Nwagu et al. Annals of Microbiology (2020) 70:20
https://doi.org/10.1186/s13213-020-01564-x

Annals of Microbiology

UNIVERSITÀ DEGLI STUDI DI MILANO

ORIGINAL ARTICLE Open Access

Evaluation of the probiotic attributes of *Bacillus* strains isolated from traditional fermented African locust bean seeds (*Parkia biglobosa*), "daddawa"

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Abstract

Background: The involvement of probiotic cultures in food fermentation guarantees enhanced organoleptic properties and maximum consumer health benefits. In this study, isolated *Bacillus* cultures used in the fermentation of African locust bean seeds "Parkia biglobosa" into the food condiment "daddawa" were evaluated for probiotic attributes. *Bacillus cereus* strains BC1 and BC2 were tested for tolerance to acid, common salt (NaCl), and bile salt. Auto-aggregation and adhesion to epithelial cells, antibiotic sensitivity profile, hemolytic pattern, and antibacterial activity were also evaluated. To demonstrate further health benefit, spores of strain BC1 were investigated for anti-inflammatory potential employing the rat paw edema technique.

Results: Both *Bacillus cereus* strains showed antagonistic activity against pathogenic *Escherichia coli* and *Staphylococcus aureus*. BC1 was more acid-stress tolerant than BC2, maintaining 107.6% viability after 3 h incubation in MRS broth of pH 2.5. However, at 97.74% viability after incubation for 3 h, BC2 was more tolerant to 0.4 % bile salt. The *Bacillus cereus* strains were susceptible to all antibiotics tested with the exception of norfloxacin and thrived under high saline stress. Both strains were protease producers and non-hemolytic on sheep blood agar. The edema inhibition study revealed that spores of *Bacillus cereus* strain BC1 had anti-inflammation potential and produced no physiological toxicity on the animals.

Conclusion: These results indicate that the *Bacillus* cultures for "daddawa" production are good candidates for probiotics and have the potential for application in both animal and human formulations for increased health benefit to consumers.

Keywords: Probiotic, Daddawa, *Bacillus*, *Parkia biglobosa*, Food quality, African locust bean

Background

Fermented foods abound in the native African cuisine either as main course meals, beverages, or food condiments, and in most cases, constitute the main source of nutrition for the rural dwellers. These foods are fermented with no prior knowledge of the exact microbial population, diversity and succession, or their individual roles during fermentation; many are either contaminants or pathogens, serving minor/major roles in fermentation without any probiotic quality or effect (Franz et al. 2014). One major class of these fermented foods is the legume-based fermented foods such as ugba (Nwagu et al. 2011), daddawa (Ezeokoli et al. 2018), and okpeye (Oguntoyinbo et al. 2007). *Bacillus* species are widely documented as primary agents in the alkaline fermentation of these legumes (Tamang et al. 2016). However, there is paucity of studies on the possible probiotic quality of these foods or the probiotic nature of the

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microbial strains actively involved in their fermentation especially *Bacillus*.

Probiotics have been defined as "live microorganisms that when administered in adequate amounts, confer health benefit to the host" (FAO/WHO 2002; Hill et al. 2014). A lot remains to be unraveled about probiotic microorganism, their sources, role in the biological system, and mode of function. This is due to the importance of this group of organisms, their versatility in numerous fermented foods and healthy human systems, and also their enormous health benefits whether in preventive (Horosheva et al. 2014), or in promotive and curative (Sazawal et al. 2006) health care. According to Burgain et al. (2011), probiotics represent about 65% of the global functional food market and have been incorporated into numerous foods amongst which are dairy and non-dairy products (chocolates, cereals and juices). Though probiotics are living organisms, dead bacteria and bioactive compounds produced by live cells can also exhibit probiotic qualities (Chugh and Kamal-Eldin 2020; Plaza-Diaz et al. 2019).

To qualify as a probiotic, a microbial strain must have the ability to exert beneficial effect on the host animal, e.g., increased growth or resistance to disease, be non-pathogenic and non-toxic, be found in the finished product as viable cells in large numbers, be able to survive and metabolize in the gut environment, and show stability in storage and field conditions. These properties are attributed to the ability of the microorganism to produce acids and/or bacteriocins, and other metabolites which not only have the capacity to boost the host immune systems but also favorably impacts its competitiveness against other microbes. Some probiotics are known to have the ability to stimulate, modulate, and regulate immune response in the host, modulate the release of hormones in the gastrointestinal tract (Kristensen et al. 2016), and regulate acute and chronic inflammation in intestinal mucosal tissue caused by inflammatory bowel disease (IBD) progression (Bakirtzi et al. 2016). In addition to the qualities mentioned above, the microorganism has to be microbiologically characterized and subjected to randomized clinical trials (Singh et al. 2011; Plaza-Diaz et al. 2019).

Contrary to what was previously thought (that probiotics consist of merely lactic acid bacteria), it is now common knowledge that other bacteria including *Bacillus* species, *Clostridium* and *Escherichia coli*, and even yeasts such as *Saccharomyces* species (*S. cerevisiae* and *S. boulardii*) possess probiotic qualities (Sanders et al. 2019). Due to the importance of probiotics to both humans and animals, researchers are constantly in search of new species/strains with more specific features as new probiotic candidates (Reid et al. 2019; Ryu and Chang 2013; Suez et al. 2019).

