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Characterization of potential probiotic bacteria isolated from sorghum and pearl millet of the semi-arid tropics

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The present study was conducted to isolate and characterize probiotic properties of bacteria isolated from flour and batter samples of sorghum and pearl millet. A total of five different selective media including plate count agar, yeast glucose chloramphenicol agar, Bifidobacterium agar, actinomycetes isolation agar and de ManRogosa and Sharpe agar were used and the most prominent bacteria (which were found abundantly in the plate) were isolated and maintained on the respective media slants at 4°C for further studies. The bacteria were characterized for various traits including Gram staining, morphology (color, size, shape, elevation, margin, form and surface), biochemistry (urease, catalase, oxidase, hydrogen sulphide, nitrogen reduction, gelatin liquefaction, starch hydrolysis and carbohydrate utilization), IMViC tests (indole, methyl red, Voges Proskauer and citrate utilization), probiotic potentials [acid (pH 2, 3), bile (0.5%), NaCl (6 and 9%)], phenol tolerance [0.4%], antibiotic tolerance (tetracycline, streptomycin, kanamycin, chloramphenicol, ciprofloxacin, ampicillin, penicillin, erythromycin and vancomycin) and antimicrobial activity against human pathogens (*Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhi*). A total of nine probiotic bacterial isolates were short listed based on these traits. The sequences of 16s rDNA gene of the nine isolates were found matched with *Bacillus subtilis* (two isolates), *Bacillus cereus* (three isolates), *Bacillus pumilus* (one isolate), *Bacillus amyloliquefaciens* (one isolate), *Sphingobacterium thalpophilum* (one isolate) and *Brevibacterium* sp. (one isolate) in BLAST analysis. The sequences of the nine bacteria were submitted to NCBI and accession numbers obtained. This study indicated that the selected bacteria could be exploited to develop new probiotic foods.

Key words: Probiotics, prebiotics, sorghum, pearl millet, product development.

INTRODUCTION

Cereals such as rice (*Oryza sativa*) and wheat (*Triticum aestivum*) are presently the predominant staple food for millions across the world that lead not only to an array of emerging life style diseases but also challenges human health and nutrition. Thus, there is an urgent need for identifying and recommending diversity in diets through

inclusion of other cereals in our diets in order to enhance the overall nutritional status as well as address malnutrition across the world. Two of ICRISAT's mandate crops, sorghum (*Sorghum bicolor*) and pearl millet (*Pennisetum glaucum)*, can serve this purpose. Sorghum and pearl millet are an important food for millions of people inhabiting the semi-arid tropics in Africa and Asia and are a major source of calories and vital component of food security in the semi-arid areas in the developing world (Amadou et al., 2013). Sorghum and pearl millet are often ground into flour and consumed as roti and/or porridge with milk and sometimes prepared as beverages (Obilana and Manyasa, 2002; Amadou et al., 2011). These crops can serve as the source of prebiotics (total dietary fiber, resistant starch, total oligosaccharides and β-glucan) for functional food (Awika and Rooney, 2004). Functional foods are referred as food ingredients or the food as such that influence beneficial effect on the host and/or reduce the risk of chronic diseases (Huggett and Schliter, 1996; Charalampopoulos et al., 2002). Probiotic microorganisms have been used for preparation of dairy food for thousands of years. Non-dairy based probiotic drinks utilizing cereals, including pearl millet, are also reported (Mridula and Sharma, 2015). Probiotic foods contain a single or mixed culture of probiotic microbes that improves the health of the host by improving intestinal microbial balance (Fuller, 1989). Information on the probiotic microorganisms associated with sorghum and pearl millet cultivars are inadequate (Badau, 2006). Hence, the present investigation was aimed to isolate and identify bacteria from flours and batter of sorghum and pearl millet cultivars and to further characterize these bacteria for their probiotic traits, in order to understand the probiotic potential of sorghum and pearl millet which are grown extensively in the Semi-Arid Tropics of the world.

METHODOLOGY

Collection of sorghum and pearl millet grain samples

A total of eight sorghum grain samples including K648 stress, K648 control, 6040 stress, 6040 control, R16 stress, R 16 control, K359 stress and K 59 control and two pearl millet grain samples including dual purpose hybrid (DPH) and high Fe hybrid were grown at ICRISAT and used for this study.

