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Research Article
Fortification of Rice Gruel into Functional Beverage and Establishment as a Carrier of Newly Isolated *Bifidobacterium* sp. MKK4

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Abstract

Background and Objective: Traditional fermented rice beverages consort many health beneficial microbes and impose properties to be potential industrial strain for bioprocessing engineering. Here, the main aim was to evaluate the probiotic properties of a *Bifidobacterium* strain and its functional features and to study its fermentation behavior. **Materials and Methods:** A traditional fermented beverage origin isolate MKK4 was identified by the 16S rDNA sequencing and tested for tolerance to gastric acid and bile salts, antimicrobial susceptibility. The isolate was tested for survivability in gastric acid suppressive pharmaceutical formulations and stability. The fermentation and acidification kinetics were also studied with evaluating the amylolytic byproducts and mineral content of the rice gruel. **Results:** The isolate MKK4 was identified as *Bifidobacterium* sp. and durably survived in simulated gastrointestinal environment, stable with the gastric acid suppressive pharmaceutical formulations and susceptible to most of the commercial antibiotics. The fermentation dynamics revealed the well growth of the organism (specific growth rate 1.174) with the Monod's constant (K_s) of 5.721 g L^{-1} in rice gruel by degrading starchy substrate ($Y_{x/s}$ 0.233) with a peak of amylase production (3.758 U mL^{-1} min^{-1}) at 5 h of incubation. Lactic acid accumulation was 0.483% log^{-1} CFU mL^{-1} h^{-1} ($v_{lactate}$) with $Y_{lactate/s}$ 0.087 and acidification rate was 0.11 U pH h^{-1}. The isolate was able to fortify the gruel with maltoligosaccharides as well as multivalent essential minerals like zinc, calcium and iron. The isolate showed significant viability (91.30 and 95.65%, $p<0.05$) in fermented rice gruel supplemented with inulin and fructooligosaccharide tested as per the modified ICH stability guidelines. **Conclusion:** Results of this study suggested the formulated rice gruel as a potential non-dairy food adjunct for delivery of the probiotic isolate *Bifidobacterium* sp. MKK4 as well as nutraceuticals and may help in human wellness to combat life-style related diseases.

Key words: Rice gruel, bifidobacteria, fermentation dynamics, functional beverage, stability

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Nutraceutical-based therapies have become the holy grail to protect and prevent genesis of different health related issues including gastrointestinal ailments. Nutraceuticals are normally associated with human nutrition by consisting a variety of dietary supplements like probiotics, prebiotics, minerals and phytochemicals, bioactive metabolites, etc. Foods are naturally enriched by traditional microbial fermentation which offers improved quality over the raw ingredients. Microbial fermentation brings the desirous biochemical changes in food with unique microbial compositions and as a result, food qualities are being modified with calorie, vitamins, flavorsome, palatable, functional, health beneficial food component by incredible medicinal properties. Microbial fermentation is an age-old process which was adopted to preserve and process the food ingredients and till date people are following such processes traditionally or with inclusion of technological modifications for the improvement of house-hold process[1].

Microbes play the critical role in food fermentation which is carried out either by natural microflora or by desired consortium. Many investigations are being carried out to find out such targeted microbial strain(s) with desired biochemical features. In many cases, selection of microbes is a sequential screening process in order to their ability to survive in gastric acid and intestinal bile conditions, synthesis of enzymes, organic acids, antimicrobial substances, vitamins and to exert health beneficial impacts like improvement in overall digestion or specific dietary components, managing the immunity, prevention of diseases, etc. All together such properties indicate a microbial strain as probiotic. Traditional fermented foods are the natural source of microbes those exert numerous health benefits as well as qualify the probiotic checklists.

In a recent trend, non-dairy based functional food preparations have gained more interest over dairy based food products as choice of veganism due to the pragmatic issues like lactose intolerance, allergy, fat and cholesterol associated with milk based products. Rice based fermented foods and beverages are the major non-dairy based food preparation and a part of cultural heritage particularly in Asian sub-continent. In general, it is believed that the fermented rice is nutritionally enriched than the raw rice and confers more health benefits over dairy-based products[1]. Till date there is no such rice-based fermented food or beverage commercially produced or marketed in India, though it is the second largest producer and consumer of rice. We have explored the occurrence of a group of edible microbes like *Lactobacillus* spp., *Bifidobacterium* spp., *Lactococcus* spp., *Bacillus* spp. and *Saccharomyces* spp., in the Haria, a popular rice beer among tribal of East-central India[2]. One isolate *Lactobacillus fermentum* KKL1 from this beverage has been established as a potent probiotic organism[3]. The role of other dominant microbes in this beverage as well as their health beneficial impacts is now determining to establish this as a national ethnic drink. The exploitation and consumption of *Bifidobacteria* from this beverage is safe that supported by the long historical consumption of this fermented product and the deep study on bifidobacterial taxonomy, physiology and health beneficial impacts[4].

Food originated probiotic bacteria are become popular because these are generally regarded as safe (GRAS), biochemically active, secrets different bioactive molecules and do not show pathogenecity. Probiotic foods are the fastest growing area of functional food development. The market of probiotics, in sale terms, the probiotic[5] put the market at $27.9 billion in 2011 and it will reach $44.9 billion in 2018. Indian probiotic market is valued at $12 million in 2011, is expected to witness a Compound Annual Growth Rate (CAGR)[6] of 11% by 2016. Considering the mounted demand of this natural product, scientists are searching for a delivery vehicle or carrier system which could stabilize the organism for a longer duration and organism will be biochemically active in this carrier. Different exogenous or endogenous factors affected the viability of probiotic bacteria including the nature of strains, pH, presence of hydrogen peroxide and dissolved oxygen, the concentration of metabolites such as lactic and acetic acids, the medium buffering capacity, storage temperature, the nature of the added ingredients and food matrices[7]. Cereal-based carrier system is compatible, safe and provides constant support for active growth of the organism up to consumption and in gastrointestinal tract as it contains prebiotic saccharides. It is also evident from many studies that cereal-based beverage with reliable probiotic cultures could reduce diarrhoea and malnutrition in weaning children and help in reducing fatalities and improve well-being[8].

Thereby, the functional combination of indigenous probiotic bacteria, prebiotic food components and bioenrichment of food quality will be the best trilogy for improving the beverage into functional drink[2]. Considering this, the objective of this study was to prepare a nutraceutical rich probiotic drink from rice gruel with the catalysis of a locally isolated non-human origin bifidobacterial strain. The probiotic characteristics of the isolate were evaluated with the stability in product formulation. The fermentation kinetics and bioenrichment ability along with the stability in commercially available acid suppressive pharmaceutical drugs formulation were also investigated.

MATERIALS AND METHODS

Isolation of *Bifidobacterium* sp., through selective enrichment: Fresh fermented rice beer (Haria) was collected from different sites of West Bengal, India and immediately carried out into the laboratory under chilled condition. Sample was diluted (1:10, v/v) inoculated (1%) into the sterile selective *Bifidobacterium* enrichment broth (Himedia, India) and incubated under anaerobic conditions (5.0% CO_2) at 37°C for 24 h. After that growth of the organism and selection of *Bifidobacterium* sp., was made using pour plate technique in *Bifidobacterium* agar (HiMedia, India) following the same incubation conditions. A dominant *Bifidobacterium* sp. MKK4 was isolated and selected according to colony and cellular characteristics. The culture was purified by repeated sub-culturing and preserved into glycerol stock (20% v/v) at -20°C for further use.