Bacillus species are Gram-positive spore formers, and some strains are known to have probiotic qualities (Cutting 2011). Though not yet fully explored, *Bacillus* strains as probiotic agents have potential benefits over widely used LAB due to their higher acid tolerance and better stability during heat processing, drug formulation, and low temperature storage (Bader et al. 2012). This stability is due to their spores, known to survive extremely adverse conditions of growth and germinate when the environmental condition improves. The species of *Bacillus* that have been extensively examined for probiotic attributes include *Bacillus subtilis*, *Bacillus clausii*, *Bacillus cereus*, *Bacillus coagulans*, and *Bacillus licheniformis* (Cutting 2011). Spore probiotics are currently being used in humans as dietary supplements, in aquaculture, and in animals as growth promoters (Kuebutornye et al. 2019). Safety issues surround the use of *Bacillus* as probiotics. Some strains like *Bacillus anthracis* are pathogenic to humans. Another species, *Bacillus cereus*, appears to be a cause for concern on a case-by-case basis. In other words, there are probiotic/safe *B. cereus* strains (Cutting 2011; Zhao et al. 2016; Jiang et al. 2019), as well as pathogenic strains. Many works have reviewed the safety of *Bacillus* species (Lakshmi et al. 2017; Lefevre et al. 2017; Metlakunta and Soman 2020). Animal and in vitro toxicity studies on *Bacillus subtilis* CU1 (Lefevre et al. 2017), *B. clausii* UBBC07 (Lakshmi et al. 2017), *Bacillus coagulans* SNZ 1969 (Metlakunta and Soman 2020), and *B. licheniformis* 2336 (Sorokulova et al. 2008) indicated no adverse effects associated with use. According to the Qualified Presumption of Safety (QPS) adopted by the European Food Safety Authority (EFSA), for an organism to be considered safe for use as a probiotic, it must satisfy the following criteria: be identified at the strain and species level, lack the ability to transfer antimicrobial resistances, and lack toxigenic activity (Lefevre et al. 2017). In addition, human consumption studies are to be carried out to determine if the probiotic leads to the induction of any undesirable physiological effects (FAO/WHO 2002). However, before these safety evaluation protocols, studies to assess the probiotic attributes of the species is a required first step.

Bacillus cereus strains were isolated during the traditional fermentation of African locust bean (*Pakia biglobosa*) seeds for the production of "daddawa," an important food condiment in Nigeria and in many parts of West Africa and utilized as starter cultures for controlled fermentation of "daddawa." The current study evaluated the probiotic attributes of these *Bacillus* strains.

Materials and methods

Microorganism

Two *Bacillus cereus* strains with accession numbers KY746353.1 and KX784915.1 earlier isolated in our

laboratory as active agents in the traditional fermentation of "daddawa" and identified through molecular biology techniques were used in this study. *B. cereus* strain KY746353.1 had a similarity/E-score of 99.4% while *B. cereus* strain KX784915.1 had a similarity/E-score of 87%. Both organisms were maintained on nutrient agar slants at 4 °C prior to use. Sterile MRS broth was inoculated with *Bacillus* and incubated at 37 °C for 24 h, referred to in this study as "24 h culture" or "overnight culture." This was used for some tests described below.

Antibacterial activity

Antibacterial activity was evaluated against pathogenic *Klebsiella pneumoniae*, *Escherichia coli*, and *Staphylococcus aureus* using a cut well assay diffusion method modified from Rokana et al. (2016). The pathogens were obtained from the stock culture of Medical Microbiology Laboratory, University of Nigeria, Nsukka. *K. pneumoniae* and *S. aureus* were separately grown in tryptic soy broth while *E. coli* was grown in nutrient broth. Aliquots of 100 μl of the actively growing pathogenic strains were seeded in sterilized molten tryptic soy agar (for *K. pneumoniae* and *S. aureus*) and nutrient agar (for *E. coli*), and then dispensed into plates. Wells (5 mm) were punched in the agar plates using a sterile borer. Then, 1 ml aliquots of the cell free supernatants of overnight-grown cultures of the *Bacillus* strains were dispensed in separate wells. The agar plates were kept at 7 °C to allow the supernatants diffuse into the agar. They were then incubated in inverted position until inhibition zones appeared. The diameter of the zones was measured using a caliper.

Auto-aggregation

Auto-aggregation of the *Bacillus cereus* isolates was determined according to a modified method of Lee et al. (2016). Bacterial cells were collected from a 24-h culture by centrifugation at 10,000 rpm for 10 min. The cells were washed twice with phosphate buffered saline (PBS) and re-suspended with 3 ml of PBS. The suspension was vortexed for 30 sec. Exactly, 0.1 ml was taken from the upper layer of the suspension, mixed with 2.9 ml of PBS, and the absorbance measured after 0, 1, 2, and 3 h at 600 nm using a UV/visible spectrophotometer.

Auto-aggregation (%) = $(1\text{-}A_t/A_0) \times 100$; A_t = absorbance at 1, 2, and 3 h at 600 nm, A_0 = absorbance at 0 h at 600 nm.

