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ORIGINAL ARTICLE

Evaluation of the probiotic ability of Cellulolytic *Bacillus subtilis* SBMP4 (FSP_{20}) isolated from waste-dump site in FIIRO, Lagos Nigeria

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ABSTRACT

BACKGROUND:
Current invention in the field of biotechnology includes research into the probiotic potentials of enzyme-producing bacteria species.

OBJECTIVE:
This study evaluated the probiotic ability of cellulolytic *Bacillus subtilis* SBMP4 isolated from waste dump site.

METHODS:
Isolated *Bacillus* species was screened for cellulolytic ability on carboxyl methylcellulose agar (CMCA), and hydrolysis efficiency was calculated. Probiotic ability of cellulolytic *Bacillus* species was carried out as follows: pathogenic potentials, bacteriocin producing ability, antibiotic susceptibility testing, gastric acid, and bile tolerance. Molecular identification was done by means of 16S rRNA gene sequencing, blast search analysis and phylogenetic analysis.

RESULTS:
Zone of clearance of 30 ± 2.08 mm on CMCA with hydrolysis efficiency of 114 % depicted the isolate's cellulolytic potential. *Bacillus subtilis* SBMP4 showed γ-hemolytic phenotype with negative lecithinase activity. Antibacterial activity of bacteriocin observed against *Staphylococcus aureus* ATCC 25923 reference culture, had maximum inhibition zone of 14±1.0 mm at 72 hour of incubation. Substantial susceptibility towards 9 out of 10 commonly used antibiotics further depicted the isolate's probiotic ability. *Bacillus subtilis* SBMP4 exhibited good gastric acid tolerance (pH 2.5) of 5.857 Log cycle/ml and good tolerance to 5 % and 10 % bile salt concentrations with 6.134 and 6.301 Log cycle/ml of viable bacteria cells. Molecular identification of the selected bacteria species revealed it to be *Bacillus subtilis* SBMP4 with RefSeq accession number NR_118383, showing maximum sequence similarity of 98%.

CONCLUSION:
Cellulolytic *Bacillus subtilis* SBMP4 possesses good probiotic ability and has the potentials of being applied as additive in livestock feeding to enhance overall productivity.

KEYWORDS:
Cellulolytic, Probiotic, CelA gene, Bacteriocin, *Bacillus subtilis*

INTRODUCTION

The International Scientific Association for Probiotics and Prebiotics, with the backing of the Food and Agriculture Organization of the United Nations/World Health Organization (FAO/WHO), defined probiotics as "live microorganisms that, when administered in suitable amounts, impart a health benefit on the host".[1] Probiotics serve as growth promoters and thus provide conditions that allow the host to efficiently utilize nutrients. The probiotic capacity of numerous different microbial species has been related, including the *Lactobacillus* species, *Bifidobacterium* species, *Saccharomyces* species, *Candida* species, *Lactococcus* species, *Enterococcus* species, *Pediococcus* species, *Leuconostoc* species, *Escherichia* species, *Streptococcus* species, and *Bacillus* species, of which *Bacillus cereus*, *Bacillus coagulans*, *Bacillus licheniformis*, *Bacillus clausii*, and *Bacillus subtilis* are notable.[2,3,4,5] Amongst these, the lactic acid bacteria e.g. *Lactobacillus, Bifidobacterium* and *Enterococcus* species are the most widely studied and are inherent members of the gastrointestinal tract of humans and animals.[6]

The capability of a probiotic microorganism to remain alive and transit through the stomach and make it to the gastrointestinal tract is highly dependent on its capacity to withstand the acid and bile salt concentrations. The probiotic microorganisms must exhibit their health-promoting effect when they enter the colon as being metabolically active living cells, hence, this requirement is extremely important and critical.[7] Despite the vast research studies that has been carried out on the lactic acid bacteria as potential probiotics for ruminants over the *Bacillus* species, [8,9,10] *Bacillus* species possess other distinctive qualities that makes them sourced candidates for probiotics. *Bacillus* produces thermo-stable spores, which can endure temperatures as high as 235 °F (113 °C) for eight minutes. Due to their thermostability, spores can be spray-dried as well as added to feed during milling and pelletizing.[11,12,13] They can survive the harsh, low pH of the gastric barrier and bile before they reach the small intestine, where they exert their probiotic characteristics since they are resistant to desiccation and other stress conditions.[14,15,3,16] Also, the genetic potential of *Bacillus* species, which ranges from 3000 to 6000 genes (Big Tool Box) in fully sequenced *Bacillus* species, is another crucial trait.[17,18] Based on these distinctive qualities, there is therefore need to carry out further studies on *Bacillus* probiotics in order to discover the untapped potentials inherent in them. Research into the probiotic potentials of enzyme-producing bacteria species is a current invention in the field of biotechnology [19] and only a few reports are available on probiotic ability of cellulolytic *Bacillus* species. The application of cellulolytic probiotics as additives in livestock feeding demands the exploration of these attributes, hence the need for this current research.

On the other hand, cellulases are collections of extracellular enzymes that hydrolyze glycosidic bonds in cellulose and generate fermentable glucose monomers which are easily absorbable in the body thus improving the utilization of dietary carbohydrate and enhancing digestion. The bye-products formed after enzyme action serves as prebiotics for probiotics thus enhancing feed digestion rich in cellulose in herbivorous animal species.[20]

This study therefore described a *Bacillus subtilis* strain that in addition to having cellulolytic ability also possessed probiotic attributes which could possibly be harnessed and thus applied as additive in livestock feeding with a view of improving productivity and overall performance.