Identification of *Bifidobacterium* sp. MKK4: The axenic culture of *Bifidobacterium* sp. MKK4 was subjected to molecular identification through 16S rDNA profiling. The genomic DNA was isolated from overnight grown culture of strain MKK4 using QIAamp DNA Purification Kit (Qiagen) and electrophorized on 1.2% agarose gel. Amplification of 16S rDNA was done using following reaction mixture of final volume of 25 µL containing 0.1 U Taq polymerase, 10 pmol of each primer (Bif164f (164-181), 5-GGGTGGTAATGCCGGATG-3 and Bif662r (679–662), 5-CCACCGTTACACCGGGAA-3), 1 ng of template DNA, 200 pmol dNTPs and sterile distilled water. Fragment of the 16S rDNA gene was amplified by PCR (initial denaturation at 95°C for 5 min; 10 cycles of denaturation at 94°C for 30 sec, annealing at 66-56°C for 30 sec, extension at 72°C for 30 sec; another 20 cycles of denaturation at 94°C for 20 sec, annealing at 56°C for 30 sec final extension at 72°C for 30 sec, final extention at 72°C for 7 min, store at 4°C) from the isolated DNA (Eppendorf, India). A single discrete PCR amplicon band was resolved on agarose gel (2%) and contaminants were removed by using a Qiagen Mini elute Gel extraction (Qiagen) kit. Forward and reverse DNA sequencing reaction of the pure PCR amplicon was carried out with primers using a BDT v3.1 cycle sequencing kit and an ABI 3730xl genetic analyzer consisted denaturation at 96°C for 10 sec, annealing at 52°C for 5 sec and extension at 60°C for 4 min. Consensus sequence of the partial 16S rDNA gene was generated from forward and reverse sequence data using BioEdit. The BLAST (nr-database) was done and based on the maximum identity score, the first 10 sequences were selected and aligned using multiple sequence alignment software (Clustal W). Phylogenetic tree was built by neighbour Joining method in PHYLIP.

Preparation of rice gruel and inoculum: The washed whole rice (*Oryza sativa*) grain (500 g) was soaked in drinking water (1500 mL) for 1 h and boiled for 30 min. The boiled grain was filtered to get the colloid rice gruel which was further sterilized by autoclaving. This was cooled down to room temperature and used for further analysis followed by the centrifugation (at 6,000×g for 8 min).

For preparation of active inoculum, 25 mL of sterile *Bifidobacterium* broth, (Himedia, India) was inoculated with 1 mL of thawed glycerol stock culture and incubated at 37°C for 14 h in CO_2 incubator. This was then centrifuged at 5000 rpm for 10 min and the cell pellet was collected after a gentle wash with phosphate buffered saline (pH 6.8) and serially diluted to obtain a working culture containing 6-7 log CFU mL^{-1} cells as determined by plate counts.

Fermentation: To study the optimum growth, fresh inoculum (1% v/v) was inoculated in different concentration of rice gruel (10-100% v/v) and incubated at 37°C for 22 h under anaerobic conditions (CO_2 5% v/v). Samples were collected after every 1 h interval and used for biochemical analysis. Growth of the organism was determined on the basis of colony forming unit (CFU mL^{-1}).

Acidification kinetics: For acidification kinetics study, the pH of the collected samples was measured by a glass probe-based digital pH meter (Systronics, India). Obtained pH data were plotted to get the curve where from kinetic parameters were calculated for maximum acidification rate (pH_{max}), calculated as the dpH/dt, expressed as pH U h^{-1} and identified in the acidification curve, time to reach pH_{max} (T_{pHmax}) in hours, final pH in pH U and fermentation time in hours.

Fermentation kinetics: The fermentation kinetic parameters were calculated in terms of viable activity (log_{10} CFU mL^{-1}). Specific growth rate (m) and specific rate of product (lactate) formation (v) were calculated respectively as (1/X)×(dX/dt) and (1/X)×(dP/dt), where X is the cell population. Maximum specific growth rate (µmax) and rate of specific lactate formation (vp) were determined from the curves (m vs time) and (v vs time). Growth and lactate yields ($Y_{x/s}$ and $Y_{lactate/s}$, respectively) were calculated as the slope of the linear

regressions of either population or lactate vs residual substrate (as total sugars) during the exponential growth phase.

Analytical methods: The cell-free supernatant of fermented rice gruel was used for the determination of total titratable acidity (equivalent to percent of lactic acid) was determined by the standard titration procedure according to AOAC[9]. Briefly, 10 g of sample was dissolved in 90 mL of distilled water and then titrated by 0.1 N NaOH. Phenolphthalein (0.1% w/v in 95% ethanol) was used as an indicator. The amount of acid produced was calculated as % and w/v of lactic acid according to the following equation:

$$\text{Total tritratable acidity} = \frac{\text{Milliliter of 0.1 (N) NaOH} \times \text{Normality of NaOH} \times \text{Molecular weight of lactic acid}}{\text{Milliliter of sample} \times 10}$$

The quantification of lactic acid and acetic acid was done by high performance liquid chromatography (HPLC) as described by Ghosh *et al.*[3]. The total starch content and amylase activity was quantified by the iodine-complexation method and the actual content (g L^{-1}) was recovered from the standard curve. The amylase activity was determined on the basis of DNS method[10] and expressed as U mL^{-1} min^{-1}.

Determination of amylolytic byproducts: The occurrence of starch hydrolytic end products was initially evaluated by paper chromatography. The residual free clear supernatant was spotted onto Whatman No. 1 paper (chromatography paper) and was run in a descending solvent system with a butanol-acetic acid-water (5:5:3 v/v/v). The starch hydrolytic products were identified on the basis of Rf values after silver nitrate staining (1.2% $AgNO_3$+0.1% KOH+5% $Na_2S_2O_3$)[11].

Determination of mineral contents: The bioavailability of essential minerals like zinc (Zn), manganese (Mn), magnesium (Mg), iron (Fe), copper (Cu), calcium (Ca) were determined in the fermented rice gruel beverage by atomic absorption spectrophotometer (AAS) [Shimadzu Analytical, India]. The fermented broth was centrifuged at 12,000 rpm for 10 min and the supernatant was used for analysis[3].

***In vitro* probiotic characterization**

Tolerance to simulated gastrointestinal juice: The *Bifidobacterium* sp. MKK4 was tested for the survivability test in simulated gastric juices and small intestinal juices. The simulated gastric juice was prepared by PBS buffer solution with pepsin (0.3%, w/v) with different values of pH viz., pH 2.0, 3.0 and 6.8 (control) and sterilized by autoclaving at 121°C for 15 min[12]. Whereas, simulated small intestinal juice was prepared by suspending 1.0 g of pancreatin in 100 mL of sterile solution containing 0.680 g di-potassium hydrogen phosphate and 0.089 g of sodium hydroxide (pH 6.8 and 8.0) and sterilized by filtration (0.2 mm) and autoclaving at 10 lb $inch^{-2}$ for 15 min[13]. The survivability of the isolate was checked up to 4 h of incubation at 37°C in anaerobic condition (5% CO_2), respectively, for simulated gastric and small intestinal juices ($N_0 = 10^9$ CFU mL^{-1} in 10 mL of different aqueous solutions). The final viable cell populations were determined by serial dilutions and spread plate methods.

Bile salt tolerance: The bile tolerance ability of the isolate was examined following the method described by Vinderola and Reinheimer[14]. Cell suspensions ($N_0 = 10^9$ CFU mL^{-1} in 10 mL of bile solution) were added into different strengths (0.3, 1 and 2%) of bile salt solutions and incubated up to 4 h at 37°C in anaerobic condition (5% CO_2). The final viable cell populations were determined by serial dilutions and spread plate methods.