Antibiotic susceptibility

To evaluate the antibiotic susceptibility of the *Bacillus cereus* strains, the method of Patel et al. (2009) was used. The fresh culture of the *Bacillus* sp. was streaked densely on Mueller-Hinton agar by a sterile cotton swab. Paper discs impregnated with streptomycin (10 μg), erythromycin (30 μg), ampiclox (10 μg), rifampicin (30 μg), norfloxacin (30 μg), gentamicin (10 μg), amoxil (30 μg), and ciprofloxacin (5 μg) were loaded on the plates. The diameters of the clear zones were measured after incubation for 48 h at 37 °C.

Acid tolerance

Following the method of Lee et al. (2013), a 2-ml aliquot of *Bacillus cereus* culture grown overnight in MRS broth was incubated in 10 ml of freshly prepared MRS broth (pH 2.5) at 37 °C. Samples were collected after various time intervals (0–3 h). After collection, each sample was spread onto MRS agar plates and incubated for 24 h at 37 °C; then, viable cells were enumerated. The relative survival of the organisms was calculated with the formula below:

$$\begin{aligned}\text{Viability }(\%) &= \left[\frac{\text{Nt}}{\text{N0}}\right] \text{ x } 100; N\text{t} \\ &= \text{ log CFU at intervals } 1, 2, \text{and } 3 \text{ h and } N0 \\ &= \text{ log CFU at } 0 \text{ h}\end{aligned}$$

Bile salt tolerance

Bile salt tolerance of the *Bacillus* strains was determined using a slight modification of the method of Lee et al. (2013). A 2-ml aliquot of *Bacillus* culture grown overnight in MRS broth was incubated in 10 ml of freshly prepared MRS broth containing 0.4 % bile salt at 37 °C for 0–3 h. After every hour, samples were collected and spread onto MRS agar plates using a glass rod, then incubated for 24 h at 37 °C, after which viable cells were counted. The relative survival of the organisms in the MRS broth containing 0.4 % bile salt was calculated with the formula below:

$$\begin{aligned}\text{Viability }(\%) &= \left[\frac{\text{Nt}}{\text{N0}}\right] \text{ x } 100; N\text{t} \\ &= \text{ log CFU at intervals } 1, 2, \text{and } 3 \text{ h and } N0 \\ &= \text{ log CFU at } 0 \text{ h}\end{aligned}$$

Cell hydrophobicity

The cell hydrophobicity of the isolates was determined according to Lee et al. (2016). A 24-h culture was centrifuged at 10,000 rpm for 3 min. The cells were washed twice with PBS and re-suspended with 2 ml of PBS. Its absorbance was measured at 600 nm and this was used as the value of A_0 to determine hydrophobicity in percentage. Cell suspension was mixed separately with equal volumes of ethyl acetate and chloroform, then vortexed for 5 min. The mixture was allowed to separate into two phases for 30 min. Then, the absorbance of the aqueous phase was measured at 600 nm and used as the value of A_1.

$$\text{Hydrophobicity}\ (\%) = (1 - A_1/A_0) \times 100.$$

Sodium chloride tolerance

The method of Lee et al. (2013) was adapted for the determination of the level of tolerance to sodium chloride by the *Bacillus cereus* strains. Two milliliters of a 24-h culture was incubated in MRS broths containing varying percentages (1, 4, 10, and 15 %) NaCl for 24 h at 37 °C. Each sample was then spread onto MRS agar and viable cells counted.

Tolerance to sodium chloride was calculated as:

$$\begin{aligned}\text{Viability}\ (\%) &= \left[\frac{\text{Nt}}{\text{N0}}\right] \text{x } 100; Nt \\ &= \text{log CFU at 24 h and } N0 \\ &= \text{log CFU at 0 h}\end{aligned}$$

Amylase and protease production test

For protease production, overnight culture (1%) was added into the protease production medium containing (g/L): casein, 10; glucose, 5.0; MgSO4.7H2O, 5.0; KH2PO4, 5.0; and FeSO4.7H2O, 0.1, and incubated at 37 °C for 24 h. The culture was then centrifuged at 10, 000 rpm for 15 min. The clear, cell-free supernatant was used for protease assay by a method modified from Nwagu et al. (2015).

For amylase production, a medium containing: soluble starch (20 g/L), peptone (5 g/L), (NH4)2SO4 (2 g/L), KH2PO4 (1 g/L), K2HPO4 (2 g/L), and MgCl2 (0.01 g/L) was inoculated with overnight grown bacterial culture. The inoculated medium was incubated at 37 °C for 24 h. After the incubation period, the culture medium was centrifuged at 10,000 rpm for 15 min to obtain the crude enzyme extract. Amylase assay was carried out following the method of Bernfeld (1955) using 3,5–dinitrosalicylic acid.

Hemolysis

The *Bacillus cereus* strains were streaked on 7% sheep blood agar and incubated at 37 °C for 48 h in line with Anand et al. (2000). The sheep blood was obtained aseptically from the Faculty of Veterinary Medicine Animal Research Laboratory, University of Nigeria, Nsukka. Isolates that formed a green zone around the colony were designated as alpha hemolytic while those that formed a clear zone were denoted as beta hemolytic.