Collection of human pathogenic bacteria

Human pathogenic bacteria such as *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 6538), and *Salmonella typhi* (ATCC 14028) were acquired from American Type Culture Collection (ATCC), USA.

Preparation of the grain samples

The grain samples of both sorghum and pearl millet were dried at room temperature (30 to 32°C) for 3 days and milled in Cyclotech™

- mill. The ground samples were sieved through 0.2 mm sieve, in order to get fine flour. The batter samples were prepared, by mixing 5 g of flour in 5 ml of sterilized distilled water in a sterilized beaker and incubated/fermented at 28°C for 12 h. At the end of fermentation, the batter samples were immediately used for isolating the bacteria.

Isolation and maintenance of bacterial isolates

Ten grams of flour/batter from each of sorghum/millet grain sample was separately suspended in 90 ml of sterilized physiological saline (0.85% of NaCl in water) in a flask and placed on an orbital incubator shaker (at 120 rpm) for 30 min. At the end of shaking, the flour/batter samples were serially diluted up to 10^6 dilutions with physiological saline. Dilutions 10^4 to 10^6 were spread plated (0.1 ml) on five different selective media including plate count agar, yeast glucose chloramphenicol agar, Bifidobacterium agar, actinomycetes isolation agar and de ManRogosa and Sharpe (MRS) agar and incubated at 30°C for 24 h. Bacteria were enumerated and the most prominent colonies which were found abundantly in the plate isolated and maintained on the respective media slants at 4°C for further studies.

Morphological and biochemical characterization of bacterial isolates

The bacteria were streaked on respective media and incubated at 30°C for 24 h. At the end of incubation, the colonies were observed for its morphological traits including form, surface, texture, color, elevation and margin. Gram staining reaction of the bacteria was done as per the protocols of Pelczar et al. (2008). The bacteria were also characterized for their biochemical traits including hydrogen sulphide, urease, catalase, oxidase, nitrate reduction, gelatin liquefaction, starch hydrolysis and IMViC (Indole, Methyl red, Voges Proskauer and simmons Citrate) tests as per the methods of Holt (1984). Utilization of carbohydrates such as lactose and sucrose were determined as per Forouhandeh et al. (2010).

Antibiotic resistance pattern and antimicrobial activity of bacterial isolates

Antibiotic resistance pattern was conducted by disc diffusion method. The resistance or susceptibility to antibiotics of bacterial isolates to ampicillin (10 µg), chloramphenicol (30 µg), ciprofloxacin (10 µg), erythromycin (15 µg), kanamycin (30 µg), penicillin (10 µg), streptomycin (10 µg), tetracycline (30 µg) and vancomycin (10 µg) (HiMedia, Mumbai, India) were determined according to the guidelines of Clinical and Laboratory Standards Institute (CLSI) (Wikler, 2006). In brief, antibiotic discs were placed on the Muller-Hinton agar plates immediately after swab with actively grown bacterial cultures. The plates were incubated at 30°C for 24 h. At the end of incubation period, zone of inhibition was measured.

The antimicrobial activity of the bacterial isolates was done by ditch assay method as per the protocols of Aswathy et al. (2008) with slight modifications. In brief, the bacterial isolates were grown in Muller-Hinton broth at 30°C for three days. At the end of incubation period, the culture filtrates were collected by centrifugation at 10,000 × *g* for 20 min and concentrated on a rotary evaporator until one fifth of the original volume. The concentrated culture filtrate samples were filter-sterilized through 0.2 µm membrane filter. Human pathogens such as *E. coli*, *S. aureus* and *S. typhi*, grown separately on nutrient broth at 37°C for 24 h, were amended with sterilized nutrient agar (2.5%) at 45°C, poured on sterile Petri plates and allowed to solidify. Upon solidification, a ditch of 0.5 mm was cut in the Petri plate and filled with membrane-

filtered concentrated culture filtrate of bacterial isolate (0.2 ml). The plates were initially placed at 4°C for 1 h, for diffusion of metabolites present in the culture filtrate, and further incubated at 37°C for 18 h. At the end of incubation, zone of inhibition was noted.

Probiotic traits of bacterial isolates

The bacterial isolates were characterized for their probiotic traits including tolerance to acid (low pH), bile salt, phenol and NaCl as per the standardized protocols used at ICRISAT.

