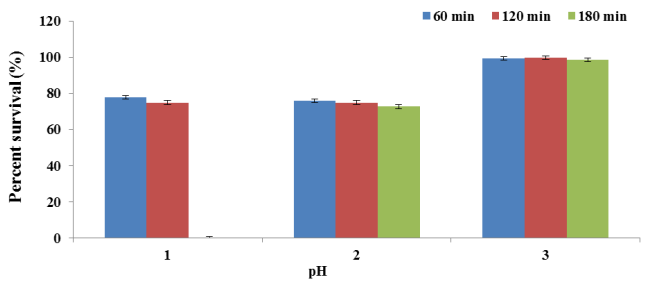

Figure 5: Tolerance of *P. acidilactici* Ch-2 to low acidity.

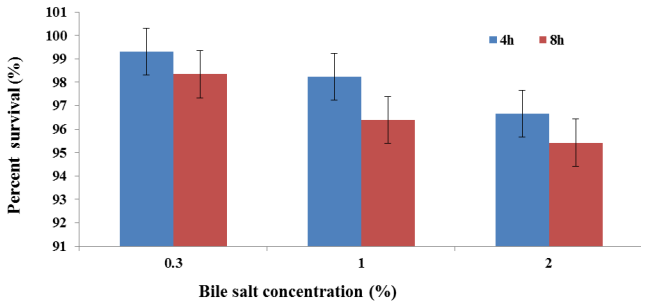

Figure 6: Tolerance of *P. acidilactici* Ch-2 to Bile salt concentrations.

Survival in simulated gastrointestinal conditions

Isolate Ch-2 when exposed to gastric juice (pH 2), exhibited 74.32% survival upto 1h while percent survival at pH 3 was found to be 77.22 and 57.18% for 1 and 4 h, respectively (as shown in Table 2). Resistance to intestinal transit was studied at pH 8.0 and found that the isolate Ch-2 successfully survived the intestinal juice with 96.13 and 79.50% survival for 1h and 4h, respectively. The data obtained reveal that the isolate was efficient in surviving the harsh conditions of gastrointestinal conditions, thus can be used for further study and recommended for its use as successful probiotic.

BSH activity

Survival under simulated gastrointestinal conditions indicates the resistance of the selected strain to bile salts, which could be ascribed to bile salt hydrolase (BSH) activity. BSH catalyses the deconjugation of bile salts and free (deconjugated) bile salts resulted after the deconjugation have lower solubility at low pH and thus, precipitate as a result of the fermentative metabolism of LAB [30]. But in our investigation, isolate Ch-2 was unable to deconjugate the bile acids (no growth of cells was observed on plates) though it exhibited good survival under high concentration of bile salts.

Autoaggregation and Coaggregation

The autoaggregation ability of isolate Ch-2 was determined during a period of 5 h. Autoaggregation percentage of bacterial isolate was measured by comparing the initial absorbance at 600 nm with absorbance of 1st, 2nd, 3rd, 4th and 5th h of incubation. It was found that the cells of isolate Ch-2 efficiently aggregated (97.0% aggregation) with each other over a period of 5 h. The minimum required autoaggregation percentage for good probiotic strains has been recommended more than 40% as the time proceeds more than 5 h [14].

As depicted by the results described above, isolate Ch-2 exhibited strong autoaggregation potential as the strain has autoaggregation more than 40% over a period of 5 h Ch-2 has a coaggregation percent of 20.83, 16.32% and 6.25 with L. monocytogenes, C. perfringens and B. cereus (Figure 7). As the isolate showed maximum coaggregation % with L. monocytogenes, a serious food borne pathogen, it depicts its ability to inhibit the serious pathogen to the maximum extent.

Survival percentage (%)				
Gastrointestinal juices	1h		4h	
	log CFU/ml	% Survival	log CFU/ml	% Survival
Pepsin (pH 2)	7.7(± 0.20)	62.0	-	-
Pepsin (pH3)	8.0(± 2.00)	64.51	5.97(± 0.02)	48.1
Pancreatin (pH8)	10.08 (± 0.04)	81.29	8.3 (± 0.20)	66.90

Table 2: Resistance of isolate Ch-2 to simulated gastrointestinal juices. *Log cfu/ml: Mean (± SD) of results from three separate experiments. **% Survivability = (log cfu 4th, 1st h / log cfu 0th h) × 100.