Phenol tolerance

Phenol tolerance was determined by inoculating 100 μl of 24 h-old *Bacillus* culture into MRS broth containing 0.2 % and 0.5 % of phenol. The optical density (OD) of the broths was measured at 600 nm before (0 h) and after 24 h of incubation. Values obtained were used to calculate viability (%):

Viability (%) = $\frac{OD_{24}}{OD_0}$ x 100; OD_{24} = optical density at 24 h and OD_0 = optical density at 0 h

In vivo anti-inflammatory activity of spores of *Bacillus* BC1

Spores of *Bacillus* spp. BC1 were produced by cultivating vegetative cells of the strain in a sporulation medium containing: 16 g/L Difco nutrient broth, 2 g/L KCl, and 0.7 g/L MgSO4.7H2O (Gashtasbi et al. 2014) for 1 week. After harvesting, the spores were treated to kill the vegetative cells, washed thrice, and stored in deionized water at freezing temperature. The spore suspension was adjusted to 10^8 spores/ml.

The male Wistar rats (200–250 g) were housed in plastic shoe-box cages (4 per cage) with wire mesh tops, at the animal house facility of the Department of Biochemistry. The animal housing facility had restricted access. Softwood shavings were used as the bedding material. The animals had adequate access to conventional standard laboratory diet (corn, soybean pulp, shorts, bonquality flour, alfalfa pellets, molasses, meat and bone meal, poultry meal, sepiolite, inorganic DCP, marble dust, vitamins, minerals) bought from the Vital Feed Company and potable drinking water. The temperature of the housing facility was kept at room temperature, and the lighting cycle was 12 h light, 12 h dark. Their feeding behaviour was regularly monitored.

All experimental protocols were approved by the Ethics Committee, Faculty of Biological Sciences, University of Nigeria. Ethics Committee reference number for the study is UNN-IRB/FBS/2019_006. The animals were fed with food and water while they acclimatized for 1 week to the experimental environment.

To assess anti-inflammatory activity, animals were divided into two categories. Each category had the following groups (n = 4/group):

1. Group 1 (negative control) received 500 μl of distilled water orally.
2. Group 2 received 200 μl of probiotic *Bacillus* spores suspension (10^8 spores/ml).
3. Group 3 received 500 μl of probiotic *Bacillus* spores suspension (10^8 spores/ml).
4. Group 4 (positive control) received 150 mg/kg of diclofenac sodium.

Carrageenan-induced inflammation model of Sudjarwo (2005) was used to assess the anti-inflammatory potential of *Bacillus* BC1 spores. The Wistar rats in category one were orally administered with their respective treatments (vehicle, *Bacillus* spores, or diclofenac sodium);

30 min after, 100 μl of freshly prepared carrageenan solution (1 %) was injected into the left hind paw of each rat. The extent of inflammation was monitored by measuring the paw thickness before and after injection of carrageenan at 0, 4, and 24 h with the help of a vernier caliper. A method modified from Solanki et al. (2015) was adopted and modified in evaluating the motility of the rats 24 h after carrageenan injection. The movement of the rats was observed for 5 min and scored on a scale of 0–5 depending on the degree of movement: 0, if it did not walk and 5, if the rat moved about easily.

After the experiments, the animals were anaesthetized using chloroform (300 μl in a 500-ml jar) by "drop jar" inhalation technique.

Statistical analysis

Data are expressed as mean ± standard deviation of replicates. An analysis of variance (ANOVA) and Tukey's mean comparison test were performed to determine significant difference ($P < 0.05$) in the in vivo anti-inflammatory activity test results using the Minitab 16.0 software. The P values less than 0.05 were considered to be statistically significant.

Results and discussion

Antibacterial activity

Probiotic microorganisms including LAB, *Bacillus* species, *Clostridium*, and yeasts have been found in various fermented foods (Angmo et al. 2016); however, more researches have been done on LAB than any other group (Wang et al. 2016; Son et al. 2018). In this study, *Bacillus cereus* strains, KY746353.1 and KX784915.1 (henceforth referred as BC1 and BC2, respectively), isolated from traditional fermented "daddawa" were studied for antibacterial activity. *Bacillus* strains are antibacterial, antifungal, and antiviral (Sumi et al. 2015) though these properties are strain-specific. *Bacillus cereus* strains BC1 and BC2 showed antimicrobial activity against *Escherichia coli, Klebsiella pneumoniae*, and *Staphylococcus aureus* (Table 1). With inhibition zone diameters (IZDs) of 13 mm, 12 mm, and 14.5 mm for *E. coli*, *K. pneumoniae*, and *S. aureus*, respectively, BC1 showed more antibacterial potential than BC2, which showed corresponding IZD values of 12 mm, 10.5 mm, and 11.25 mm. Earlier studies have demonstrated the antagonistic effect of *Bacillus* strains against pathogenic bacteria. Manhar et al. (2015) reported the inhibition of *K. pneumoniae* by *B. amyloliquefaciens* AMS1. Probiotic *Bacillus* strain DET6 from food wastes and MKSK-E1, MKSK-J1, and MKSK-M1 from Korean traditional soy sauce inhibited the growth of *E. coli* (Patel et al. 2009; Lee et al. 2016). In the same vein, Sumathi et al. (2017) reported the antagonistic activity of probiotic *B. megaterium* from fish gut towards *Streptococcus mutans* responsible for oral diseases. Pathogen inhibition by bacterial strains has been attributed to a variety of factors including secretion of certain digestive enzymes and inhibitors, competitive exclusion, and cell-to-cell signaling (Hughes and Sperandio 2008). The antimicrobial activity of these *Bacillus* cultures could inhibit the proliferation of certain pathogens which may accidentally contaminate these fermented foods. This will inadvertently reduce the risk of food infection or intoxication especially during traditional food processing. This may explain the paucity of incidences of food intoxications from consumption of these foods which are often poorly stored due to incessant power failures.