Acid tolerance

This test was done as per the protocols of Liu et al. (2007) for identifying the bacterial isolates which could tolerate simulated gut acidic conditions. In brief, the Muller-Hinton broth was adjusted to pH 2.0, 3.0 and 4.0, and inoculated with one ml of log phase bacterial isolate. The inoculated broth was adjusted to 0.6 OD at 620 nm in UV spectrophotometer and incubated at 37°C for 120 min. The samples were withdrawn at the interval of 30 min and absorbance was measured at 620 nm.

Bile tolerance

Bile tolerance test was determined based on Aswathy et al. (2008). Brain heart infusion (BHI) broth was amended with various concentrations of bile salt (0.3, 0.5 and 0.8%) and inoculated with one ml of log phase bacterial isolate. The inoculated broth was adjusted to 0.6 OD at 620 nm in UV spectrophotometer and incubated at 37°C for 120 min. The samples were withdrawn at the interval of 30 min and absorbance was measured at 620 nm.

NaCl tolerance

NaCl tolerance was done as per the methods of Graciela and Maria (2001). Muller-Hinton broth was adjusted to different concentration of NaCl (3, 6, 9, and 12%) and inoculated with 1 ml of log phase bacterial isolate. The inoculated broth was adjusted to 0.6 OD at 620 nm in UV spectrophotometer and incubated at 37°C for 120 min. The samples were withdrawn at the interval of 30 min and absorbance was measured at 620 nm.

Phenol tolerance

The phenol tolerance of the bacterial isolates was assessed using the protocols of Teply (1984) with slight modifications. Log phase of bacterial isolate was inoculated in Muller-Hinton broth containing 0.2 and 0.4% of phenol. Absorbance was measured at 620 nm using UV spectrophotometer initially at 0 h and after 24 h of incubation at 37°C.

Molecular identification of the bacterial isolates

Pure cultures of probiotic potential bacteria were grown until log phase and genomic DNA were isolated according to Bazzicalupo and Fani (1995). The amplification of 16S rDNA gene was done by using universal bacterial primer 1492R (5'-TACGGYTACCTTGTTACGACTT-3') and 27F (5'-AGAGTTTGATCMTGGCTC AG-3') according to the conditions by Pandey et al. (2005). The polymerase chain reaction (PCR) product was sequenced at Macrogen Inc. Seoul, Korea. The sequences were compared with those from GenBank using the BLAST

program (Alschul et al., 1990), aligned using the ClustalW software (Thompson et al., 1997) and phylogenetic trees inferred using the neighbor-joining method (Saitou and Nei, 1987).

Submission of sequences to NCBI

The 16S rDNA gene sequences of potential probiotic isolates were submitted to NCBI and the GenBank accession numbers were obtained.

Compatibility of the nine probiotic potential bacteria

Compatibility study was done as per the published protocols of Gopalakrishnan et al. (2011). In brief, the nine probiotic potential bacteria were streaked on Luria Agar in 10 cm diameter plate in a specific pattern that was first drawn on a paper as template. The template was prepared as follows: A 5 cm long vertical line at a distance of 5 mm from the margin was drawn on one side of the circle. Five lines, each 5 mm away and as perpendicular to the verticalline, were drawn. Each perpendicular line is 5 cm long and 1 cm apart from each other. Keeping this as a template below the Petri dish, the nine test bacteria were streaked over the vertical line and over the perpendicular lines. Care was taken that the cultures did not touch wall of the Petri plate. The plates were incubated for 24 h and observed for compatibility. If zone of inhibition was found between the two bacteria both of them were considered as not compatible whereas if no zone was found, these were considered as compatible.

RESULTS

Isolation and morphological characterization of bacterial isolates

In the present investigation, a total of 218 bacteria were isolated from the five different selective media (plate count agar, yeast glucose chloramphenicol agar, Bifidobacteria isolation agar, actinomycetes isolation agar and MRS agar), of flour and fermented batter samples of sorghum and pearl millet. A total of nine probiotic bacterial isolates were short listed based on morphological and biochemical traits, IMViC tests, probiotic potential, resistance to antibiotics and antimicrobial activity against human pathogens. The selected nine bacteria (PHFB-22, PHFF-11, S6SF-44, S8CF-32, S8SF-4, SKSB-14, SKSF-55, SKSF-7 and SKSF-8) were found in different forms (circular/irregular), size (big/medium/punctiform), surface (rough/veined/glistening), texture (moist/mucoid/dry), color (cream/pink/pale-pink/translucent/yellow/white), elevation (flat/raised/umbonate), margin (entire/lobate) and Gram staining (Gram positive/Gramnegative; rod/cocci) (Table 1).