Antibiotic susceptibility assay: The selected *Bifidobacterium* isolate was checked for the antibiotic susceptibility using Kirby and Baueragar diffusion method[3]. The overnight culture of the isolate (10^6-10^7 CFU mL^{-1}) was spread over the solid BSC propionate agar medium surface and preincubated at room temperature for 1 h. Next, antibiotic discs (Himedia, India) were placed over the culture inoculated medium surface and incubated under anaerobic conditions (5% CO_2) at 37°C for 24 h. At the end of the incubation, inhibition zones were measured and expressed in millimeter.

Survivability against Gastric Acid Suppressive (GAS) pharmaceutical formulations: The survival stability of the *Bifidobacterium* sp. MKK4 was examined against several commonly used gastric acid suppressive pharmaceutical formulations like OMEZ, Ranitidine, ENO, Diovol and Pantop. For this, cells ($N_0 = 10^9$ CFU mL^{-1}) were suspended in the 10 mL of GAS contained phosphate buffer saline (pH 7.4) and incubated at 37°C under anaerobic condition (5% CO_2). The cell viability was checked after 1 and 2 h of incubation by standard serial dilutions and spread plate methods.

Stability studies: The shelf life of fermented rice gruel was studied by following the slightly modified International Conference on Harmonisation (ICH) Q1A (R1) stability guidelines through determining the viability of *Bifidobacterium* sp. MKK4. Briefly, 100 mL of fermented rice

gruel (FRG-C) was aseptically transferred into the glass bottle, sealed and kept at refrigerated condition (4±1°C). Simultaneously, stability test was carried out in the bifidobacterial broth (BB) and sterile inulin (FRG-I) and fructooligosaccharide (FRG-FOS) (1% w/v) were mixed separately with the fermented rice gruel in order to know the impact of prebiotics on the stability of the probiotic. The stability studies conditions were same as was in control and was conducted up to 30 days. The viable count of *Bifidobacterium* sp. MKK4 was determined as per standard microbiological method as discussed earlier.

Statistical analysis: Collected data were presented as the arithmetic mean of three replicates (Mean±SD). Significant variation was accepted at the level of 5 and 1% (i.e., $p<0.05$ and $p<0.001$) which was measured by student's t-test using Sigmastat 11.0 (USA) statistical software.

RESULTS

Isolation and identification of *Bifidobacterium* sp. MKK4: A total 10 different types of *Bifidobacterium* colonies were isolated from 29 Haria samples. Among them, MKK4 strain was shown to be dominant after the specific enrichment and selective isolation on *Bifidobacterium* selective agar. Initially, colonies were screened by Gram staining characteristics and shape of the cell. The presumptive identification of the isolate *Bifidobacterium* sp. MKK4 was done by biochemical test and 16S rRNA sequencing. A 649 bp fragment of the partial 16S rRNA sequence was analyzed for interference of evolutionary history using the Neighbor Joining method. *Bifidobacterium longum* subsp., C118 (GenBank accession No.: gb|KC160497.1|) was found to be closer to the strain MKK4 based on nucleotide homology and phylogenetic analysis (Fig. 1). The partial 16S rDNA sequence was submitted to GenBank and allotted accession No. KU555422.1 as reference.

In vitro evaluation of probiotic characteristics of *Bifidobacterium* sp. MKK4

Survivability in simulated gastrointestinal environment: The survivability profile of the newly isolated *Bifidobacterium* sp. MKK4 was examined for 240 min (4 h) against Simulated Gastric Juice (SGJ) and Simulated Intestinal Juice (SIJ). A relatively high survivability (respectively, 91.5 and 94.8%) of the isolate was recorded in SGJ and SIJ at pH 6.8 (Fig. 2). A moderate level of relative survivability (70.8%) was noticed for SIJ (pH 8.0), whereas relative survivability was reduced to 52.7% for SGJ at pH 3.0 ($p<0.05$) after 4 h of treatment.

Survivability in simulated gastrointestinal bile environment: The *in vitro* bile tolerance of the *Bifidobacterium* sp. MKK4 showed a significant growth impact in the time and dose-dependent manner. The isolate was retained above 80% viability up to 30 min in all the tested combinations of bile salt and oxgall (0.3%). The relative viability of the isolate was reduced down by 40% for bile salts and oxgall after 4 h of exposure (Fig. 3).

Antibiotic resistance/susceptibility profile: *Bifidobacterium* sp. MKK4 was susceptible to most of the tested antibiotics

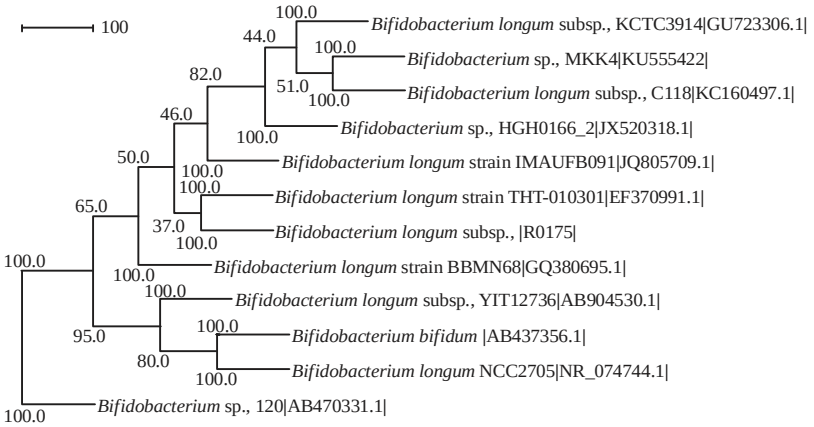

Fig. 1: Phylogenetic tree (Neighbour Joining method) of the isolate MKK4. According to the tree, the isolate belongs to *Bifidobacterium* sp.

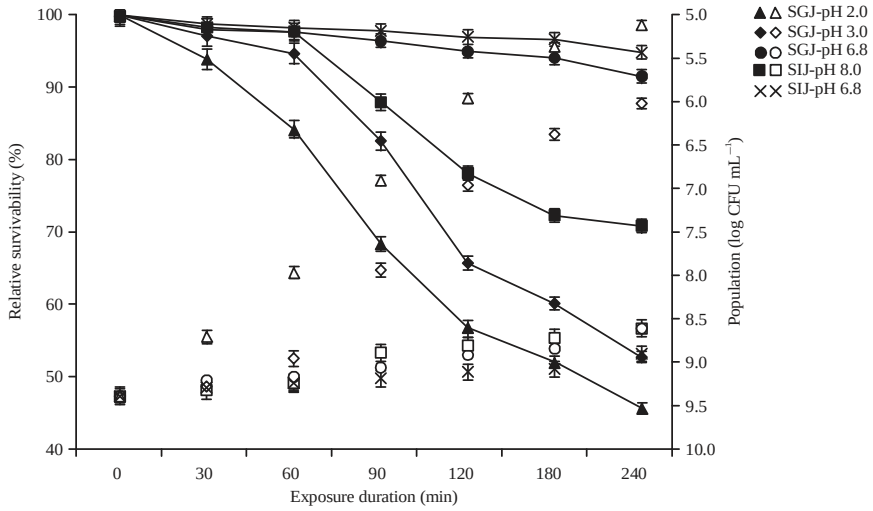

Fig. 2: *In vitro* probiotic characteristics study by examining the survivability of the *Bifidobacterium* sp. MKK4 in Simulated Gastric Juice (SGJ) and Simulated Intestinal Juice (SIJ) for different time durations. Expression of relative survivability (%) is expressed using the line with markers and alteration in population (log CFU mL^{-1}) is expressed with scattered markers. Seven time-point results were plotted for different samples