Table 1 Antibacterial activity of *Bacillus* strains

	inhibition zone diameter (mm)	
Pathogen	BC2	BC1
Escherichia coli	12.00 ± 0.00	13.00 ± 0.00
Klebsiella pneumoniae	10.50 ± 0.71	12.00 ± 2.83
Staphylococcus aureus	11.25 ± 2.11	14.50 ± 1.34

Mean ± standard deviation ($n = 3$)

Auto-aggregation

Figure 1 shows the results of the auto-aggregation test; BC1 showed higher (53.7%) auto-aggregation over 3 h than BC2 (48.69%). Manhar et al. (2015) reported probiotic *Bacillus amyloliquefaciens* strains which had the highest degree of auto-aggregation (65.5–75.5%) observed after 24 h incubation. The auto-aggregation percentages of the *Bacillus cereus* strains BC2 and BC1 are less than that reported by Lee et al. (2016) for *Bacillus* strains MKSK –E1, MKSK-M1, and MKSK-J1 isolated from Korean traditional soy sauce. Auto-aggregation is related to the ability of the microbial cells to adhere to the gut epithelial cells (Patel et al. 2009), a key factor in microbial colonization and persistence in the host's gastrointestinal tract.

Antibiotic susceptibility

Probiotic bacteria are reservoirs of antibiotic resistance genes; therefore, the possibility of their transfer of these genes to pathogenic organisms does not only exist but is a constant concern in dietary use of probiotics (Das et al. 2019), as this may lead to the proliferation of pathogens resistant against commonly used antibiotics. Antibiotic susceptibility test showed that the *Bacillus cereus* strains, BC1 and BC2, were susceptible to streptomycin, erythromycin, ampiclox, gentamycin, and ciprofloxacin, and only resistant to norfloxacin (Table 2). There were differences, however, in the degree of susceptibility to the antibacterial agents as determined by their inhibition zone diameters (IZDs) in mm. Both isolates were highly susceptible to gentamycin with high IZD (mm) of 34.0 and 31.5 for BC1 and BC2, respectively, observed. However, while BC1 was the most susceptible to amplicox (35.5 mm), and ciprofloxacin (34.0 mm), BC2 was the most