Biochemical characterization of the selected nine bacterial isolates

When the nine selected bacterial isolates were characterized for their biochemical traits, all were found

Table 1. Morphological characterizations of the nine probiotic potential bacterial isolates.

Isolate	Form	Size	Surface	Texture	Color	Elevation	Margin	Gram staining[*]
PHFB-22	Irregular	Medium	Rough	Moist	Cream	Flat	Entire	G$^+$ rod
PHFF-11	Irregular	Medium	Veined	Moist	Pale pink	Flat	Lobate	G$^+$ rod
S6SF-44	Circular	Medium	Glistening	Mucoid	Cream	Raised	Entire	G$^+$rod
S8CF-32	Circular	Big	Rough	Moist	Cream	Flat	Entire	G$^+$rod
S8SF-4	Circular	Punctiform	Glistening	Mucoid	Pink	Raised	Entire	G$^-$rod
SKSB-14	Circular	Punctiform	Rough	Mucoid	Translucent	Flat	Entire	G$^+$ rod
SKSF-55	Circular	Punctiform	Glistening	Mucoid	Yellow	Raised	Entire	G$^+$rod
SKSF-7	Circular	Medium	Veined	Moist	White	Flat	Entire	G$^+$rod
SKSF-8	Circular	Punctiform	Rough	Dry	Translucent	Umbonate	Lobate	G$^+$ rod

*G$^+$rod = Gram positive rod; G$^-$ rod= Gram negative rod.

Table 2. Biochemical characterization of the nine probiotic potential bacteria.

Isolate	HS	U	C	O	NR	GL	SH	I	MR	VP	Cit	Carbohydrate utilization test			
												L	LG	S	SG
PHFB-22	-	-	-	-	+	-	+	-	-	+	+	+	+	+	-
PHFF-11	-	-	-	-	+	-	+	-	+	+	-	+	+	+	-
S6SF-44	-	+	-	-	+	-	+	-	-	+	+	+	+	+	-
S8CF-32	-	+	-	+	+	-	+	-	-	-	+	+	+	+	-
S8SF-4	-	+	-	-	-	-	+	-	+	-	+	+	+	+	+
S6SF-44	-	+	-	-	+	-	+	-	-	+	+	+	+	+	-
SKCF-55	-	+	-	-	-	-	-	+	-	+	-	+	+	+	-
SKSF-7	-	-	-	-	+	-	+	-	+	+	-	+	+	+	-
SKSF-8	-	+	-	-	-	-	+	-	+	+	-	+	+	+	-

HS= Hydrogen sulfide test; U = urease test; C=catalase test; O= oxidase test; NR= nitrate reduction test; GL= gelatin liquefaction; SH = starch hydrolysis; I = indole test; MR = methyl red test; VP= Vogues Proskauer test; Cit= citrate utilization test; L= lactose; LG= lactose gas production; S= sucrose; SG= sucrose gas production.

positive for lactose utilization, lactose gas production, sucrose utilization, sucrose gas production (only for S8SF-4), urease test (except for PHDB-22, PHFF-11 and SKSF-7), nitrate reduction (except for S8SF-4, SKCF-55 and SKSF-8), starch hydrolysis (except for SKCF-55), Vogues Proskauer (except for S8CF-32 and S8SF-4), citrate utilization (except for PHFF-11, SKCF-55, SKSF-7 and SKSF-8), methyl red (only for PHFF-11, S8SF-4, SKSF-7 and SKSF-8) and iodine (only for SKCF-66). However, none of the isolates were positive for gelatin liquefaction, oxidase, catalase and hydrogen sulfide tests (Table 2).

Antibiotic resistance pattern and antimicrobial activity of the selected bacterial isolates

In the present investigation, the nine bacteria were evaluated for their antagonistic traits against human pathogens. It was noted that all the nine selected bacterial isolates were found to inhibit all the three tested pathogens, viz. *E. coli*, *S. aureus* and *S. typhi* (Table 3).