METHODS

Sample collection

Soil samples were aseptically retrieved from waste dump site of groundnut shell and spent mushroom substrate located in Federal Institute of Industrial Research Oshodi (FIIRO) Lagos State, Nigeria. The samples were collected from surface and at 5, 10 and 15 cm depth in sterile screw-capped containers from three distinct locations on the same site using sterile stainless-steel spatulas and thereafter conveyed to the laboratory for further analysis. To create the composite sample for isolation, all soil samples retrieved from the three locations were thoroughly and aseptically mixed together.

Isolation of bacteria

Serial dilution of ten grams (10 g) of soil sample was carried out using 90 ml of sterile distilled water in a 100 ml screw-capped test tube and further dilutions made to 10^{-6}. One milliliter (1ml) of the diluent from 10^{-5} and 10^{-6} dilutions were cultured on nutrient agar (prepared according to manufacturer's instruction) using the pour-plating method and incubated for 20±4 hours at 37 ºC. Based on morphology, size, and color presented on the nutrient agar plate, different colonies that emerged after incubation were selected and series of sub-culturing was done to obtain pure cultures which were maintained on Luria Bertani agar (LBA) slants and used in subsequent experiments.

Screening of isolated bacteria for cellulase producing ability

Twenty-four-hour (24 h) pure cultures of bacterial isolates on nutrient agar were point inoculated on Carboxyl Methyl Cellulose (CMC) agar screening medium (Composition (g/L): KH_2PO_4 1.0; CMC 26.0; KCL 0.2; Yeast extract 1.0; $MgSO_4.7H_2O$ 1.0; Agar 12.0) and incubated at 37 ºC for 20±4 hours. The plates were flooded with 2 ml of Grams iodine solution and allowed to stand for 2 minutes after which it was decanted. It was thereafter observed for cellulase activity indicated by halo/clear zone around growing microbial colonies. Clear zones which appeared were indicative of the microorganism's ability to produce cellulase. The clear zones were measured and recorded accordingly.[21]

Determination of percentage hydrolysis efficiency of isolated bacteria

All isolated bacteria exhibiting cellulase ability were plated out on CMC agar screening medium by point inoculation, and after the prescribed incubation period of 20±4 hours at 37 ºC, plates were flooded with 2 ml of Grams iodine solution and both their halo diameters (H) and colony diameters (C) were measured. Thereafter, the percentage hydrolysis efficiency was calculated for all isolated bacteria using the formula below [22].

$$\% \, Hydrolysis \; efficiency = \frac{H - C}{C} \times 100$$

Where:
H = Halo Diameter
C = Colony Diameter

Based on the diameter of clear zone exhibited on CMC agar screening plate and the percentage hydrolysis efficiency obtained via calculation, isolate FSP_{20} was selected and its probiotic ability ascertained.

Screening for the probiotic properties of selected bacteria isolate

Pathogenicity: haemolytic and lecithinase activity of Isolate

Haemolytic activity of isolate

Twenty-four (24) hour pure culture of bacteria was inoculated by streaking on blood agar medium (5% sheep blood in nutrient agar) and incubated for 20±4 hours at 37 ºC. Observation was made for the presence of hemolysis after the prescribed incubation period [23].

Lecithinase producing ability of isolate

Twenty-four (24) hour pure culture of gamma hemolytic cellulolytic bacteria was inoculated on egg yolk agar (EYA) medium by single line streaking, incubated for 48 hours at 37 °C, and lecithinase activity monitored at 24 hours interval by observing for an opaque diffuse zone extending from the colony's edge into the medium [24].

Screening for production of antimicrobial metabolite (Bacteriocin)

Bacteriocin production was carried out in modified TY medium (composition g/L: Tryptone 10.0; Yeast extract 5.0; NaCl 5.0; pH 7.0). After reconstitution, the medium was sterilized at 121 ºC for 15 minutes. 100 ml of inoculum was cultivated in this medium and incubated for 24, 48, 72, and 96 hours, respectively at 37 ºC. After each incubation period, the broth was transferred to an incubator shaker and swirled for 10 minutes at 150 rpm. The cells were harvested by centrifuging the mixture at 4100 rpm for 20 minutes at 4 ºC. A 0.22 µm filter membrane was used to filter the cell-free supernatant under sterile conditions, and the resultant solution (bacteriocin) was stored in a bio-freezer at -40 ºC for further studies. To monitor the growth pattern of cultivated bacteria, one sample was withdrawn after each respective incubation period and used to measure the optical density (OD) at 600 nm [25].

Determination of the antibacterial activity of the produced cell-free supernatant (Bacteriocin)

The reference strains (*Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923), procured from the Biotechnology Department of Federal Institute of Industrial Research, Oshodi, Lagos, Nigeria, were prepared by growing them in nutrient broth at 37 °C for 24 hours. The cells were harvested and washed twice in normal saline using the refrigerated centrifuge at 4100 rpm for 20 minutes at 4 °C. The reference strain cultures were thereafter adjusted to 0.5 McFarland standards, comprising 1.5 x 10^8 cfu/ml of microbial isolates. The antibacterial activity assay of the produced cell-free supernatant (bacteriocin) was carried out using the agar well diffusion technique and employing the reference strain cultures. One hundred (100) µl suspensions of each reference strain, comprising 1.5 x 10^8 cfu/ml, was spread on nutrient agar plates using sterile glass rod. Cell-free supernatants (50 µl) from various incubation intervals (24, 48, 72, and 96 hours) were introduced into 5 mm wells made on the previously inoculated nutrient agar plates using sterile cork borers. Observations were made after 24 hours incubation at 37 °C. Results obtained were the mean of the observations as all tests were carried out in triplicate [26].