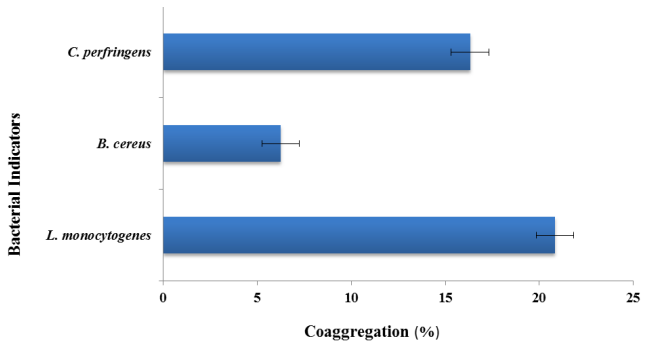

Figure 7: Coaggregation percent of *P. acidilactici* Ch-2.

Antimicrobial activity

The antagonistic potential of Ch-2 was assessed against most challenging and serious food borne pathogens. The inhibitory spectrum of isolate Ch-2 has been described in Figure 8. Isolate Ch-2 exhibited a broad spectrum of inhibition against test pathogens with zones of inhibition ranging from 17.6-23.6 mm. Efficacy and effectiveness of antimicrobial potential of Pediococcus spp. have been studied widely.

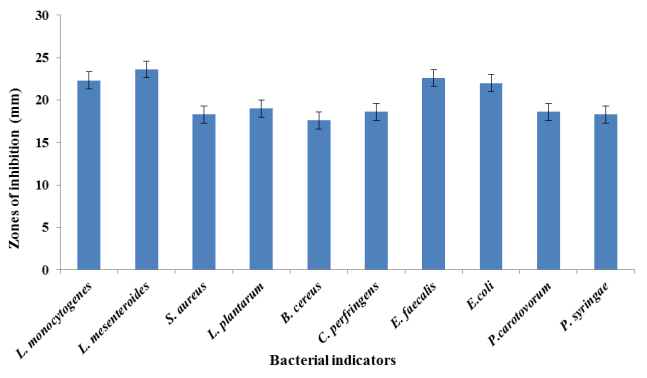

Figure 8: Antagonistic spectrum of *P. acidilactici* Ch-2.

HPLC- determination of Lactic acid

P. acidilactici Ch-2 isolated from Chuli was able to inhibit the tested indicator organisms with varied zones of inhibition. The inhibitory spectrum of strains against pathogenic bacteria is shown in Fig 9. Some species of *Staphylococcus aureus*, *Enetrococcus*, *Leuconostoc* and *Listeria* are highly pathogenic to human beings. The isolated *P. acidilactici* Ch-2 showed strong bactericidal activity against these species. The pH and titratable acids in the cell free culture supernatant of *P. acidilactici* Ch-2 were 3.63 and 0.07%, respectively. *Pediococcus* are homofermentative bacteria which produce more than 85% lactic acid from glucose being the major product of fermentation. *P. acidilactici* Ch-2 being a homofermentative culture was evaluated for lactic acid production which was confirmed by HPLC analysis (Novapak C-18) and has been found to be 7.911 mg/L after 24 h of incubation. Figure 8 shows typical HPLC chromatograms of standard and lactic acid extracted from culture supernatant of *P. acidilactici* Ch-2 revealing its antimicrobial potential Figure 9.

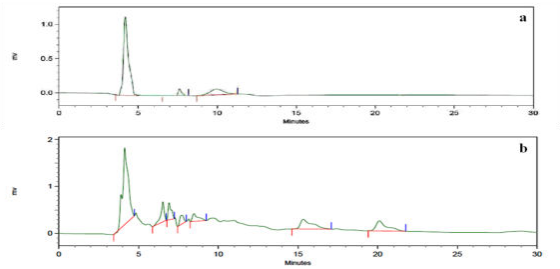

Figure 9: HPLC Chromatogram of a) standard solution of lactic acid b) culture supernatant of *P. acidilactici* Ch-2.

Production of bacteriocin

The antagonistic pattern of *P. acidilactici* Ch-2 was evaluated against test pathogen viz. *L. monocytogenes* and has been found to inhibit it efficiently (Figure 10).

H_2O_2 production

Probiotic isolate P. acidilactici was evaluated for H_2O_2 production and has been reported to produce 0.61g/L.