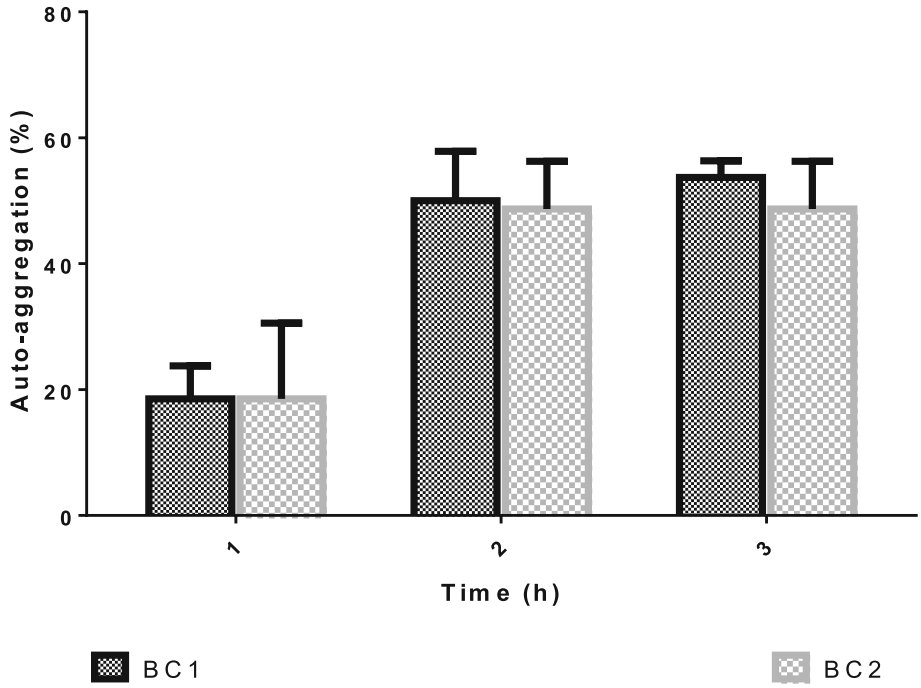

Fig. 1 Auto-aggregation of *Bacillus* strains

susceptible to ciprofloxacin only (35.00 mm). Sorokulova et al. (2008) reported that *B. licheniformis* strain included in a popular east European probiotic was resistant to chloramphenicol and clindamycin. Nithya and Halami (2013) reported a potential probiotic *Bacillus coagulans* which was resistant to the penicillin group of β-lactam antibiotics. *Bacillus amyloliquefaciens* showed sensitivity to all antibiotics tested except penicillin G and ampicillin (Manhar et al. 2015). Hoa et al. (2000) also reported the resistance of probiotic *Bacillus* against ampicillin and penicillin. Probiotic *Bacillus* strains, MKSK-E1, MKSK- J1, and MKSK-M1, from Korean traditional soy sauce were susceptible to all antibiotics tested including erythromycin, chloramphenicol, gentamicin, and cephalexin (Lee et al. 2016). The resistance of our test strains to norfloxacin was not very surprising since Frimodt-Moller et al. (1983) earlier reported that norfloxacin was poorly active against Gram-positive bacteria and inactive against anaerobes. Their susceptibility to a wide range of antibiotics suggests that these *Bacillus* strains might not carry antibiotic resistant genes which can be transferred to pathogenic microorganisms, subject to further detailed investigations.

Table 2 Antibiotic susceptibility of *Bacillus* isolates

	inhibition zone diameter (mm)	
Antibiotic	BC1	BC2
Streptomycin	21.00 ± 1.41	21.50 ± 0.71
Erythromycin	16.00 ± 1.41	20.00 ± 0.00
Ampiclox	35.50 ± 2.12	20.00 ± 1.41
Rifampicin	17.00 ± 1.41	18.50 ± 2.12
Norfloxacin	Nil	Nil
Gentamicin	34.00 ± 1.41	31.50 ± 2.12
Amoxil	20.50 ± 2.12	20.00 ± 1.41
Ciprofloxacin	26.00 ± 5.66	35.00 ± 2.83

Mean ± standard deviation ($n = 3$)

Acid and bile salt tolerance

Acid and bile stability are important parameters and basis for the selection of a probiotic strain; acid resistance is an indication of the potential of the strain to survive the gastric and duodenal juices (Jena et al. 2013). To evaluate the resistance of the *Bacillus* strains to acidic environment, the strains were cultivated at pH 2.5 for varying hours (Fig. 2). *Bacillus cereus* strain BC1 maintained over 150% viability after 1-h incubation and over 100% viability after 3 h. BC2 was not as acid stable as BC1 over the 3-h exposure to the acidic environment. The increase in viability, rather than decline, demonstrated by the organisms in the initial hour of incubation shows that it takes longer exposure for an acid-stressed environment to affect their growth. BC1 experienced a decline in viability after 2 hours of exposure; subsequent increase in viability at 3 h implies the strain's ability to favorably readjust the acid-stressed environment and resume growth. This could be by a combination of

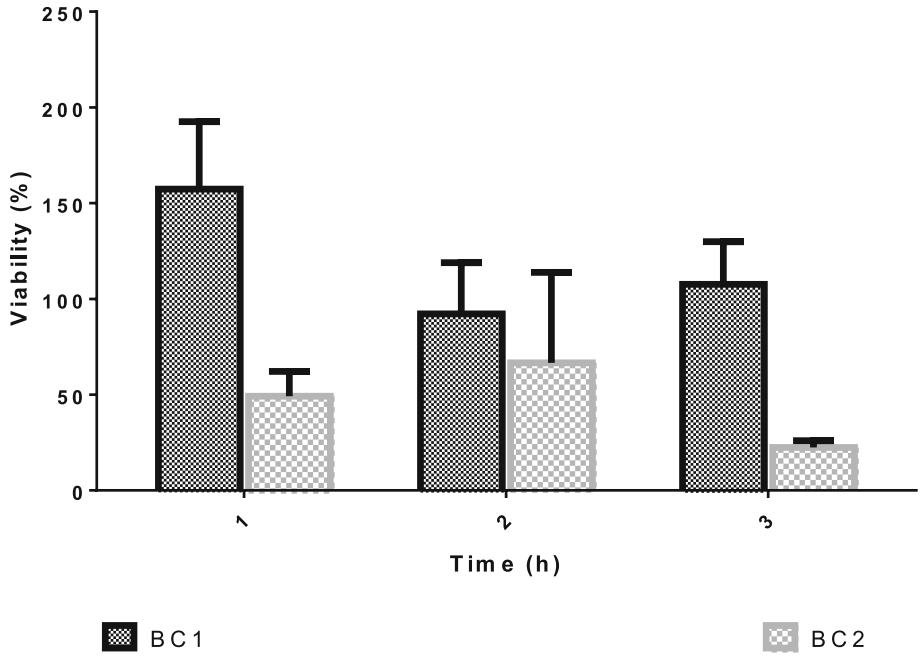

Fig. 2 Acid tolerance of *Bacillus* strains

genetic and physiological mechanisms, common with acidophilic microorganisms. Elsewhere, after exposure to 0.1% pepsin solution (pH 2.0) for 3 h, probiotic *Bacillus* strains, *MKSK*-E1, MKSK-J1, and MKSK-M1, showed relative survival ratios of 93.1%, 91.9%, and 96.0% (Lee et al. 2016).