Gastrointestinal stress tolerance:

Tolerance to gastric acid

Eighteen to twenty-four (18-24) hour pure culture of cellulolytic bacteria in nutrient broth was harvested by centrifugation (4100 rpm, 20 minutes at 4 °C). The cells were washed twice using sterile distilled water, adjusted to 0.5 McFarland standards and 1 % was inoculated into nutrient broth that was previously acidified to pH 2.5 using concentrated HCl having 1,000 units of pepsin and incubated for 2 hours at 37 °C. A 10-fold serial dilution in sterile distilled water was used to quantify the viable cells, and a 0.1 ml aliquot was evenly spread on nutrient agar plates. Colony forming units (CFU) were estimated after 24 hours of aerobic incubation at 37 °C [27].

Tolerance to bile

Eighteen to twenty-four (18-24) hour pure culture of bacteria isolate in nutrient broth was harvested via centrifugation (4100 rpm at 4 °C for 20 minutes). Using sterile distilled water, the cells were washed twice, adjusted to 0.5 McFarland standards, and 1% was inoculated into nutrient broth comprising either 5 % or 10 % bile salt concentrations respectively. The inoculated broth cultures were incubated at 37 °C for 24 hours. A 10-fold serial dilution in sterile distilled water was made, and a 0.1 ml aliquot was evenly spread on nutrient agar plates. The inoculated plates were incubated aerobically for 24 hours at 37 °C and colony-forming units (CFU) estimated.[27]

Antibiotic susceptibility testing

The Kirby Bauer disc diffusion method was used to carry out the antibiotic susceptibility assay[28]. A solution of the test isolate previously washed and suspended in normal saline (0.85% NaCl) corresponding to the McFarland No. 0.5 standard was prepared by sequentially diluting the sample ten-fold using normal saline and comparison made on a wickerham card until a cell density of 1.5 × 10^8 cfu/ml was achieved. Using sterile micropipette, 100 µl each of the test isolate culture was placed on Mueller Hinton agar, and with the aid of sterile swab stick, lawn culture of the isolate was made on the agar surface. Gram-positive antibiotic disc (MAXI DISC) supplied by Maxicare Medical Laboratory, a subsidiary of Global Maxicare Nigeria Ltd was placed aseptically on the inoculated plate using sterile forceps and incubated at 37 °C for 20 ± 4hours. The diameter of the inhibition zones was measured following the completion of the incubation time and comparison made with standard tables to determine if the tested bacterial species was susceptible or resistant to the antibiotics.

Molecular identification of isolate

Molecular identification of the selected isolate was investigated by means of the 16S rRNA gene sequence, BLAST search analysis and phylogenetic analysis. DNA was extracted using the Nigerian Institute of Medical Research (NIMR) Biotech DNA extraction kit 2, following the manufacturer's instructions. Polymerase chain reaction was carried out to amplify the 16S rRNA gene of the bacteria using the universal primer pair 799F (5'-AACMGGATTAGATACC -3') and 1193R (3'-ACGTCATCCCCACCTTCC- 5'). The PCR reaction was carried out using the Solis Biodyne 5X HOT FIREPol Blend Master mix. PCR was performed in 25 µl of a reaction mixture, and the reaction concentration was brought down from 5X concentration to 1X concentration containing 1X Blend Master mix Buffer (Solis Biodyne), 1.5 mM $MgCl_2$, 200µM of each deoxynucleoside triphosphates (dNTP)(Solis Biodyne), 25pMol of each primer (StabVida, Portugal), 2 unit of Hot FIREPol DNA polymerase (Solis Biodyne), Proofreading Enzyme, 5µl of the extracted DNA, and sterile distilled water was used to make up the reaction mixture. Thermal cycling was conducted in a Techne 3Prime thermal cycler for an initial denaturation of 95 °C for 15 minutes followed by 35 amplification cycles of 30 seconds at 95 °C; 1 minute at 61°C and 1 minute 30 seconds at 72 °C. This was followed by a final extension step of 10 minutes at 72 °C.

The amplification product was separated on a 1.5% agarose gel and electrophoresis was performed at 80V for 1 hour 30 minutes. DNA bands were visualized after electrophoresis using ethidium bromide staining and a 100bp DNA ladder (Solis Biodyne) was used as DNA molecular weight marker. All PCR products were purified with a QIAquick PCR Purification kit based on manufacturer's instructions and subsequently sent to Epoch Life science (USA) for Sanger sequencing. Using the online blast search at http://blast.ncbi.nlm.nih.gov/Blast.cgi, the corresponding sequences were identified. Furthermore, based on comparison of the 16S rRNA gene sequences of the cellulolytic probiotic *Bacillus* species with related reference strains, neighbour-joining phylogenetic tree analysis was done using the maximum likelihood method.