Metabolic fingerprinting

The metabolite composition of P. acidilactici Ch-2 provides a window for elucidating its safety and possibility of finding novel compounds with nutritive as well as health properties. In the current study, intra- as well as extracellular metabolites were assessed by GC-MS analysis and the isolate revealed the presence of 41 compounds (four were obtained in aqueous fraction while 38 were observed in solvent fraction and 1compound was common to both) of which 14 compounds were found to have biological activities. These compounds were confirmed based on their peak area, retention time and molecular formula and are represented in Table 3a and b.

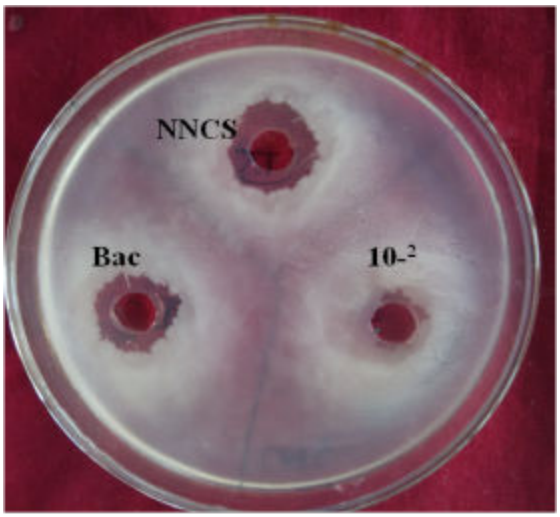

Figure 10: Bacteriocins production by *P. acidilactici* Ch-2.

The compounds identified using GC-MS possessed many biological activities helpful in food industry as well as in pharmaceutical industry with therapeutic potential such as L-Lactic acid (85.19%), Methyltartronic acid (7.47%), Pyrrolo [1,2a] pyrazine1,4dione, hexahydro (5.89%) and Phenol, 2,4bis (1,1dimethylethyl) (8.32%) exhibited good antibacterial activity. n-Hexadecanoic acid (6.61%) possesses anti-inflammatory, Geranyl-isovalerate (0.33%), Benzaldehyde, 2,4 dimethyl (1.23%) and Hexadecane, 2,6,11,15 tetramethyl (0.71%) were reported to be flavor agents and are being used as food additives. Hexadecane (2.00%), Pyrrolo [1,2a] pyrazine1,4 dione, hexahydro3 (2methylpropyl) (0.72%), Hexadecane (1.70%), Tetradecane (2.11%) and Dodecane (1.73%) possess good antioxidant properties. Two rare compounds with therapeutic potential have been reported to be produced by this isolate for the very first time from any probiotic bacteria i.e. 9Octadecenamide, (Z) (6.06%) and Squalene (0.54%) (Figure 11).

9 Octadecenamide, (Z) has a therapeutic potential and is having a strong activity against sleep and mood related disorders. Squalene, in addition to have antimicrobial activity, has also been reported to have anticancer, anti-oxidant, chemo-preventive, anti-tumor and sunscreen properties [31]. Squlene, a triterpine compound, was originally found in shark liver oil. It can also be obtained from plant sources; however the production at the desired purity level for food and pharmaceutical applications cannot be achieved.

The use of microbial production of squalene may be a suitable alternative to address the issue. Currently, *Saccharomyces cerevisiae* has been used successfully for its production while other food grade probiotic lactic acid bacteria or other bacilli have not been reported so far to produce squalene [32,33]. This study becomes of paramount importance by revealing the production of Squalene from probiotic bacteria 1st time and can open a gateway for assessing the different probiotics for unique bioactive compounds of special pharmacy properties. Thus potential of this isolate for Squalene production can be exploited by optimizing the yield and can further be used for commercial production of Squalene along with its good probiotic capabilities.

S. No.	Compound Name	Retention Time	Molecular Formula	Peak Area	Area %	Peak height	Biological activity
1	L-Lactic acid	6.13	C3H6O3	1823198239.01	85.19	80403220.79	Antimicrobial
2	Pyrrolo[1,2a] pyrazine1,4dione, hexahydro	13.39	C7H10N2O2	126120542.69	5.89	9336208.17	Antimicrobial, antioxidant
3	Dibutyl phthalate	14.60	C16H22O4	61085191.70	2.85	8439561.87	-
4	*9Octadecenamide, (Z)	17.52	C18H35NO	129710489.48	6.06	16525865.53	Anti-sleep disorder agent

Table 3a: Metabolic profile of *P. acidilactici* Ch-2 (aqueous fraction).