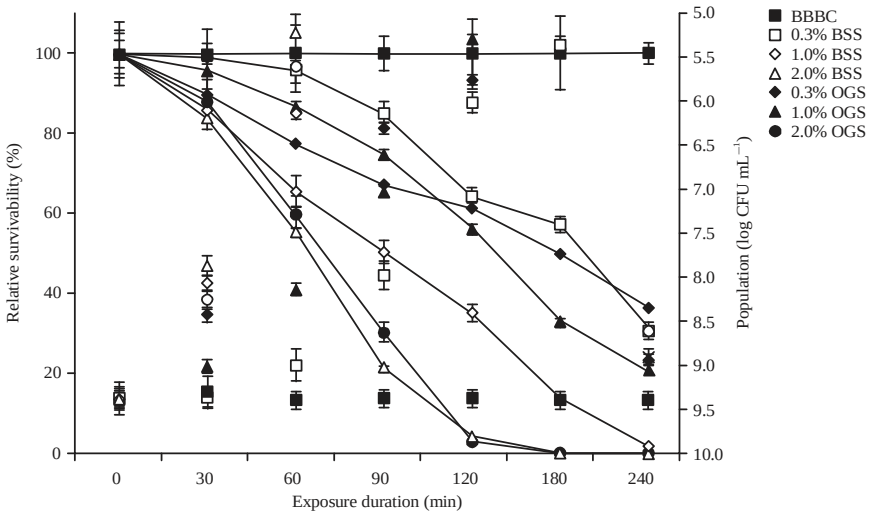

Fig. 3: *In vitro* bile-tolerance competency of probiotic *Bifidobacterium* sp. MKK4 in Bile Salt Solution (BSS) and oxgall solution (OGS) for different time durations. Expression of relative survivability (%) is articulated using the line with markers whereas alteration in population (log CFU mL^{-1}) is expressed with scattered markers. Bifidobacterial Basal Broth (BBB) with the culture (-bile/oxgall) was served as control (BBBC) with other time-point experimental samples

like amikacin (10 µg), gentamycin (30 µg), erythromycin (10 µg), streptomycin (25 µg), nitrofurantoin (300 µg), ciprofloxacin (10 µg), norfloxacin (10 µg), cefixime (5 µg), ampicillin (2 µg), penicillin (2 U), tetracycline (10 µg) and chloramphenicol (10 µg) (Fig. 4). A low and variable resistance pattern has been seen against cotrimazole, polymyxin and cefuroxime.

Impact of gastric acid suppressive (GAS) drugs on bacterial survivability: In this study we have examined for the first time the impact of common antacid and acid reducing formulations on the relative survivability of the *Bifidobacterium* sp. MKK4 and it was noted that the organism exhibited significant stability (91-100%) for the tested formulations for 1 h. Relative survivability of the

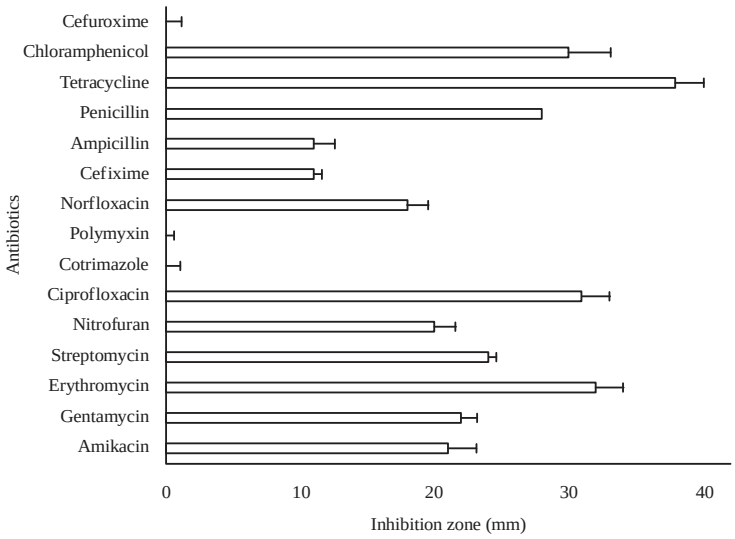

Fig. 4: Susceptibility of *Bifidobcterium* sp. MKK4 against different antimicrobial antibiotics, represented by the respective zone of inhibition headed with standard error of mean bar

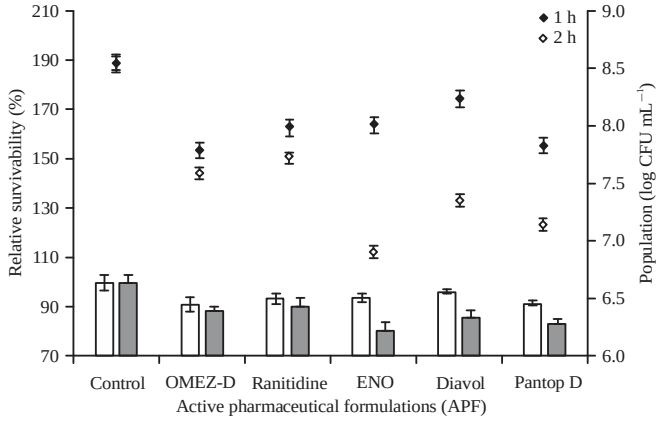

Fig. 5: Impact of active pharmaceutical formulations on the survivability of the isolate *Bifidobacterium* sp. MKK4 recovered for rice gruel fermentation. The relative survivability (%) of the isolate was examined at different time points like after 1 and 2 h of incubation. Alterations in the bacterial population (log CFU mL^{-1}) were also pointed at the same time point (1 and 2 h) by falling directions

organism was slightly reduced about 19.17% after 2 h of incubation with ENO, which contains salts of sodium bicarbonate, carbonate and citric acid. In compare to ENO, another salt dependent antacid, diovol (contains aluminium hydroxide, magnesium hydroxide, magnesium carbonate and dimethicone) showed the least effect on the survivability of the isolate. The significant survivability of the probiotic against Proton Pump Inhibitors (PPI) containing formulations like omez (omeprazole) and pantop (pantoprazole) and H_2 histamine receptor antagonist like rantac (ranitidine HCl) indicated its feasible combined administration of these drugs (Fig. 5).

Fermentation kinetics and bioenrichment of rice gruel: The rice gruel is starch rich substrate (~22.01 g L^{-1}) that utilized by *Bifidobacterium* sp. MKK4 during the course of fermentation. The starch content was rapidly broken down with a rate of starch hydrolysis of 1.294 g L^{-1} h^{-1} and after 9 h of fermentation starch content reached to 2.208 g L^{-1} (Table 1, Fig. 6).