The selected nine isolates were also found resistance to tetracycline (except for S8SF-4) at 30 µg, streptomycin (except for S6SF-44 and S8SF-4) at 10 µg, kanamycin (except S8SF-4 and SKSB-14) at 30µg, chloramphenicol (except for S8SF-4) at 30 µg, ciprofloxacin (except for S8SF-4) at 10 µg, ampicillin (only for PHFB-22, S8CF-32 and SKSF-8) at 10 µg, penicillin (only for PHFB-22, S8CF-32 and SKSF-8) at 10 µg, erythromycin at 15 µg and vancomycin (except for S6SF-44 and SKSB-14) at 10µg (Table 3).

Probiotic traits and molecular identification of the selected bacterial isolates

When the selected nine bacterial isolates were tested for their probiotic properties, all of them were found to tolerate acidic pH (2-3), bile (at 0.3 to 0.5%), NaCl (3 to 9%) and phenol (0.2%; only for PHFB-22 and PHFF-11) (Table 4).

Phylogenetic analysis of 16S rDNA sequences of the seven of the nine probiotic potential bacterial isolates

Table 3. Antibiotic resistance pattern and antimicrobial traits (zone of inhibition in μg) of the nine probiotic potential bacteria.

| Isolate | T | ST | K | Chl | Cip | Amp | Pen | Ery | Van | Antimicrobial activity | | |
										E. coli	*S. aureus*	*S. typhi*
PHFB-22	27	18	21	26	27	18	15	22	18	9	13	10
PHFF-11	23	22	22	20	27	0	0	21	18	11	10	21
S6SF-44	22	0	10	14	31	0	0	23	0	10	10	10
S8CF-32	27	26	22	27	27	12	10	28	20	11	10	11
S8SF-4	0	0	0	0	0	0	0	21	17	14	12	10
SKSB-14	14	14	0	13	21	0	0	21	0	11	10	14
SKSF-55	22	23	23	16	24	0	0	16	18	28	10	11
SKSF-7	20	20	18	17	24	0	0	19	15	11	11	22
SKSF-8	26	27	22	16	31	16	15	21	17	10	12	14
Mean	20	17	15	17	24	5	5	21	14	13	11	14
SE±	1.0***	0.6***	1.1***	0.6***	0.7***	1.0***	1.1***	0.8***	0.3***	0.5***	0.3***	0.4***
LSD (5%)	2.9	1.9	3.3	1.8	1.9	3.1	3.2	2.3	1.0	1.4	0.8	1.3
CV%	8	7	13	6	5	35	40	6	4	6	4	5

SE = Standard error; ***= Statistically significant at 0.001; LSD = Least significant difference; CV = Coefficient of variance; T = Tetracycline (30 μg); ST= Streptomycin (10 μg), K= Kanamycin (30μg), Chl= Chloramphenicol (30μg), Cip= Ciprofloxacin (10μg), Amp= Ampicillin (10 μg), Pen= Penicillin (10 μg), Ery= Erythromycin (15 μg), Van= Vancomycin (10 μg),*S. aureus=Staphylococcus aureus*, *S. typhi= Salmonella typhi*

Table 4. Probiotic properties, identity and NCBI accession numbers of the nine probiotic potential bacteria.

Isolate	Acid tolerance (pH)	Bile tolerance (%)	Phenol tolerance	NaCl tolerance (%)	Identified isolate	Accession number
PHFB-22	2	0.3	0.2%	9	*Bacillus subtilis*	–
PHFF-11	3	0.5	0.2%	6	*Bacillus cereus*	KM624626
S6SF-44	2	0.3	Nil	3	*Bacillus amyloliquefaciens*	KM624628
S8CF-32	2	0.5	Nil	9	*Bacillus subtilis*	KM624629
S8SF-4	2	0.5	Nil	3	*Spingobacterium thalpophilum*	KP326566
SKSB-14	3	0.3	Nil	3	*Brevibacterium* sp	KM817772
SKSB-55	3	0.3	Nil	6	*Bacillus cereus*	KM658265
SKSF-7	2	0.3	Nil	6	*Bacillus cereus*	KM658262
SKSF-8	3	0.3	Nil	9	*Bacillus pumilus*	KM658263

belonged to *Bacillus* but different species. The isolates PHFB-22 and S8CF-32 had maximum sequence similarities with *Bacillus subtilis*, PHFF-11, SKSB-55 and SKSF-7 showed maximum sequence similarities with *Bacillus cereus*; whereas S6SF-44 and SKSF-8 showed maximum sequence similarities with *Bacillus amyloliquefaciens* and *Bacillus pumilus*, respectively (Table 4 and Figure 1). The sequences of the other two isolates S8SF-4 and SKSB-14 were found to be similar to *Spingobacterium thalpophilum* and *Brevibacterium* sp., respectively (Table 4 and Figure 1).