Table 1: Zone of clearance of cellulase enzyme and hydrolysis efficiency exhibited by isolated *Bacillus* species

Isolate code	Clear zone diameter (H)* (mm)	Colony diameter (C) (mm)	Hydrolysis efficiency Z-C/C (%)
FSP_1	21±2.08	14	40
FSP_2	20±1.53	12	57
FSP_4	7±1.00	8	100
FSP_{17}	28±2.08	16	75
FSP_{18}	10±2.08	16	11
FSP_{20} (*Bacillus subtilis* SBMP4)	30±2.08	14	114

*Mean of three replicates ± standard deviation

RESULTS

Isolation, screening and determination of the % hydrolysis efficiency of isolated cellulolytic bacteria

In Table 1, the data presented depicts the diameter of the measured zones of clearance (mm) by isolated cellulolytic bacteria on CMC agar screening medium as revealed by Gram's iodine staining. The range of clear zone obtained from the six isolated cellulolytic bacteria was between 7 mm and 30 mm showcasing their individual abilities to hydrolyze cellulose as a sole carbon source in the medium. *Bacillus subtilis* SBMP4 (FSP_{20}) displayed the highest clearance zone of 30±2.08 mm, as revealed by the existence of a halo zone (zone of hydrolysis) surrounding the microbial colonies on CMC agar screening medium (Figure 1), while the least zone of clearance of 7±1.00 mm was observed in isolate FSP_4. Studies carried out to determine the percentage hydrolysis efficiency of these six isolated bacteria species, also revealed *Bacillus subtilis* SBMP4 (FSP_{20}) as possessing the highest efficiency of 114% which further re-echoes its high cellulose degrading capabilities. The lowest percentage hydrolysis efficiency of 11% was however seen in isolate FSP_{18}.

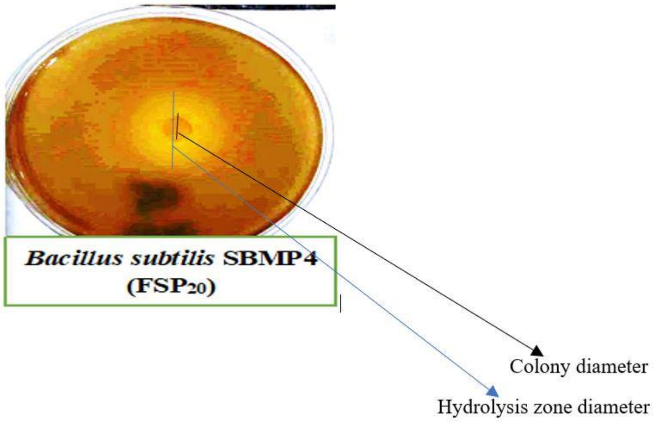

Figure 1: Screening for cellulase activity of *Bacillus subtilis* SBMP4 (FSP_{20}) on CMC agar medium.

Determination of probiotic properties of selected bacteria (***Bacillus subtilis*** **SBMP4 - FSP$_{20}$**) *haemolytic and lecithinase activity*

Result of studies carried out to ascertain the hemolytic ability of *Bacillus subtilis* SBMP4 (FSP$_{20}$) is shown in Figure 2. *Bacillus subtilis* SBMP4 (FSP$_{20}$) displayed γ-hemolytic phenotype (absence of hemolysis) on sheep blood agar which depicts its inability to lyse red blood cells. On the other hand, Figure 3 shows the lecithinase activity testing carried out on *Bacillus subtilis* SBMP4 (FSP$_{20}$) on EYA medium. A negative lecithinase activity was observed, depicted by the absence of an opaque diffuse zone from the colony's edge into the medium.

Figure 2. γ-hemolytic activity of *Bacillus subtilis* SBMP4 (FSP$_{20}$) on blood agar

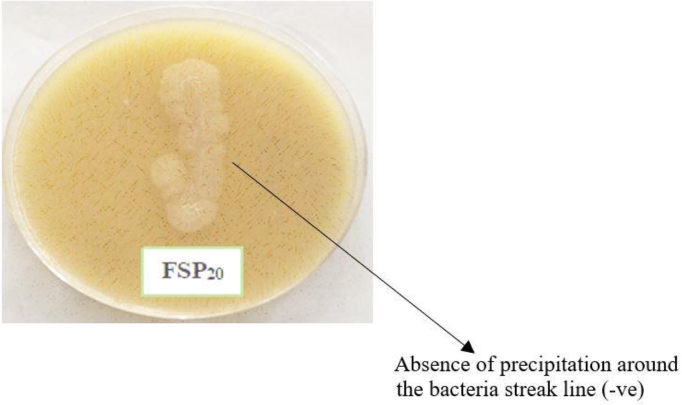

Figure 3. Negative lecithinase activity of *Bacillus subtilis* SBMP4 (FSP$_{20}$) on EYA medium depicted by the absence of an opaque diffuse zone (halo/precipitation) surrounding the streak line of the bacteria in the medium.

Antibacterial activity of antimicrobial metabolite (Bacteriocin) produced by Bacillus subtilis **SBMP4 (FSP$_{20}$)**

Observation made from the studies carried out on the antibacterial activity of bacteriocin produced by *Bacillus subtilis* SBMP4 (FSP$_{20}$) against the gram-positive and gram-negative reference strain cultures (*Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922) as seen in Table 2, shows that clear zone was visible from 48 hours incubation period for *Staphylococcus aureus* ATCC 25923 depicting the commencement of bacteriocin production at the said incubation period. Based on results obtained, bacteriocin production was seen to be highest at 72 hours of incubation with a value of 14±1.0 mm, but with further increases in incubation period to 96 hours, a decline was observed with a value of 13.7±0.6 mm. In contrast, no inhibition zone was observed for the gram-negative counterpart *Escherichia coli* ATCC 25922 at all hours of incubation period ranging from 24 – 96 hours respectively.