The maximum amylase activity (3.508 U mL^{-1}) was observed at 13 h of fermentation, prior to that, it was inclined sharply and proportional to the logarithmic cell population ($Y_{amylase/x}$-0.558 U log^{-1} CFU mL^{-1}) (Table 1, Fig. 6). However, amylase synthesis was declined with the sharp reduction of

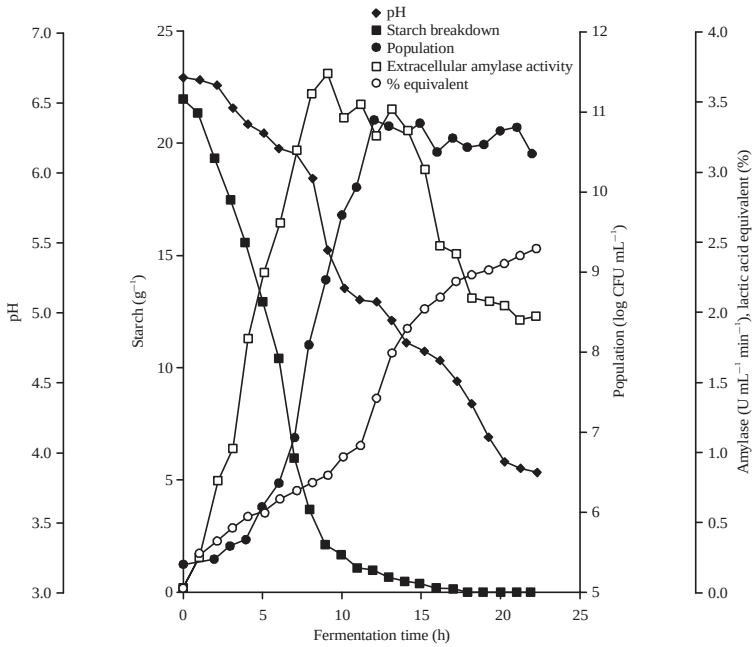

Fig. 6: In-process fermentation dynamics of rice gruel by *Bifidobacterium* sp. MKK4, considering parameters like changes in pH, starch breakdown (g L^{-1}), population (log CFU mL^{-1}), extracellular amylase activity (U mL^{-1}) and accumulated lactic acid content (% equivalent) in the fermentation medium. The parameters were studied over the regular interval of gruel fermentation (h)

Table 1: Kinetic parameters for rice gruel fermentation by *Bifidobacterium* sp. MKK4

Parameters	Kinetic values
Initial starch content in rice gruel (g L^{-1})	22.01
Maximum acidification rate (-dpH/dt) (U pH h^{-1})	0.11
Maximum acidification rate at time point	0.153 at 10 h
Initial/final pH	6.73/3.85
Initial/final population (log CFU mL^{-1})	5.349/10.491
Final lactic acid concentration (% equivalent)	2.484
Maximum/final amylase concentration (U mL^{-1} min^{-1})	3.758/1.990
Rate of starch hydrolysis (g L^{-1} h^{-1})	1.294
Rate of lactic acid production (% equivalent h^{-1})	0.112
$Y_{x/s}$ (increase in log CFU mL^{-1} mg^{-1} of starch consumption)	0.233
$Y_{x/s}$ in log phase of isolate	0.402
$Y_{lactate/s}$ (percentage lactate produced per gram of starch breakdown)	0.087
$Y_{lactate/s}$ in log phase of isolate	0.073
$Y_{amylase/x}$ (U log^{-1} CFU mL^{-1} population)	0.387
$Y_{amylase/x}$ in log phase	0.558
μ_{max} (h^{-1})	1.174
Monod's constant, K_s (g L^{-1} substrate for ½ μ_{max})	5.721
$v_{lactate}$ (percentage lactate log^{-1} CFU mL^{-1} h^{-1})	0.483

starch (R^2 = 0.75, $p<0.05$) and 0.693 times lower than log phase metabolism of the isolate (0.387 U log^{-1} CFU mL^{-1}) (Fig. 6).

Acidification kinetics of rice gruel: The medium pH became acidic and it was shifted from 6.73-3.85 with a maximum rate of medium acidification (-dpH/dT, 0.11 U pH h^{-1}). However, the highest acidification rate was recorded at the 10 h of fermentation (0.153 U pH h^{-1}) (Table 1), when the isolate was at the end of its log phase (Fig. 6). The change in medium pH was found to be in significant correlation with the increased population (R^2 = 0.93, $p<0.05$) and course of fermentation (R^2 = 0.99, $p<0.05$). The decrease in medium pH indicated the increase in the production of organic acids like lactic acid and acetic acid (Fig. 7), which also happened up to the end of the fermentation. At the end of the fermentation the concentration of accumulated lactic acid was (Percentage of equivalent) 2.484 (Table 1). The response surface analysis clearly demonstrated that during course of fermentation (time), both quantity of cell population (log CFU mL^{-1}) and acidification rate (pH U h^{-1}) were correlatively increased (actual R^2 = 0.86 and adjusted R^2 = 0.82) (Table 2, Fig. 8). However, the conversion of starch to organic acid was maintained at a steady state rate ($Y_{lactate/s}$ 0.087% lactic acid equivalent), even in log phase of the growth ($Y_{lactate/s}$ 0.073% lactic acid equivalent) throughout the fermentation period (Table 1). The rate of lactic acid production was 0.112% h^{-1} which is 11.55 times slower than the rate of starch breakdown (1.294 g L^{-1} h^{-1}). The starch breakdown

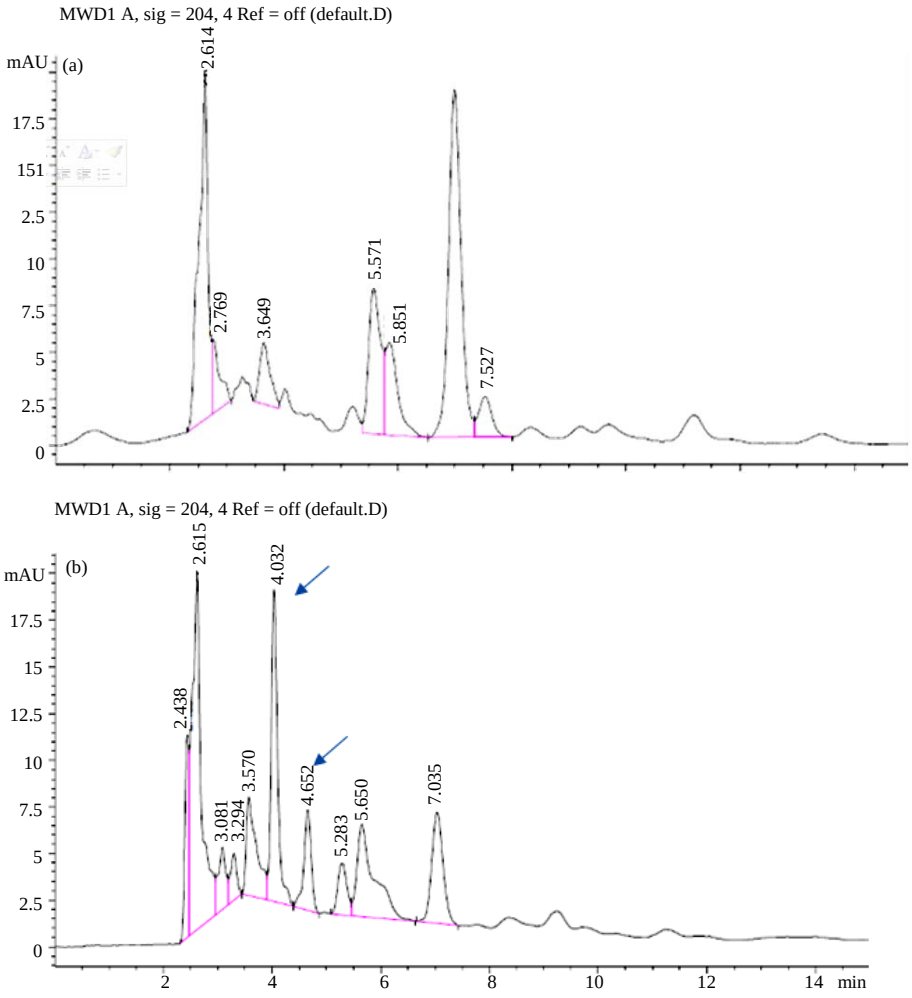

Fig. 7(a-b): Production of (a) Lactic acid and (b) Acetic acid during rice gruel fermentation by *Bifidobacterium* sp. MKK4. The taller peak is of lactic acid than the short one is acetic acid (b) and both are absent in control samples (a)