Bile tolerance is also important for the survival of the probiotic strain in the small bowel. Bacteria growth is inhibited by bile which enters through the duodenal

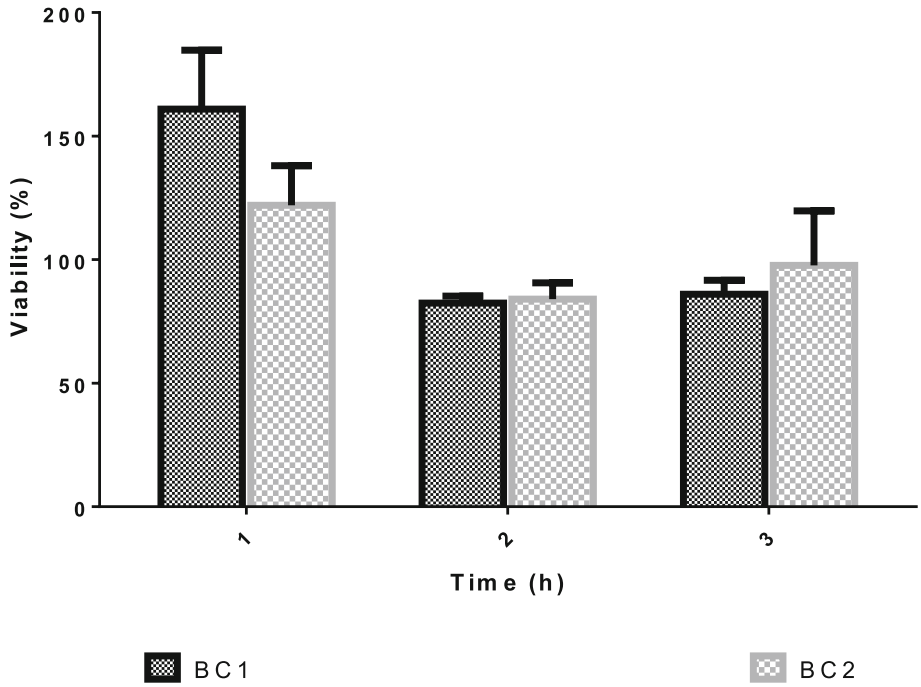

Fig. 3 Bile salt tolerance of *Bacillus* strains

section of the small intestine; this is possible as the bacteria cell membrane is made up of lipids and fatty acids which are sensitive to bile salts. To determine the ability of these strains to survive the intestinal bile, bile tolerance studies were carried out, and results are shown in Fig. 3. BC1 was highly bile tolerant, maintaining above 150% viability after 1 h incubation in MRS broth containing 0.4% bile salt and above 85.0% after 3 h. BC2 also showed above 100% viability (122.0 %) after incubation at 1 h; after 2 h and 3 h incubation, the strain maintained 83.0% and 97.7% viability, respectively. Kavitha et al. (2018) reported that *Bacillus* strain FC6 retained 91.62% viability, 3 h after exposure to 1% bile salt. Our findings further agree with the reports of Jini et al. (2011) and Giri and SukumaranV (2012) that probiotic strains are able to survive a range of bile concentrations. Our current result is an indication that these organisms when consumed with the fermented food has the potential to survive the acid- and bile-rich environments, a pre-requisite necessary to reach and survive in the intestinal gut in order to confer its benefits to the host.

Cell hydrophobicity

Hydrophobicity is an important feature which aids the attachment of probiotic microorganisms to the intestinal epithelium (Lee et al. 2013). Probiotic microorganisms, through their adhesion capability, can prevent pathogen access by steric interactions or specific blockage of cell receptors (Otero et al. 2004). The cell surface hydrophobicity of the *Bacillus cereus* strains was evaluated by determining the rate of bacteria adhesion to ethyl acetate and chloroform as shown in Fig. 4. For ethyl acetate, BC1 had lower surface hydrophobicity (52.8 %) than BC2 (58.5 %). However, BC1 had higher adhesion to chloroform (70.54 %) compared to 49.96 % for BC2. The *Bacillus* strains from "daddawa" showed very high hydrophobicity when compared to results obtained from similar studies. Hydrophobicity of the isolates in ethyl acetate was remarkably higher than that of probiotic *Bacillus* spp. DET9, JHT3, and DET6 from food waste with values in the range of 6–12% (Lee et al. 2013) and MKSK-J1, MKSK-E1, and MKSK-M1 from Korean traditional soy sauce with values less than 35% (Lee et al. 2016). However, percentage hydrophobicity in chloroform for the *Bacillus* strains is comparable with results obtained by Kavitha et al. (2018) for *Bacillus* strains FC6 (65.7 %) and FS1 (45.08 %). Cell surface hydrophobicity is reported to increase the propensity of microbial cells to adhere to surfaces; adhesion is the primary stage in microbial colonization, making the cell surface hydrophobicity a crucial property in cell attachment to surfaces (Krasowska and Sigler 2014). Auto-aggregation ability and cell surface hydrophobicity are directly correlated, and according to Manhar et al. (2015),