Table 2. Inhibition zones of bacteriocin produced by *Bacillus subtilis* SBMP4 at varying incubation temperatures

Isolate Code	**Isolate Identity**	**Inhibitory Zones at Varied Incubation Temperatures (mm)***							
		***Staphylococcus aureus* ATCC 25923**				***Escherichia coli* ATCC 25922**			
		24h	**48h**	**72h**	**96h**	**24h**	**48h**	**72h**	**96h**
FSP$_{20}$	*Bacillus subtillis* SBMP4	_	12.7±0.6	14±1.0	13.7±0.6	_	_	_	_

*Mean of three replicates ± standard deviation; "-" indicates no inhibitory effect to indicator organism.

Gastrointestinal tolerance test

Table 3 shows the gastrointestinal tolerance test result obtained for the study isolate. *Bacillus subtilis* SBMP4 (FSP$_{20}$) showed resistance at acidic condition with a value of 5.857 log cycle per milliliter of bacteria corresponding to 7.2×10^{5} cfu/ml after 2 hours of incubation at pH 2.5. At 5 % and 10 % bile salt concentrations respectively, *Bacillus subtilis* SBMP4 (FSP$_{20}$) also showed stability with 6.134 log cycle/ml (1.36×10^{6} cfu/ml) and 6.301 log cycle/ml (2.04×10^{6} cfu/ml) of bacteria after incubation for 24 hours respectively.

Antibiotic susceptibility test

Antibiotic susceptibility test carried out revealed that *Bacillus subtilis* SBMP4 (FSP$_{20}$) showed varying degrees of susceptibility to nine (9) out of the ten (10) commonly used antibiotics utilized for the screening with the exception of Ampicox (Table 4). *Bacillus subtilis* SBMP4 (FSP$_{20}$) showed complete susceptibility to Amoxacillin (AM), Rocephin/Ceftriaxone (R), Streptomycin (S), Septrin/Cotrimoxazole (SXT) and Gentamycin (CN). Intermediate susceptibility was observed for Ciprofloxacin (CPX), Erythromycin (E), Pefloxacin/Cephalexin (PEF) and Zinnacef/Cefuroxime (Z) while the only resistance observed was to Ampiclox (APX).

Table 3. Gastric Acid and Bile Salt Tolerance of *Bacillus subtilis* SBMP4 (FSP$_{20}$)

Gastric Acid (pH 2.5)		**5 % Bile Salt**		**10 % Bile Salt**	
Cfu/ml	**LC/ml**	**Cfu/ml**	**LC/ml**	**cfu/ml**	**LC/ml**
7.2×10^{5}	5.857	1.36×10^{6}	6.134	2.04×10^{6}	6.301

Cfu - Colony forming units; LC/ml – Log cycle/ml

Molecular identification of isolate

Analysis of the PCR products of *Bacillus subtilis* SBMP4 (FSP$_{20}$) on 1.5 % agarose gel electrophoresis using 100bp DNA ladder revealed the presence and the size of the expressed genes of the said microorganism to be approximately 450 bp (Figure 4). The isolate showed blast homology value of about 98 % and was named *Bacillus subtilis* SBMP4 with the RefSeq Accession number NR_118383 (Table 5). Based on comparison of the 16S rRNA gene sequences of the cellulolytic probiotic *Bacillus* species with related reference strains, neighbour-joining phylogenetic tree analysis was done using the maximum likelihood method. Result obtained revealed that *Bacillus subtilis* SBMP4 (FSP$_{20}$) is closely associated with the genus *Bacillus* and clustering perfectly with other *Bacillus* strains, including *Bacillus lequilensis* strain 10b as shown in Figure 5.

Table 4. Antimicrobial Susceptibility Pattern of *Bacillus subtilis* SBMP4 (FSP_{20})

Antimicrobial agent	Inhibition zone based on CLSI standard (mm)			Inhibition zones obtained from *B. subtilis* SBMP4	Interpretation based on CLSI standard
	(R)	(I)	(S)		
AM	≤13	14-17	≥18	20	S
R	≤13	14-17	≥18	18	S
CPX	≤15	16-20	≥21	19	I
S	≤11	12-14	≥15	25	S
SXT	≤10	11-15	≥16	20	S
E	≤13	14-22	≥23	21	I
PEF	≤18	19-20	≥21	20	I
CN	≤12	13-14	≥15	28	S
APX	≤13	14-16	≥17	11	R
Z	≤14	15-22	≥23	22	I

Keys: R-(Resistant); I-(Intermediate susceptibility); S-(Susceptible); AM-(Amoxacillin); R-(Rocephin/Ceftriaxone); CPX-(Ciprofloxacin); S-(Streptomycin); SXT-(Septrin/Cotrimoxazole); E-(Erythromycin); PEF-(Pefloxacin/Cephalexin); CN-(Gentamycin); APX-(Ampiclox); Z-(Zinnacef/Cefuroxime).