Table 2: ANOVA results for response surface analysis acidification kinetics of rice gruel fermentation by *Bifidobacterium* sp. MKK4 keeping time (h) as independent variable and population (log CFU mL^{-1}) and acidification rate (pH U h^{-1}) as dependent continuous variable ($R^2 = 0.86$, Adj $R^2 = 0.82$)

Factors	SS	Df	MS	F	P	Standard error coefficient
Acidification rate (AR-L)	0.104	1	0.10375	0.012450	0.912463	8.77843
Acidification rate (Q)	1.282	1	1.28233	0.153875	0.699734	11.86701
Population (log CFU mL^{-1}) (Pop-L)	12.564	1	12.56424	1.507665	0.236226	7.22682
Population (log CFU mL^{-1}) (Q)	0.519	1	0.51900	0.062278	0.805921	6.55609
AR-L by pop-L	0.000	1	0.00002	0.000002	0.998815	19.01213
Error	141.671	17	8.33358			
Total SS	1012.000	22				

was negatively correlated with the lactic acid production ($R^2 = 0.83$, $p<0.05$), however, a strong positive correlation with the fermentation course ($R^2 = 0.98$, $p<0.05$) revealed that the lactic acid production is continuous and time-dependent[15].

Bioenrichment rice gruel during course of fermentation: It was evident from the paper chromatogram that the starch content of rice was hydrolyzed in duration dependent manner and produced different saccharides like maltotetraose (G2) and glucose (G1) at 10 h, whereas, maltose (G2), maltotriose (G3), maltotetraose (G4), maltopentaose (G5) and maltohexaose (G6) at 20 h of fermentation (Fig. 9). The calcium, iron and zinc content (1.02, 2.03 and 4.27 ppm, respectively) was found higher in the fermented rice gruel (Fig. 10).

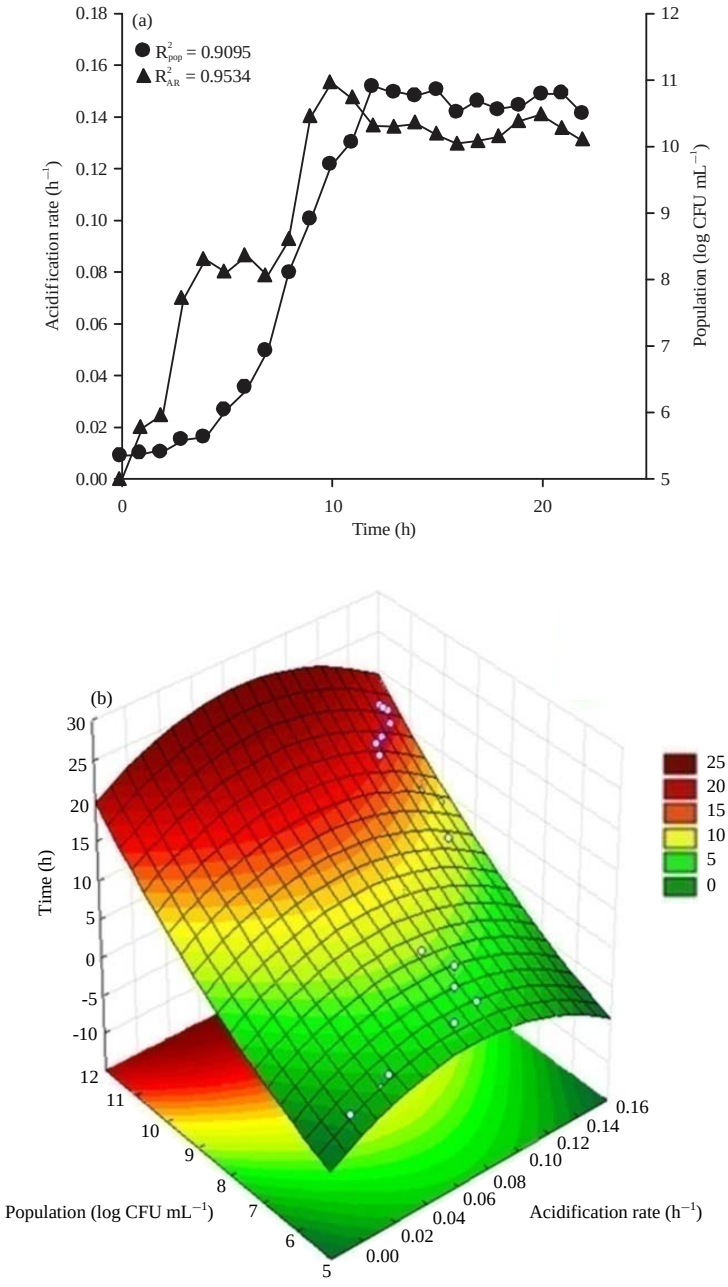

Fig. 8(a-b): (a) Population (log CFU mL^{-1}) and acidification rate (μ h^{-1}) of Haria isolate MKK4 during the rice gruel fermentation. A well-fit polynomial regression line was used for strong correlation between time and population $\left(R^2_{pop} = 0.9095\right)$ and acidification rate $\left(R^2_{AR} = 0.9534\right)$ and (b) Response surface analysis plot keeping time as independent variable and population and acidification rate as dependent continuous variable

Stability studies: In this study, the fermented rice gruel was tested for the shelf life by determining the viability of the probiotic *Bifidobacterium* sp. MKK4 in presence of media (BB), fermented rice gruel (FRG), inulin (FRG-I) and fructooligosaccharide (FRG-FOS). The isolate was found to retain 76.09% viability after 30 days of storage at refrigerated conditions. This durable viability of the probiotic *Bifidobacterium* sp. MKK4 in rice gruel is due to the adequacy of maltooligosaccharides which may acts as a growth enhancing prebiotic substance formed during the course of fermentation[16]. The viability was improved significantly (91.30 and 95.65%, $p<0.05$) when the fermented rice gruel

Table 3: Stability studies of rice gruel fermented by *Bifidobacterium* sp. MKK4. Mean population values (n = 3) are indicating the viable cell activity with factor ($\times 10^8$ CFU mL^{-1})

Stability test	1st	5th	10th	15th	20th	25th	30th
	(Days)						
FRG-C	4.6±0.3	4.4±0.2	4.2±0.3	4.0±0.3	4.0±0.3	3.9±0.3	3.5±0.4
BB	4.6±0.4	4.4±0.3	4.1±0.4	3.9±0.3	3.4±0.2	3.3±0.4	3.1±0.2
FRG-I	4.6±0.4	4.5±0.3	4.3±0.2	4.3±0.2	4.2±0.2	4.2±0.2	4.2±0.2
FRG-FOS	4.6±0.4	4.6±0.3	4.6±0.2	4.5±0.2	4.5±0.2	4.5±0.2	4.4±0.2

FRG: Fermented rice gruel, BB: *Bifidobacterium* broth, I: Inulin, FOS: Fructooligosaccharide

