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Assessment of efficacy of a potential probiotic strain and its antiuremic and antioxidative activities

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SUMMARY

Background & aims: Kidney disease requires dialysis or kidney transplantation. No generally applicable therapies to slow progression of renal disease are available. The aim of this study was to characterize *Sporosarcina pasteurii* (MTCC 1761, Type strain) as an effective probiotic strain for acetaminophen induced uremic patent.

Methods: Antibacterial and antioxidative activities, bile salt and pH tolerance, starch and protein digestibility, hydrophobicity, opsonophagocytic assay and antibiotics sensitivity of the strain was performed to investigate its probiotic potentialities. Blood uremic profiles, DNA fragmentation assay of kidney tissue and kidney histological studies were investigated on acetaminophen-induced nephrotoxic rats (Wister strain albino male).

Results and discussion: The cell free extract of *S. pasteurii* showed high *in vitro* antioxidative property and potential antibacterial activity (average diameter of 6 mm) against some reference enteropathogenes. The strain can survive at highly acidic environment (pH 3.0) and showed bile resistance upto 0.8% (w/v) along with 8% (w/v) salt and 0.8% (w/v) phenol. The strain able to digest starch and milk protein and show medium hydrophobic attachment with non-polar solvent. Bacterial strain completely destroyed in the presence of blood components and sensitive to all tested 20 antibiotics. After oral administration of the strain significantly lowered the level of blood urea, creatinine, and uric acid level and minimized the glomerular necrosis, DNA damage of uremic rats.

Conclusion: Therefore, the strain *S. pasteurii* may be exploited as a potent probiotic organism and oral ingestion of bacteria decrease uremic syndrome.

1. Introduction

Renal insufficiency leads to uremia and each year, the number of patients with chronic kidney failure increases by an astounding 11%.[1] Globally, kidney transplantation is opted by a very few kidney failure patients for effective treatment, due to shortage of donor because of high cost and high probability of organ rejection. Therefore, there is a great necessity for an unconventional, affordable therapy for patients who cannot afford expensive dialysis or kidney transplant to keep them alive.

Acetaminophen is the major metabolite of phenacetin and may contribute to kidney injury through a specific mechanism different from other kidney failure due to other type of analgetics. Acetaminophen (APAP) overdose can result in nephro-toxicity. APAP also interact with cytochrome P450 (CYP)-and produce the toxic electrophile N-acetylp-benzoquinone imine (NAPQI) both in liver and kidney.[2] APAP overdose produces extremely large amount of NAPQI and only a part of it can form GSH conjugate with subsequent depletion of cellular GSH. The remaining part of NAPQI binds to cellular proteins and induces oxidative stress, leading to renal injury.

"Enteric dialysis" is an adaptive physiological process for removal of solutes from the body. High concentration gradients can facilitate diffusion of solutes from plasma to intestinal lumen. The intestinal barrier functions as a semipermeable membrane. Concentration gradient makes solutes scatter from plasma into the lumen. When amount of uremic toxins become larger in plasma than lumen, a large amount of uremic solutes become distributed

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throughout the intestine. Recent studies indicated that uremic toxins like urea, creatinine etc. can excrete through enteric dialysis.[3] "Enteric dialysis" thus become an alternative therapy for solute removal in uremia. The live bacteria which degrade uremic toxins within the gut have been acceptable therapy of today.[4] Probiotics by the generally accepted definition, are "live microorganisms which when administered in adequate amounts confer a health benefit on the host". To shift the microecological balance in the intestines in favor of probiotic bacteria prebiotics, such as dietary oligosaccharides, are used as nutritional supplements. Combination nutritional products comprised of probiotics and prebiotics named synbiotics are also available. Probiotics and prebiotics evaluate the impact on solute concentration in serum or on their fecal or urinary excretion.[4] Semipermeable microcapsules containing genetically engineered live cells of *Escherichia coli*DH5 lower the high plasma urea level to normal in uremic rats when orally administred.[5] Oral administration of *Bifidobacterium longum* to hemodialysis patients is effective in reducing the serum toxin levels of by correcting the intestinal microflora.[6]

Sporosarcina pasteurii (Sp) formerly is known as *Bacillus pasteurii* from older taxonomies and is a non-pathogenic spore-forming ureolytic gram positive bacterium and it has been isolated from human feces.[7] It can use urea as sole nitrogen source, and according to literature, needs urease only to generate a nitrogen source, ammonia,[7] which prompted us to initiate the present study. Our previous study demonstrated that feeding with *S. pasteurii* attenuates blood urea-nitrogen levels (BUN) improves in the life span of uremic animals.[8] In this present study we want to establish *S. pasteurii* as a potent probiotic organism and antiuremic agent in uremia.

2. Materials and methods

2.1. Microorganisms

Sporosarcina pasteurii (MTCC 1761) was collected from IMTECH Chandigarh, India. Some pathogenic reference strains like *Escherichia coli* (ATCC 8739), *Bacillus subtilis* (ATCC6633), *Staphylococcus aureus* (ATCC 25093), *Shigella dysentery*, *Pseudomonas aeruginosa* (ATCC 27853), *Klebsiella pneumonia* (ATCC 10145), were also collected from Department of Microbiology, Vidyasagar University, West Bengal, India.