Table 5. NCBI BLAST results of 16S rRNA gene sequence of *Bacillus subtilis* SBMP4

Isolate code	Strain	16S rRNA identity	Blast homology value	RefSeq accession number
FSP_{20}	**SBMP4**	***Bacillus subtilis***	**98 %**	**NR_118383**

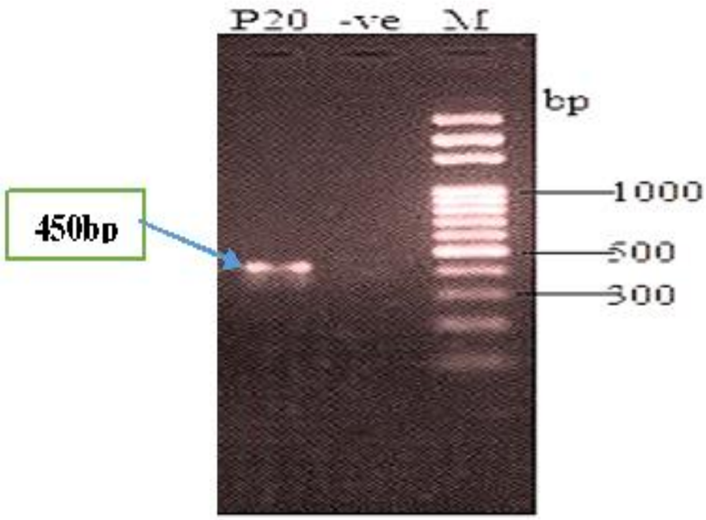

Figure 4: PCR products of *Bacillus subtilis* SBMP4 (FSP_{20}) on 1.5 % agarose gel electrophoresis using 100bp DNA ladder showing the presence and the size of the expressed genes to be approximately 450 bp. **Keys**: M: DNA Ladder, -Ve: Negative Terminal, P20: Lane1 (FSP_{20}), bp; Base pair.

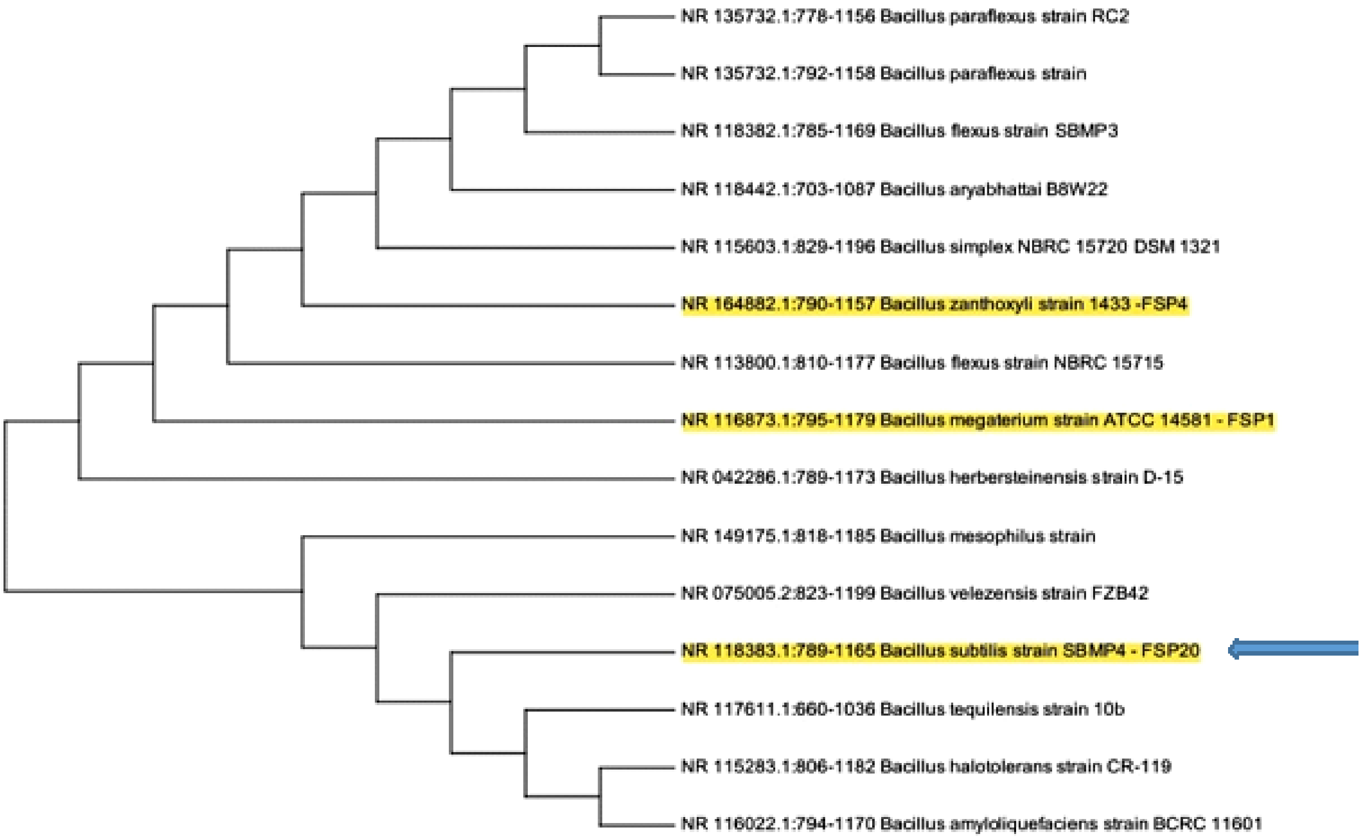

Figure 5. Neighbour-joining phylogenetic tree analysis centered on comparison of the 16S rRNA gene sequences of *Bacillus subtilis* strain SBMP4 (FSP_{20}) with related reference strains showing their relationship with closely related species using the maximum likelihood method.