S. No.	Compound Name	Retention Time	Molecular Formula	Peak Area	Area %	Peak height	Biological activity
1	Trichloromethane	3.09	CHCl3	2151864601.56	13.80	377516301.26	-
2	Butane, 2ethoxy2methyl	3.56	C7H16O	1296781662.84	8.32	101201149.67	-
3	Diethyl carbonate	4.29	C5H10O3	1716108545.09	11.01	202279957.97	-
4	Butane, 2,3dichloro2methyl	4.68	C5H10Cl2	125154855.13	0.80	14275282.52	-
5	Benzene, chloro	5.01	C6H5Cl	893423584.87	5.73	82472224.20	-

6	Methyltartronic acid	5.96	C4H6O5	1164627846.18	7.47	89720023.58	Antibacterial
7	Cyclopentane, 1,3dichloro, cis	6.63	C5H8Cl2	484275080.34	3.11	49194324.93	-
8	Propanal, 2,3dichloro2methyl	7.30	C4H6Cl2O	400387071.13	2.57	41587372.49	-
9	Undecane	7.98	C11H24	183319732.83	1.18	33643465.46	-
10	Benzoic acid	8.87	C7H6O2	139633781.42	0.90	21482083.85	-
11	Dodecane	9.02	C12H26	269670925.56	1.73	59154152.22	Antioxidant
12	Benzaldehyde, 2,4dimethyl	9.31	C9H10O	192127881.29	1.23	41299139.71	Flavor agent
13	Benzene, 1,3bis(1,1dimethylethyl)	9.62	C14H22	647249953.19	4.15	169956544.53	-
14	Trichloroacetic acid, hexadecyl ester	10.07	C18H33Cl3O2	68316277.38	0.44	7818007.08	-
15	Dodecane, 2,6,11trimethyl	10.23	C15H32	84724504.08	0.54	14572730.71	-
16	Tetradecane	10.90	C14H30	329137555.64	2.11	78166898.57	Antioxidant
17	Phenol, 2,4bis(1,1dimethylethyl)	11.92	C14H22O	1297998762.54	8.32	318041677.69	Antimicrobial
18	Hexadecane, 2,6,11,15tetramethyl	12.10	C20H42	111256301.00	0.71	20177977.66	Natural food additive
19	Dodecanoic acid	12.33	C12H24O2	119319719.97	0.77	19561701.25	-
20	Hexadecane	12.55	C16H34	265278473.56	1.70	60185485.26	Antioxidant
21	Geranyl isovalerate	12.94	C15H26O2	51700961.39	0.33	6917915.01	Flavor agent
22	Tetradecane, 2,6,10trimethyl	13.33	C17H36	77962782.28	0.50	11468334.41	-
23	Tetradecanoic acid	13.85	C14H28O2	282944336.98	1.81	20902215.25	-
24	10Methylnonadecane	14.05	C20H42	295915206.74	1.90	39204553.25	-
25	iPropyl 12methyltridecanoate	14.24	C17H34O2	50533687.90	0.32	13277202.93	-
26	Pyrrolo[1,2a]pyrazine1,4dione, hexahydro3(2methylpropyl)	14.56	C11H18N2O2	111498511.60	0.72	10373887.81	Antioxidant
27	n-Hexadecanoic acid	15.23	C16H32O2	1030968696.76	6.61	80644345.90	Anti-inflammatory
28	Z5Methyl6heneicosen11one	16.19	C22H42O	166958679.19	1.07	11246215.74	-
29	Eicosanoic acid	16.69	C20H40O2	349862423.03	2.24	19708800.55	-
30	1Hexadecanol, 2methyl	16.98	C17H36O	83780395.92	0.54	9128309.20	-
31	*9Octadecenamide,(Z)	18.17	C18H35NO	395784730.00	2.54	48534273.34	Anti-sleep disorder agent
32	Pyrrolo[1,2a] pyrazine1,4dione, hexahydro3(phenylmethyl)	18.61	C14H16N2O2	125921545.39	0.81	10456092.22	-
33	Tetratriacontane	20.16	C34H70	37940689.16	0.24	4004069.70	-
34	Phthalic acid, di(2propylpentyl) ester	20.31	C24H38O4	53198945.89	0.34	8477434.89	-
35	Tetracosane	20.90	C24H50	40257312.22	0.26	4304905.66	-
36	Hexadecanoic acid, hexadecylester	24.00	C32H64O2	64912603.62	0.42	3021684.96	-
37	Octadecane, 3ethyl5(2ethylbutyl)	25.16	C26H54	67973082.92	0.44	4149687.97	-

38	Squalene	25.89	C30H50	53908277.01	0.35	7539688.23	Anti-cancerous

Table 3b: Metabolic profile of *P. acidilactici* Ch-2 (organic solvent fraction). *Common compounds.