2.2. Probiotic characteristic of S. pasteurii

Composition of basal media: Basal media containing several components such as Yeast extract-20.0 g, $(NH_4)_2SO_4$-10.0 g. All ingredients were dissolved in 1000 mL of 0.13 M Tris buffer (pH 9.0).

Tolerance to inhibitory substances: The organism was grown at 37 °C in different medium pH (1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0) different NaCl concentration [1, 2, 3, 4, 5, 8, 9 and 10% (w/v)] Bile salt concentrations (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7 and 0.8 g sodium taurocholate/100 mL) Phenol level (0.1–1.0 g phenol/100 mL) were monitored at 620 nm after 24 h.[9]

Survival under conditions simulating the human GI tract: Tolerance to stomach condition and small intestine condition was tested.[10]

Determination of bacterial hydrophobicity: Hydrophobicity was determined using Bacterial Adherence to Hydrocarbons (BATH) Percentage bacterial adhesion to hydrocarbon (toluene) is calculated as shown below: Adhesion (%) = [(OD_{600} cells free of hexane) − (OD_{400} aqueous)]/(OD_{600} cells free of n − octane) × 100%.[11]

Antibiotic resistancy study: Antibiotic resistancy of the bacteria was done using Icosha Disc (HiMedia, India). The plates were incubated at 37 °C overnight, and diameters of the zone of inhibition around the discs were measured.

Antimicrobial activity and nature of antimicrobial substances: One mL cell free culture supernatant (CFCS) of *S. pasteurii* was retained as untreated filtrate. To determine the organic acid function, 1 mL CFCS was adjusted to pH 6.5. In order to test the heat sensitivity, 1 mL CFCS was incubated at 100 °C for 15 min and treated with protease-K. The antimicrobial activity of all samples was tested using the Agar-well diffusion assay.[12]

Detection of enzymatic activities: Modified nutrient agar containing skimmed milk (HiMedia, India), tributyrin and soluble starch was used for detecting the protein, lipid and starch digesting capabilities of selected strain, respectively. The digesting capability of the tested strain was classified as positive when the diameters of clear zone were more than 1 mm. Each assay was performed in triplicate.[13]

Urease assay: The urease activity was determined for *S. pasteurii*, by measuring the amount of ammonia released from urea according to the phenol-hypochlorite assay method.[14]

Antioxidative activity of bacteria strain: Cell-free culture supernatants (CFCS) were obtained by centrifugation (10,000× *g*, 4 °C, 20 min) of *S. pasteurii*. Antioxidative activity of *S. pasteurii* (MTCC 1761) was performed by the thiobarbituric acid (TBA) method via the measurement of lipid per oxidation,[15] based on the monitoring of inhibition of linoleic acid and human plasma peroxidatin by CFCS. Twenty mL of linoleic acid emulsion was made up of 0.1 mL of linoleic acid (Amersham Life Science, Cleveland, Ohio), 0.2 mL of Tween 20, and 19.7 mL of deionized water. Five tenths mL of phosphate buffer solution (0.02 M, pH = 7.4), 1 mL of linoleic acid emulsion, 0.2 mL of FeSO4 (0.01%), 0.2 mL of H_2O_2 (0.56 mM), and 0.4 mL of CFCS were mixed and incubated at 37 °C. Intracellular extract was replaced by deionized water in the control samples. After 12 h of incubation, 2 mL of the reaction solution was mixed with 0.2 mL of trichloroacetic acid (TCA; 4%), 2 mL of TBA (0.8%), and 0.2 mL of butylated hydroxytoluene (BHT; 0.4%). This mixture was incubated at 100 °C for 30 min and allowed to cool. Two mL of chloroform was then added for extraction. The extract was obtained and the absorbance was measured at 532 nm. The percentage of inhibition of linoleic acid peroxidatin was defined as follows: [1-A532 (experimental sample)/A532 (control)] × 100%. The plasma lipid peroxidation was also analyzed. Four tenths mL of plasma, 0.1 mL of $FeSO_4$ solution (50 μM), and 0.2 mL of CFCS were mixed and incubated at 37 °C in a water bath. CFCS was replaced by deionized water in the control samples. After 12 h of incubation, the reaction solution was mixed with 0.375 mL of 4% TCA and 75 μM of BHT (0.5 mM) and placed in an ice bath for 5 min. The upper phase was obtained by centrifugation at 3000× *g* for 10 min. Two tenths mL of TBA (0.6%) was then added. This mixture was incubated at 100 °C for 30 min and allowed to cool. The absorbance was then measured at 532 nm. The percentage of inhibition of plasma lipid peroxidation was also defined as [1-A532 (experimental sample)/A532 (control)] × 100%.

Quantitative determination of a,a-Diphenyl-b-Picrylhydrazyl (DPPH) radical: The DPPH radical scavenging activity was measured by the method 0f Shimada[16] with slight modification. Intact cells of 0.8 mL and CFCS were mixed separately and allowed to react for 30 min with 1 mL of freshly prepared DPPH solution (0.2 mM in methanol). Blank samples contained either PBS or deionized water. The scavenged DPPH was then monitored by measuring the decrease in absorbance at 517 nm. The scavenging ability was defined as follows: [A517 (blank) − A517 (sample)/A517 (blank)] × 100%.

2.3. Safety assays in vitro

Opsonophagocytic assay (*in vitro*): The opsonophagocytic assay has been performed as described by Huebner.[17]