DISCUSSION

The findings of this research revealed that of the six cellulolytic bacteria isolated, *Bacillus subtilis* SBMP4 (FSP_{20}) presented the highest clearing zone of 30±2.08 mm with percentage hydrolysis efficiency of 114 % showcasing its high cellulose degrading capabilities. Tests carried out to determine the pathogenicity of *Bacillus subtilis* SBMP4 (FSP_{20}), showcased the bacteria species as possessing γ-hemolytic phenotype on sheep blood agar with negative lecithinase activity on EYA medium both of which depicts the isolates inability to lyse red blood cells and break down lecithin, a component of animal tissue. Bacteriocin production by the selected *Bacillus* species was observed to be highest at 72 hours of incubation with a value of 14±1.0 mm and had antagonistic activity only against the gram-positive reference strain (*Staphylococcus aureus* ATCC 25923) utilized, an indication that the produced bacteriocin would only be effective in the treatment of infections caused by gram-positive bacteria species. The gastrointestinal tolerance test result obtained with values of 7.2×10^{5} cfu/ml (5.857 log cycle/ml) for gastric acid and 1.36×10^{6} (6.134 log cycle/ml) / 2.04×10^{6} cfu/ml (6.301 log cycle/ml) for 5% and 10% bile salt concentrations respectively, shows substantial stability to conditions within the gastrointestinal tract. On the other hand, the susceptibility pattern displayed by *Bacillus subtilis* SBMP4 (FSP_{20}) to 10 commonly used antibiotics employed, reiterates its candidature as a probiotic strain. Molecular characterization revealed the isolate to have a blast homology value of 98% with assigned reference sequence (RefSeq) Accession number NR_118383.

This study, through comprehensive analysis, investigated the various aspects of *Bacillus subtilis* SBMP4 (FSP_{20}), viz cellulolytic activity, pathogenicity, bacteriocin-producing ability, gastrointestinal tolerance and antibiotic susceptibility, thus reiterating its candidature as a probiotic feed additive in livestock feeding. Through the use of clear methodologies, this report provides detailed information on the methods used for each experiment and also presents quantitative data with standard deviations and log cycle values while characterizing the selected isolate to strain level through utilization of molecular tools. However, further studies will require conducting in vivo experiments using animal models to determine the efficacy and safety of cellulolytic probiotic *Bacillus subtilis* SBMP4 (FSP_{20}).

CMC agar applied for screening of bacteria species for their cellulolytic ability showcases their capability to utilize carboxyl methyl cellulose as a sole carbon source which is usually made visible by hydrolytic zones expressed by these bacteria species[29]. A considerable difference was seen in the abilities of the isolated bacteria species to break down cellulose as revealed by the measured zone of hydrolysis exhibited, with *Bacillus subtilis* SBMP4 (FSP_{20}) showing the highest zone of clearance of 30±2.08 mm. Research reports from literatures on some cellulase producing *Bacillus* species indicated clearing zones of between 2.1 mm – 4.5 mm[30], 24 mm -28 mm[21], and 24 – 34 mm[29] respectively. Furthermore, percentage hydrolysis efficiency of 114 % was recorded for *Bacillus subtilis* SBMP4 (FSP_{20}) while previously in 2017, 20 – 100% was observed for all *Bacillus* species screened for percentage hydrolysis efficiency[21]. From literature, most microorganisms belonging to the *Bacillus* species group are known to show β- hemolysis on rabbit and sheep blood agar respectively[31,32,23]. The study isolate, *Bacillus subtilis* SBMP4 (FSP_{20}) was observed to be γ-hemolytic on blood agar with a negative lecithinase activity on EYA agar medium. This finding agrees with a report in year 2022, which revealed that some *Bacillus* strains displayed γ-hemolytic phenotype on blood agar[33]. The antibacterial activity of bacteriocin produced by five lactic acid bacteria to four indicator bacteria reference strains of which *Staphylococcus aureus* was inclusive was studied and varying degrees of inhibition zones ranging between 10±0.2 mm and 14±0.2 mm for *Staphylococcus aureus* was observed[34]. *Bacillus subtilis* SBMP4 (FSP_{20}) produced a bacteriocin which had antibacterial activity only against the gram-positive bacteria reference strain (*Staphylococcus aureus* ATCC 25923) and the highest inhibition zone obtained for the said isolate was 14±1.0 mm and at 72 hours of incubation which is comparable with that obtained previously. In the current study, *Bacillus subtilis* SBMP4 (FSP_{20}) showed resistance at acidic condition with approximately 6 log cycle/ml of bacteria (7.2×10^5 cfu/ml) after 2 hours of incubation at pH 2.5. At 5 % and 10 % bile salt concentrations respectively, *Bacillus subtilis* SBMP4 (FSP_{20}) was seen to show stability with approximately 6.1 log cycle/ml (1.36×10^6 cfu/ml) and 6.3 log cycle/ml (2.04×10^6 cfu/ml) of bacteria after incubation for 24 hours respectively. Result obtained is comparable with that reported previously by some researchers, who worked on 170 LAB strains. The LAB strains were observed to show resistance at acidic condition, with more than 7 log cycle per milliliter of bacteria after 2 hours incubation at pH 2.5 and some of the acid-resistant strains were reported to show stability at 5% and 10% bovine bile concentrations for 24 hours[27]. The antibiotic susceptibility test carried out revealed that *Bacillus subtilis* SBMP4 (FSP_{20}) showed susceptibility to nine (9), out of the ten (10) antibiotics utilized for screening and resistance to one (1) of the antibiotics namely Ampiclox. Previous research report recorded in year 2000, strongly suggests that the resistance exhibited by *Bacillus subtilis* SBMP4 to Ampiclox, may be a defining trait of the bacterial species because it clearly states that probiotic *Bacillus* is resistant to ampicillin and penicillin [35]. Comparison of the 16S rRNA gene sequence of *Bacillus subtilis* SBMP4 (FSP_{20}) to the Genbank database using the NCBI-BLAST tool revealed the sequence of the study isolate to show high similarities to the reference sequences of the genus *Bacillus* which is in alignment with previous research report in 2021, which applied similar molecular tool in the identification of Novel Alkaline Protease producing *Bacillus subtilis* C3a-FIIRO [36].