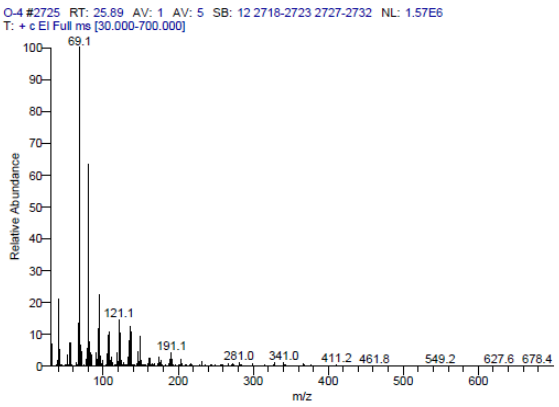

Figure 11: GC-MS chromatogram of Squalene- a novel compound secreted by *P. acidilactici* Ch-2.

Discussion

Fermented food products have a long history and they form a significant part of the diet of many indigenous communities in the developing world. Recently, there has been an increase of interest regarding the commercial utilization of probiotic LAB strains isolated from traditional and naturally fermented food products which possess medicinal and health-promoting effects. In addition to fermented food products, microbial flora of traditional fresh food sources such as fruits and vegetable samples with high nutritional as well as therapeutic characteristics have also been explored to study their potential in food and pharmaceutical sector. Sadrani et al. [34] also used fruits in addition to nine samples of fermented foods (cheese, curd and shrikhand) for the isolation of LAB using De Man Rogosa and Sharpe (MRS) broth. Probiotics lactic acid bacteria have been shown to suppress pathogenic growth through the release of a variety of antimicrobial factors viz. bacteriocins, H_2O_2, ammonia, diacetyl and organic acids such as lactic and acetic acids, which reduce the pH of the lumen, resulting in the inhibition of the growth of a variety of food-borne spoilage and pathogenic organism [35]. By producing these antimicrobial compounds, probiotic microorganisms gain an edge over other microorganisms to survive in the adverse conditions of gastrointestinal tract [36]. Similarly, Gautam et al. [37] studied the antagonistic potential of lactic acid bacteria isolated from Dulliachar-a salted pickle and found to exhibit broad spectrum activity against spoilage causing/food borne pathogens viz. *L. monocytogenes, C. perfringens, S. aureus, L. mesenteroides, L. plantarum* and *B. cereus*.

A vital issue during the selection and evaluation of new probiotics is their safety analysis. Thus, characterization of the safety criteria of the putative probiotic strains is obligatory in order to avoid their side effects [38]. Among them, some such as antibiotic susceptibility, haemolytic, gelatinase and DNase production testing have been ranked the must to ensure safety and non-virulent nature of probiotic bacteria. Klare et al. [39] studied the antibiotic susceptibility of *Pediococcus pentosaceus* (20), *Pediococcus acidilactici* (29), *Pediococcus sp.* (49) and *Lactococcus lactic* (8) and found that none of the pediococci and lactococci tested showed acquired resistance to antibiotics used in the study viz. Streptomycin, Vancomycin, Erythromycin, Clindamycin, Oxytetracyclin and Fusidic acid. Similarly, Cota-Gastelum et al. [40] studied haemolysis pattern of LAB isolated from fish and found that out of 37 presumptive *Pediococcus pentosaceus* and *Pediococcus spp.*, 26 presented β-haemolysis and 11 presented gamma haemolysis. Gamma haemolysis is displayed by bacteria that do not induce haemolysis of the blood cells while isolates with beta haemolysis were not characterized because of their capacity to lyse human and tilapia erythrocytes.

The most important factors that may affect the survival of probiotics are the high acidity in the gastric region and the high concentration of bile components in the proximal intestine [41]. Therefore, during the selection of potential probiotic isolates to assure their viability and functionality being tolerant to acidic conditions is an important criterion to be considered. Moreover, most of the probiotic bacteria exhibit unpredictable resistance to acidic conditions and this characteristic is species and strain dependent [42]. Probiotic bacteria should pass through the highly acidic stomach in order to reach the intestine and create proper conditions for residence.