Compatibility of the selected nine bacterial isolates

In the present study, when the probiotic potential bacteria were characterized for their compatibility there was no definite sign of suppression of four bacteria (S8SF-32, SKSF-55, SKSF-7 and SKSF-8) on any of the other bacteria while the remaining five bacteria showed inhibition over others, suggesting these were not compatible.

DISCUSSION

Fermentation of foods is traditionally carried out in every household in developing countries of Africa and Asia. The benefits of fermented foods include its increased nutritional value and keeping qualities. These products have a high content of probiotic bacteria with a large bio-diversity. For instance, Koko is a millet gruel produced in Northern Ghana and spontaneously fermented by lactic acid bacteria such as *Lactobacillus*
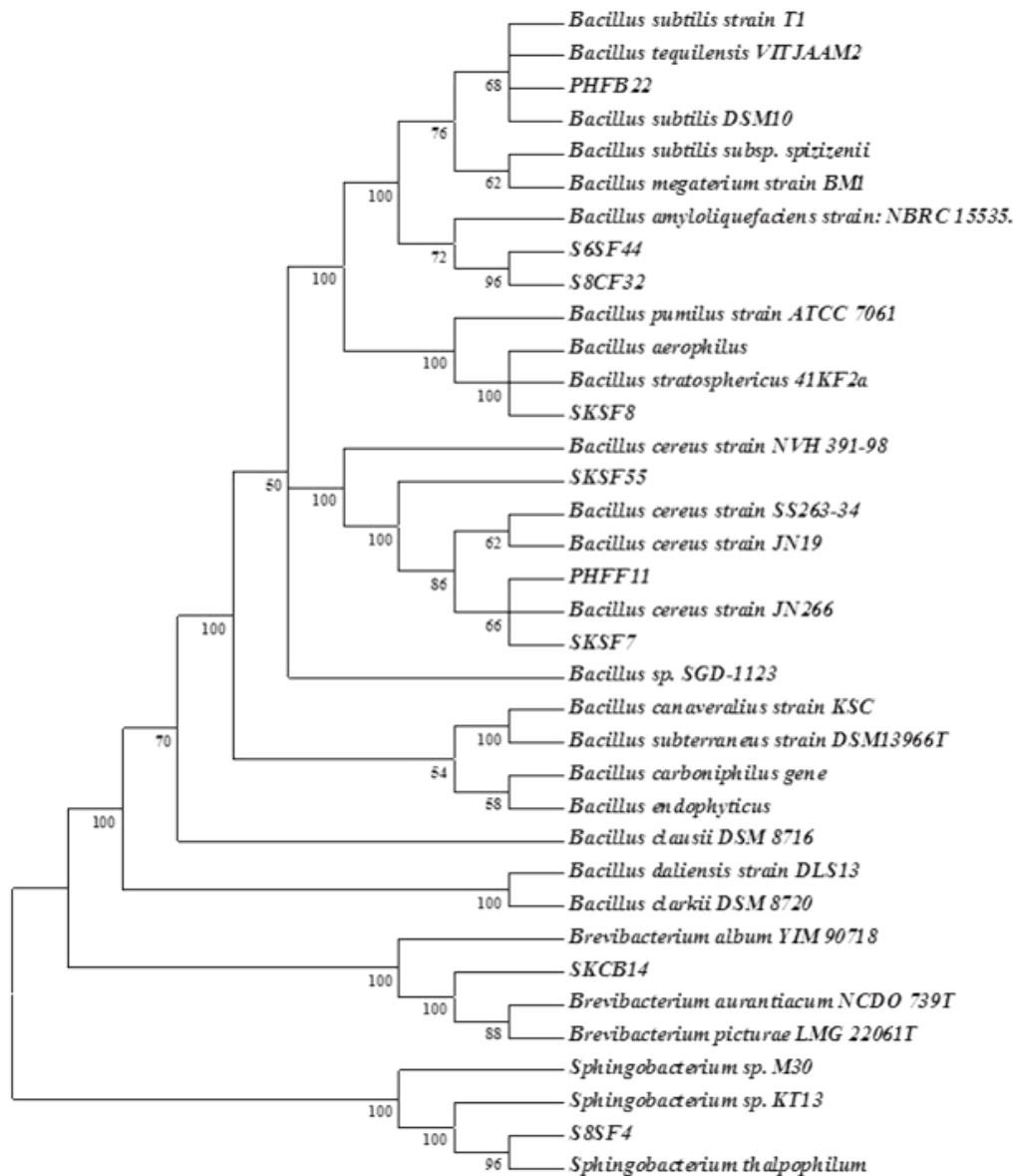
Figure 1. Phylogenetic relationship between the nine probiotic potential bacterial isolates and representative species based on full length 16 S rDNA sequences constructed using the neighbor-joining method.