Since the result obtained from current research on CMC agar screening is comparable with previous research reports, *Bacillus subtilis* SBMP4 (FSP_{20}) can be termed a good cellulase producer. Percentage hydrolysis efficiency is usually employed to select bacteria species with the best degradability when screened for enzyme-producing potentials. The obtained percentage hydrolysis efficiency of 114% seen in the current study isolate which is observed to be higher than that reported previously, clearly suggests *Bacillus subtilis* SBMP4 (FSP_{20}) to be a better cellulose degrader and further reiterates its high cellulose degrading capability. Microorganisms utilized as probiotics are grouped under the category GRS (Generally regarded as safe). The safety and suitability of *Bacillus subtilis* SBMP4 (FSP_{20}) towards its utilization as a prospective probiotics strain in livestock feeding was ascertained by its exhibition of γ-hemolytic phenotype (absence of hemolysis) on sheep blood agar depicting its non-pathogenic tendencies. Possession of γ-hemolytic phenotype by *Bacillus* species has a high tendency of occurring possibly due to genetic mutations. On the other hand, lecithinase is known to breaks down animal tissues, contributes to pathogenicity, and hence regarded as a virulent component and pathogenicity marker in microorganisms that possess it. The negative lecithinase activity by *Bacillus subtilis* SBMP4 (FSP_{20}) depicts the absence of a virulent component and further proves its non-pathogenic ability and safety for use as a probiotics candidate in livestock feeding. Depending on spectrum of activity, bacteriocins are classified either as narrow or broad spectrum. Thus, the bacteriocin produced by this study isolate, *Bacillus subtilis* SBMP4 (FSP_{20}), can be considered a narrow spectrum rather than a broad spectrum bacteriocin based on its range of activity. The ability of microorganisms inhabiting the gastrointestinal tract to survive is highly dependent on their capacity to withstand the acid and bile salt concentrations. Probiotic microorganisms must remain metabolically active living cells when they enter the colon in order to exhibit their health-promoting effects[7]. Based on the gastrointestinal stability exhibited in terms of acid resistance and bile tolerance, it is therefore pertinent to conclude that *Bacillus subtilis* SBMP4 (FSP_{20}) may certainly persist in the stomach and small intestine and inhabit the large intestine before exerting their possible health promoting effects as probiotics. On the other hand, the incapability of bacteria to transfer antibiotic resistance to other living organisms is ensured by their susceptibility to antibiotics[20]. Antibiotic susceptibility remains one of the defining abilities that must be possessed by microorganisms before they can be utilized as probiotic strains, therefore, the degree of susceptibility exhibited by *Bacillus subtilis* SBMP4 ensures its inability to transfer antibiotic resistance and thus the possibility of its utilization as a probiotic candidate in livestock feeding.

Bacillus subtilis SBMP4 (FSP_{20}) showed potential as a probiotic strain due to its gastrointestinal tolerance, non-pathogenicity and antibiotic susceptibility thereby reiterating its candidature as probiotic additive in livestock feeding. The bacteriocin produced by *Bacillus subtilis* SBMP4 (FSP_{20}) will play a vital role in the treatment of infections and diseases caused by gram positive bacteria such as *Staphylococcus aureus* when purified and used as alternative to natural antibiotics. The bacteriocin can also be used as a natural preservative to extend shelf life and ensure food safety.

CONCLUSION

Considering the results obtained from the current investigation, *Bacillus subtilis* SBMP4 has displayed great ability and potentiality to be used as feed additive in livestock production with emphasis on the cellulolytic and probiotic attributes displayed. The cellulolytic ability of *Bacillus subtilis* SBMP4 was demonstrated via its ability to break down CMC as a sole carbon source in the screening medium. Some of the desirable qualities required for a probiotic microorganism to remain alive and transit through the stomach and make it to the gastrointestinal tract which include acid and bile tolerance ability was fully displayed by this study isolate which further reiterates its candidature as a probiotic strain. The ability of *Bacillus subtilis* SBMP4 to produce antimicrobial metabolite (bacteriocin) that had lethal effect on *Staphylococcus aureus* emphasizes its potentiality to prevent animal diseases especially caused by gram-positive bacteria strains such as *Staphylococcus aureus* thus improving its efficacy and safety, and the health of livestock. The current study therefore further explores the possibility of combining both the cellulolytic and probiotic attributes of microbial species in improving digestibility of animal feed especially those comprising of cellulosic materials and also enhancing the gut health of these livestock all geared toward improving animal productivity. Although the laboratory study carried out has proven the possibility of utilizing *Bacillus subtilis* SBMP4 as feed additive in livestock production, further work involving in vivo studies with animal models needs to be carried out to further affirm its candidature.

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CONFLICT OF INTEREST

The authors declare no conflict of interest related to this paper.

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