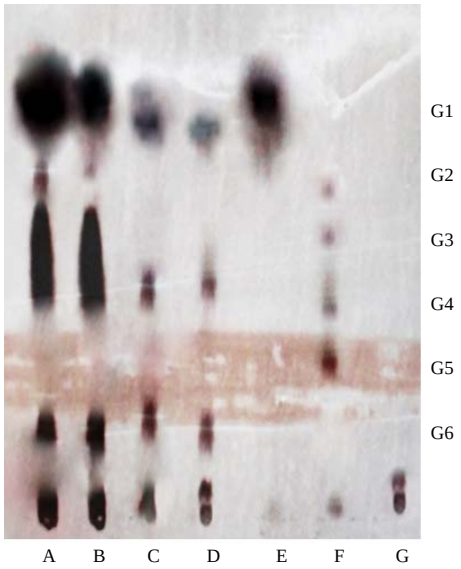

Fig. 9: Identification of the hydrolysis products obtained from rice gruel fermented by *Bifidobacterium* sp. MKK4. Time dependent samples of rice gruel fermentation like at 10 h (D), 13 h (C), 16 h (B), 20 h (A), standard glucose (G1, E), maltooligomer standard (G2-G6, F) and unfermented rice gruel (G)

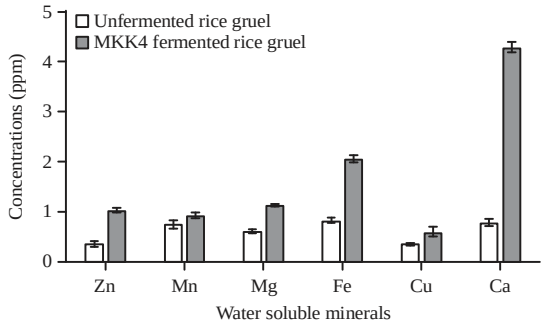

Fig. 10: Bio-fortification of rice beverage by *Bifidobacterium* sp. MKK4 with essential minerals like zinc, manganese, magnesium, ferrous, cupper and calcium

was supplemented with inulin (FRG-I) and fructooligosaccharide (FRG-FOS), respectively (Table 3).

DISCUSSION

Indian traditional fermented foods and beverages are mainly nourished by indigenous microbial strains, amongst, lactic acid and alcohol producing microbes are prevalent. Most of the microbes are unknown which are having nutritional and health beneficial impacts. Earlier studies have unveiled the microbial composition of a rice based beverage, Haria and reported about the functional interplay among *Lactobacillus* spp., *Bifidobacterium* spp., *Bacillus* sp., *Saccharomyces* spp., and others[2,3]. Here, *Bifidobacterium* sp., is evaluated for its functional role in fermentation of rice gruel and its probiotic characteristics. Generally, bifidobacteria are common resident in intestinal micro environment, fermented milk and cereal products plays important role in nutritional improvement and health beneficial properties[17]. It was evident from our earlier study that during Haria preparation, the quantity of *Bifidobacterium* was increased concomitantly with the duration of fermentation period[2]. As this beverage has long run safe consumption history, therefore, the inherited microbial consortia in this beverage could be regarded as safe and healthy. The isolate MKK4 was conformed to belong in the genus *Bifidobacterium* by the biochemical as well molecular identification.

In order to confer health benefits, the organism should be alive through many physiological barriers throughout the gastrointestinal tract, among which the presence of acid in stomach, bile and different enzymes in the small intestine which determines an important character of a potential probiotic[18]. The magnitude of Simulated Intestinal Juice (SIJ) and Simulated Gastric Juice (SGJ) toxicity on *Bifidobacterium* sp. MKK4 was found to be time and constituent dependent. However, a high degree of relative survivability (>80%) was verified after 1.5 h of exposure to different simulated juices which is within the limit of actual transit time in the human gastrointestinal tract[12]. Comparatively, a low survivability profile (12.19% at pH 2.0 and 57.56% at pH 3.0) was observed in *L. fermentum* KKL1 in SGJ over 4 h of incubation[3]. The resistance to gastric acids is a relatively inherent property in probiotic *Lactobacillus* and *Bifidobacterium* as they have special anchorage of H^+-ATPase on their cell membrane.

Gastric survival is a highly valued property with the aid of gastric transit and to ensure the delivery of viable cells into the small intestine[19].

Besides, the isolate MKK4 was seen to be resistant to bile salts on the time and doses dependent basis. Bile salts are detergent like biological substance of host defense that possess strong antimicrobial activity by disorganize the structure of cell membrane and DNA. Tolerance to bile is an essential feature for the probiotic bacterium in order to succession in the proximal small intestine[20]. Bile resistance is a multifactorial phenomenon, implicating a variety of processes including active efflux of bile acids/salts[21,22], production of bile salt hydrolase[20] and changes in the architecture/composition of cell membrane and cell wall[23] appear to be the most prevalent bile-specific resistance mechanisms in probiotic *Lactobacillus* and *Bifidobacterium*[20]. Margolles *et al*.[24] reported about the oxgall toxicity on 19 bifidobacerial isolates with less than 2.0% MIC.

Monitoring the antibiotic sensitivity profile of a probiotic bacterium is an essential to control antibiotic resistance among normal flora and opportunistic pathogens in the intestine and this testing is encouraged by major official bodies, such as the European Union (EU), the Centers for Disease Control and Prevention (CDC), Indian Council of Medical Research (ICMR) and the WHO[25]. The antibiogram profile of *Bifidobacterium* sp. MKK4 was relatively similar with other probiotics like *Bifidobacterium animalis* subsp., *lactis* and *Bifidobacterium* sp., isolated from different commercial dairy and pharmaceutical origin[26]. Charteris *et al*.[27] reported about 16 bifidobacterial strains those showed a higher antibiotic resistance profile. A comparative safe and hazard-free antibiotic profile was tested for 50 bifidobacterial strains and assured for absence of potential transferability of resistance determinants with low natural resistance[28]. Thus, with regard to a general concern about the safety of probiotics, *Bifidobacterium* sp. MKK4 was articulated to be a potential probiotic by high sensitivity to common antibiotics and appeared risk-free of antibiotics resistance.

In addition to the probiotic properties, the isolate MKK4 was seen to be resistant and retained significant cell viability in presence of Gastric Acid Suppressive (GAS) drugs. This phenomenon is an unknown feature in probiotics and eventually makes them medicinally important for the treatment of certain gastrointestinal maladies like gastro-esophageal reflux disease (GERD), barrett esophagus, peptic ulcer, dyspepsia and zollinger-ellison syndrome. The prevalence of these diseases varies throughout the world, lowest in East Asia (2.5-9.4%) and higher in central (7.6-19.4%) and Western Asia (12.5-27.6%), highest in Europe (23.7%) and the United States (28.8%)[29]. The reason behind this variation is unknown but infection of *Helicobacter pylori* is one of the common phenomenon. Antacid (acid neutralizer) and acid reducing drugs (proton pump inhibitors and H_2 receptor blockers) are the common choice of drugs to treat and control these diseases. But major side effects of these acid suppressant drugs are rising of intra gastric pH that favours the overgrowth of enteropathogens that induced persistent inflammation. Experimentally, it is established that some probiotics including *Lactobacillus* and *Bifidobacterium* can prevent the adhesion, colonization and growth of enteropathogens specifically *H. pylori* in gastric mucosa[29-31]. In this study, Del Piano *et al*.[32] adopted a new strategy by concomitant oral administration of lactobacilli along with proton pump inhibitor drugs. In this pilot study, they observed that lactobacilli supplementation significantly reduced the total bacteria and coliforms in the gastric milieu in subjects that chronically treated with proton pump inhibitor drugs. Emerging evidences suggested that *Bifidobacterium* sp., has tremendous potentialities to eradicate *Helicobacter pylori* that causes chronic gastritis, initiation of peptic ulcer and is a risk factor in the development of gastric malignancies[33]. Thus, the co-administration of gastric acid suppressive (GAS) drugs along with the *Bifidobacterium* sp. MKK4 could be a unique combination to cure peptic ulcer and to restore gastrointestinal health.