Generally, the tolerance to bile salts has been considered a condition for survival, colonization and metabolic activity of probiotic bacteria in the host's intestine. As the bile salts can influence the intestinal microflora by acting as antimicrobial molecules, consequently, it is of immense importance to evaluate their ability to tolerate bile salts [41]. The ability to survive gastrointestinal transit in high viable cell counts is an important probiotic criterion. Selection of potential probiotic strains which could survive in the GI tract was performed on the basis of the measurements of strain survival in simulated gastric (pH 2,3) and intestinal juices (pH 8).

The autoaggregation ability is one of the key factors that determine the ability of the probiotic strain to adhere to the oral cavity, gastrointestinal tract and urogenital tract and coaggregation ability helps to from a barrier that prevents colonization by pathogens. Probiotics are able to do coaggregation with pathogens will effectively inhibit and kill pathogenic bacteria as antimicrobial compounds produced can move directly on pathogens [43]. The antimicrobial action of probiotic bacteria is reportedly due to their potential to produce lactic acid, H_2O_2 and antimicrobial peptides (bacteriocins) [44]. Mandal et al. [45] studied the antagonistic potential of *P. acidilactici* LAB5 against some food spoilage and human pathogenic bacteria and proved the antagonistic efficacy of antibacterial substance secreted by the strain i.e. bacteriocin against pathogenic bacteria viz. *Listeria*, *Staphylococcus*, *Streptococcus*, *Enterococcus* and *Leuconostoc*. *P. pentosaceus* 2A2 and 1A6 isolated from Indonesian local beef exhibited antibacterial activity against *E. coli* ATCC25922, EPEC, *S. typhimurium* ATCC14028 and *S. aureus* ATCC25923 (inhibition zones 5.5-14mm) [46].

The antimicrobial peptides produced by *Pediococcus spp.* are categorized in the 2nd class of bacteriocins from LAB and are known as

antilisterial bacteriocins. In general class IIa bacetriocins have a rather narrow spectrum of activity [47,48]. All class II bacteriocins are described as being active against *Listeria*. Hydrogen peroxide is normally produced by vaginal lactobacilli isolates, but may also be associated with intestinal lactobacilli or those living in the environment [49,50]. This trait is rarely reported in probiotic LAB isolated from fermented food products. In this study, *Pediococci* were first time reported to produce H_2O_2 as an antimicrobial agent against food spoilage pathogens. Thus, this trait may be beneficial in improving vaginal health and in preventing urinogenital infections. On the basis of the results, it can be hypothesized that the antagonistic activity of the strain relies on acidity, lactic acid, H_2O_2 and other antimicrobial compounds (bacteriocins), thus revealing its potential and safe use as bio preservative agent in food and fermentation industry.

Broad spectrum antagonism by probiotic microorganism through various antimicrobial substances especially bacteriocin production is currently well accepted phenomenon [51] as compared to older concept where bacteriocin inhibition was linked against closely related species only [52-54]. There are many evidences reporting the secretory antibacterial components produced by LAB having broad range antagonism against Gram-positive and Gram-negative organisms [55] which are independent of lactic acid and hydrogen peroxide.

Probiotics play a major role in health and wellbeing beyond basic nutrition. Applications of probiotic bacteria and their metabolites in pharmaceutical preparation, medicines, food products and dietary supplements are increasing due to their potential health benefits, such as antimicrobial activity, antimutagenic, anticarcinogenic activities, regulation of immune function reducing lactose intolerance, constipation, improving gastrointestinal health and allergenic diseases such as food allergy, etc. [56-58]. These properties of probiotic lactic acid bacteria have been explained by the production of different primary and secondary metabolites that exert their biological effects either directly or by modulating the immune system. The reliability of the methodology in assessing the impact of metabolic compounds on colonic metabolic signature has been recently demonstrated [4,59,60].

Conclusions

Pediococcus acidilactici Ch-2 isolated from Chuli has been evaluated for its probiotic potential and was found resistance to low pH and bile salts (0.3%) and simulated gastric and intestinal conditions, was able to produce bacteriocin and lactic acid against a number of serious food borne and spoilage causing microorganisms. The susceptibility to selected eleven antibiotics, inability to produce gelatinase and DNase and non-hemolytic nature revealed its safe status for further use in food and nutraceutical industry. In the present study, Squalene – a rare and therapeutic anti-cancerous compound has been reported for the very first time from a probiotic bacteria isolated from fermented food product and recommended for its further investigation as a potential source of Squalene. In conclusion, most of the results in the present work revealed *P. acidilactici* Ch-2 as a potential probiotic candidate with many functional properties and novel compounds.

Conflict of Interest

Conflict of interest declared none.

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