fermentum and *Weissella confusa* (Lei and Jakobsen, 2004). Millets and sorghum are the two most important staple food for millions of poor people in Asia and Africa as it contains high carbohydrate energy and nutrition, thus making them useful components of dietary and nutritional balance in food. Probiotics help the existing microbial flora or repopulate the lost microbial flora in the colon due to antibiotics, disease and/or chemotherapy. Probiotic foods from millets and sorghum are rich in phytochemicals including phytic acid and phytates, which are known to lower cholesterol and reduce the risk of cancer, and are also reported to generate vitamins, fatty acids and other viral nutrients that improve the body's

resistance against human pathogens (FAO/WHO, 2001; Coulibaly et al., 2011; El-Salam et al., 2012). Hence, in the present investigation, millets and sorghum flour and fermented batter samples were used for isolation of probiotic bacteria.

Inhibition of human pathogenic microbes and resistance to antibiotics by probiotic microbial strains are advantageous traits. Hence, in the present study, the nine selected bacterial isolates were found to inhibit all the three tested pathogens, viz. *E. coli*, *S. aureus* and *S. typhi* (Table 3). Similar studies were conducted by Oluwajoba et al. (2013). Lactic acid bacteria isolated from millet and sorghum grains and fermented products were

demonstrated to inhibit human pathogens such as *S. aureus* 25923, *E. coli* 25922, *Pseudomonas aeruginosa* 27853 and *Enterococcus faecalis* 29212 (Oluwajoba et al., 2013). Probiotic microbial strains such as *L. fermentum*, *Bifidobacterium* sp. and *W. confusa* are also reported to help in preventing and treating acute diarrhea (Lei et al., 2006). The major aim of using these probiotic strains should be to affect beneficially the gut microbial composition and activities. Hence, in the present study, when the selected nine bacterial isolates were also tested for their antibiotic resistance pattern, these were found resistance to tetracycline (except S8SF-4), streptomycin (except S6SF-44 and S8SF-4), kanamycin (except S8SF-4 and SKSB-14), chloramphenicol (except S8SF-4), ciprofloxacin (except S8SF-4), ampicillin (only PHFB-22, S8CF-32 and SKSF-8), penicillin (only PHFB-22, S8CF-32 and SKSF-8), erythromycin and vancomycin (except S6SF-44 and SKSB-14). It is concluded that the selected nine isolates have good antagonistic potential as well as antibiotics resistance pattern.

The selection criteria of suitable probiotic microbial starter should be its ability to survive the acidic environment of the final fermented product and the adverse conditions of the gastrointestinal tract. Although, differences exist between species and specific strains, Lactobacilli (for instance, *L. casei* and *L. plantarum* showed longer shelf life than *L. acidophilus*) are generally considered to be intrinsically resistant to pH values higher than 3.0 (Hood and Zottola, 1988; Lee and Salminen, 1995). Hence, in the present investigation, the bacterial isolates were characterized for their probiotic properties including acid tolerance, bile tolerance, phenol tolerance and NaCl tolerance and all of them were found to tolerate acidic pH (2-3), bile (up to 0.5%), NaCl (up to 9%) and phenol (0.2%). Hence, it is concluded that the selected nine isolates have good probiotic properties.