Food industries are now trending in modeling the kinetics of probiotic microorganisms as a part of process development[34]. Bacterial kinetic studies help in understanding the organism's behavior like cell growth, metabolites production and substrate uptake, response to fermentation parameters, etc. These deliver a better understanding of the fermentation course and could lead to the development of improved strategies for the optimization of the fermentation process to ensure its economical viability[34]. Besides, industrially important microbes shall be stable over a period of time in order to effective formulation and exerting the physiological functions. This study was particularly aimed to develop the alternative approach of process development of an ethno-medicinal fermented beverage using the indigenous *Bifidobacterium* sp. MKK4. Further, this help to assess the nutritional quality of the *Bifidobacterium* fermented gruel-based beverage and stability profile of the isolate.

In the present experimental condition, starch was the principal carbon source in rice gruel which was used by the probiotic isolate MKK4 for its metabolism and multiplication. It was found that the changes of cell population was inversely related to the medium starch content having a significant

negative correlation (R^2 = 0.95, $p<0.05$). The average cell growth to starch consumption ratio ($Y_{x/s}$) was 0.233 log CFU mL^{-1} mg^{-1}, which was sharply accelerated during logarithmic phase ($Y_{x/s}$ 0.402 log CFU mL^{-1} mg^{-1}) (Fig. 3, Table 1). This rice gruel based low cost and simple media facilitate the growth of *Bifidobacterium* sp. MKK4 that further indicated by its specific growth rate (μ_{max}) 1.174 h^{-1} and the Monod's constant (K_s) 5.721 g L^{-1} (Table 1). This revealed the unique fermentation pattern of the isolate in rice gruel where the integrity of the substrate consumption and cell growth is uneven and dependent on the course of fermentation.

The starch hydrolysis in the fermentation medium was accomplished by the amylases, synthesized by the *Bifidobacterium* sp. MKK4 in order to the saccharification and liquefaction of rice gruel. As amylase is an inducible enzyme and starch was the main inducer, therefore, depletion of this substrate negatively influenced to the biosynthesis of amylase by the organism. This sort of negative feedback mechanism was earlier described in case of probiotic *L. acidophilus*[35], *B. breve* and *B. dentium*[36]. Generally, the content of amylase in fermented materials has excellent therapeutic potentialities related to the improve digestibility of starchy food materials and could convert them into functional maltooligosaccharides[37].

Short chain fatty acids like lactic and acetic acid are the principal health beneficial metabolites derived from lactic acid producing bacteria (LAB) during homo-or heterolactate fermentation. During rice gruel fermentation, increase in starch hydrolysis and cell population was accompanied by increase in medium acidity by the production of acidic compounds. However, organic acid analysis revealed the comparatively higher amount of lactic acid production than the acetic acid which is quite uncommon in bifidobacteria. The bifidus shunt in *Bifidobacterium* spp., generally produces more acetic acid than lactic acid. Such phenomenon may be due to the quorum sensing among other community members and co-evolutionary shifting in metabolism of the particular species. Chick *et al.*[38] reported about such rare phenomenon when *Bifidobacterium* were grown in nonfat dry milk containing medium supplemented with nectar honey. The lactic acid production was increased without affecting the acetic acid. The simultaneous increase in cell biomass and lowering of medium pH was also occurred for *Lactobacillus manihotivorans* LMG 18010^T during the starch fermentation[39]. Gupta *et al.*[40] has also shown the co-parametric evolution of cell population and lactic acid production in *Lactobacillus saccharina* and is associated with the rate of agitation. Higher lactic acid producing *Bifidobacterium* strains are particularly of importance as they inhibits tumor incidence and multiplicity in human colon[41].

The rice gruel fermentation was carried out by atypical indigenous amylolytic *Bifidobacterium* sp. MKK4 that fortified this low cost substrate with different health beneficial saccharides and minerals. These maltooligomers are important for human consumption, since, they are (i) Low calorigenic, (ii) Less sweet than sucrose (30%, using a 3% solution at 20°C), (iii) Inhibit the growth of harmful intestinal microflora, (iv) Have low viscosity, high moisture retaining capacity and low water activity, which is preferable for controlling microbial contamination and (v) Act as avour enhancers, fat replacers and bulking agents in foods[3]. Previously, Ryan *et al.*[36] also showed the strain dependent maltooligomer producing profile in *Bifidobacterium* and expression of both α-1,4 and α-1,6-glycosidic linkage degrading extracellular enzymes.

During course of fermentation, it was also noted that rice gruel was enriched with essential multivalent minerals (Ca>Fe>Mg>Zn>Mn>Cu) in comparison to its unfermented condition. This bioavailability of essential micronutrient during course of fermentation can improve its health beneficial impacts particularly to combat against anemia and osteoporosis related diseases. A significantly higher content of Zn, Ca and Mg were also documented from different non-distilled fermented alcoholic beverages of Spain[42]. In comparing to *Bifidobacterium* sp. MKK4, another isolate of the same origin, i.e., *L. fermentum* KKL1 showed a comparative low and different biomineralization ability (Mg>Na>Ca>Mn>Fe)[2]. These types of bioavailability of minerals in fermented cereals are associated with dephytinization of antinutrient phytic acid (myo-inositol-6-phosphate) by the microbial enzymes[1]. The results are clearly indicating the fermented rice gruel can uphold the better bioenrichment of essential minerals than the traditional whole grain rice.

However, shelf life of the probiotics is an important feature which ensures about the stability over the storage time to confer the desired health benefits. In order to be a good probiotic dietary adjunct, the minimum viability concentration of the probiotic organisms should be of 10^6 colony-forming units (CFU) per gram[43,44]. *Bifidobacterium* sp. MKK4 activity was increased 1.12, 1.34 and 1.41 folds in FRG, FRG-I and FRG-FOS than BB, respectively (Table 3). It is most probably because of FRG is rich in neutraceuticals and the inulin and fructooligosaccharides (FOS) are the 'non-digestible carbohydrates that selectively stimulate the growth and activity of colonic microflora' and ultimately exert health benefitial affect to the host[16]. In compare to fermented rice gruel and prebiotic supplementation, the viability was low (67.39%) in Bifidobacterial Broth (BB) which may be due to

complete depletion of available nutrients and release of metabolites. Bedani *et al.*[16] reported about the insignificant impact of inulin on the viability of *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* Bb. Saarela *et al.*[45] also mentioned about the enhanced survivability of autotrophic *Bifidobacterium* strain in acidic fruit juice (pH 3.8) for one week storage at 4°C. A different stability matrix was tested for *Bifidobacterium animalis* in cosmetic formulations where, guar gum was showed to be promising at the same temperature selected for this study also[46]. The improved viability of *Bifidobacterium* sp. MKK4 and the resistance to harsh micro environment in the gastrointestinal tract could attribute to use this fermented beverage as promising carrier. The probiotic which has high temporal and spatial stability, especially possess much importance over others with low stability[47].

CONCLUSION

The newly isolated *Bifidobacterium* sp. MKK4 from an ethno-traditional fermented rice beverage showed significant probiotic characteristics as it was able to survive in simulated gastric and intestinal conditions. The isolate was found to be stable with gastric acid suppressive pharmaceutical drugs. The rice gruel, a low cost and waste material during rice preparation can be exploited as carrier for *Bifidobacterium* sp. MKK4 with the distinct fermentation dynamic and kinetics. Interestingly, the rice gruel was fortified with a group of health beneficial metabolites including minerals, organic acids, maltooligosaccharides, etc during the fermentation. Thus, this food preparation can be exploited as functional and healthy beverage for the world community to improve individual nutritional status as well as community health.

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