In order to determine the identity of the nine potential probiotic bacteria, its 16S rDNA was sequenced and analyzed. A neighbor joining dendrogram was generated using the sequence from the nine potential probiotic bacteria (1400 bp) and representative sequences from the databases which revealed seven of the nine probiotic potential bacterial isolates belonged to *Bacillus* but different species whereas the other two isolates were found to be similar to *Spingobacterium thalpophilum* and *Brevibacterium* sp., respectively. Millet and sorghum flour have been used for isolation of lactic acid bacteria such as *Lactobacillus plantarum*, *L. cellobiosus*, *L. pentosus*, *Leuconostoc mesenteroids* and *Pedicoccus pentosaceus* (Okoronkwo, 2014). Mridula and Sharma (2015) reported a non-dairy probiotic drink from the sprouted cereals, legumes and soy milk using lactic acid bacteria. As found in the current investigation, *Bacillus subtilis* was reported to be isolated from the un-malted pearl millet grains in addition to *Lactobacillus plantarum*, *Streptococcus lactis* and *Torulopsis glabrata* (Badau, 2006) and reported to

have probiotic potential (Kamgar et al., 2013). Microbes such as *Lactobacillus* sp., *Bifidobacterium* sp. and *Weissella* sp. are reported widely as probiotic microbes. *Lactobacillus acidophilus* was used to ferment a food mixture containing sorghum flour, whey powder and tomato pulp (Jood et al., 2012). However, in the present study, we report the isolation of *Bacillus* sp., *Sphingobacterium* sp. and *Brevibacterium* sp. as potential probiotic cultures. Hence, these cultures also can be exploited for making functional foods.

For any probiotic products/functional food, the ability of the probiotic microbial strain to attain high cell population is of primary importance. The probiotic microbial cell population of about 10^7 cells ml^{-1} at the time of consumption is considered functional (Gomes and Malcata, 1999). Probiotic foods obtained using a single microbial strain are not welcomed by the consumers due to rather sour and acidic taste and therefore probiotic strains are mixed (Saarela et al., 2000). Keeping this in mind, in the present investigation, all the probiotic potential bacteria were characterized for their compatibility so that these can be used in consortia. Compatibility studies between the nine probiotic potential bacteria revealed that there was no definite sign of suppression of four bacteria (S8SF-32, SKSF-55, SKSF-7 and SKSF-8) on any of the other bacteria while the remaining five bacteria showed inhibition over others, suggesting these were not compatible. Hence, it is concluded that the four of the bacterial isolates (S8SF-32, SKSF-55, SKSF-7 and SKSF-8) can be used as consortia in functional food product development.

Conclusion

Cereals such as sorghum and millets are not only valuable sources of prebiotics and bioactive compounds useful for help in production of functional foods but also are sources of probiotic microbial cultures, which should be exploited for the production of new and innovative functional foods. Cereals and pseudocereals like Sorghum and pearl millet contain good quantities of potential prebiotic components, such as, water-soluble fibre (includes β-glucan and arabinoxylan), oligosaccharides (includes galacto- and fructo-oligosaccharides) and resistant starch important for the probiotic concept (Charalampopoulas et al., 2002). Hence, the multiple beneficial effects of cereals, especially the pseudo cereals such as sorghum and millets, can be used in association with good probiotic microbial cultures also isolated from these cereals in various ways to design novel cereal-based functional foods targeting different consumer segments having specific health requirements. Some of the important traits that need to be considered while selecting the cereals and the probiotic microbial culture include the composition of cereal grains, the substrate formulation,

the growth capability of the starter probiotic culture, the stability of probiotic culture during storage, the organoleptic properties and the nutritional value of the final functional food product (Charalamoppulos et al., 2002). Cereals not only have the ability to grow probiotic microbes such as lactic acid bacteria in the human gut but also contain potential prebiotic compounds, whose functionality needs to be explored. However, the information available on the effects of cereal composition on the growth of probiotic microbes is limited. Therefore, the importance of substrate composition in conjunction with the nutritional requirements of the specific probiotic microbial strain is absolutely essential in order to make good quality functional food with validated health claims. In summary, the present work has demonstrated that the functional foods based on cereals, such as sorghum and millets, in association with good probiotic microbial stains can be exploited for designing novel cereal based functional foods for addressing food and nutritional security for millions of malnourished people living in the poorest of the poor countries of Africa and Asia.

Conflict of Interests

The authors have not declared any conflict of interests.

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Abbreviations

PHFB, Pearl millet high Fe batter; **PHFF,** Pearl millet High Fe flour; **S6SF,** Sorghum 6040 Stress flour; **S8CF,** sorghum K 648 control flour; **S8SF,** sorghum K 648 stress flour; **SKSB,** sorghum K 359 stress batter; **SKSF,** sorghum K 359 stress flour.

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