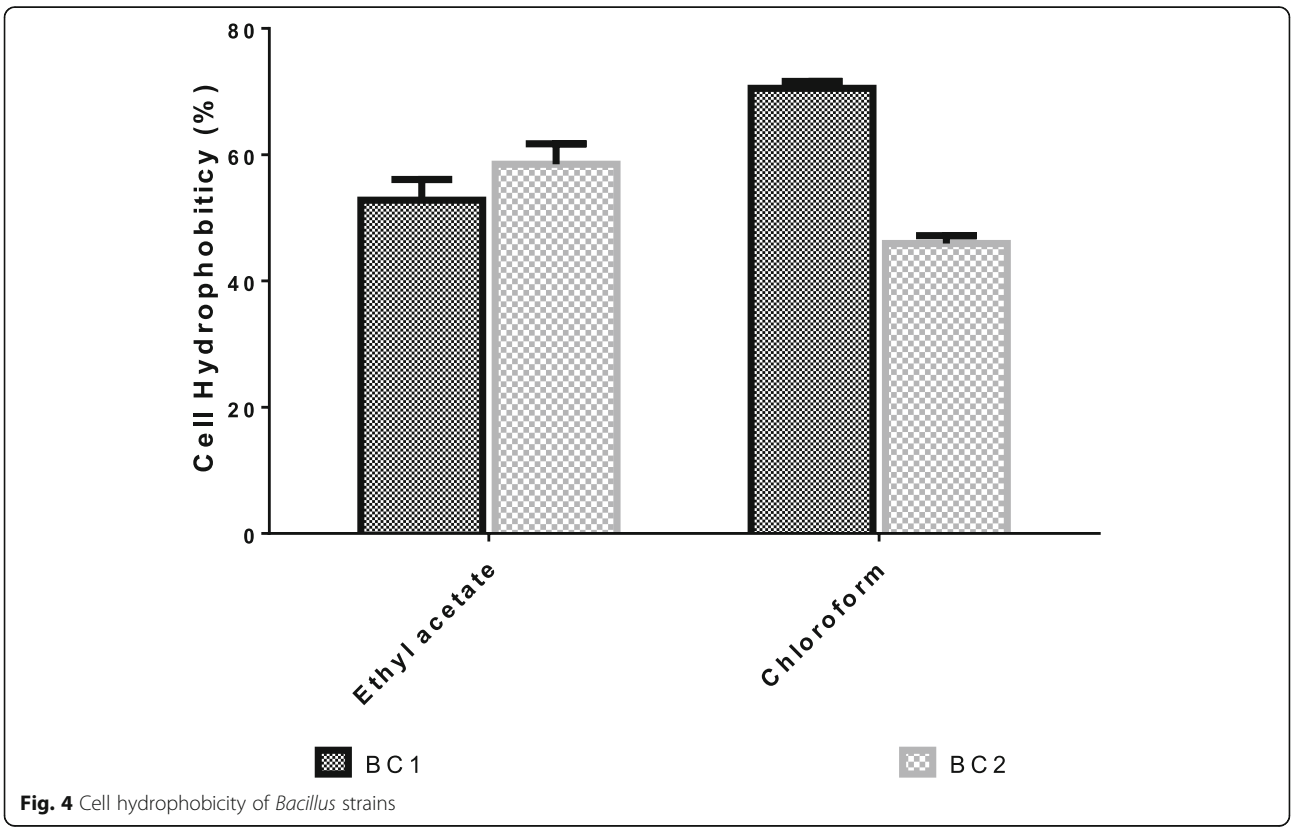

Fig. 4 Cell hydrophobicity of *Bacillus* strains

hydrophobicity could be one of the factors that determine the ability of culture to auto-aggregate.

Sodium chloride tolerance

The *Bacillus cereus* strains grew well under high salt concentrations as can be seen in Fig. 5. At 10% salt concentration isolates, BC1 and BC2 retained 64.47% and 74.4% viability, respectively, after 24 h incubation. When salt concentration was increased to 15%, 12.4% decrease in viability of BC1 was observed while BC2 had 5% loss in viability, showing that BC2 was more halotolerant than its counterpart. Pundir et al. (2013) reported tolerance of lactic acid bacteria isolates to 1–6.5% NaCl concentration. Also, *Lactobacillus* spp. isolated from yoghurts tolerated 1–9% NaCl (Hoque et al. 2010). Saline stress during microbial growth cause a loss of turgor pressure and water efflux; this adversely affects the cell physiology, enzyme synthesis and activity, water activity, and cell metabolism including carbohydrate, amino acid and fatty acid biosynthesis, and energy generation (Hoffman et al. 2013; Schroeter et al. 2013). Ability to grow well in this stress environment is an indication that it is able to circumvent the adverse effects of salt stress, achieved through proline synthesis, leading to the enhanced expression of genes for the synthesis of exopolysaccharide and capsules.

Amylase and protease production

Results obtained showed that both *Bacillus cereus* strains were capable of producing protease but not able to produce amylase. For a probiotic strain to effectively function as a food fermenter, the synthesis of hydrolytic enzymes such as amylase and protease are required to break down the complex food polymers in order to generate simpler compounds such as peptides, amino acids, reducing sugars, and oligosaccharides which will be further converted through other biological reactions to organic acids and other flavor-impacting and health benefiting compounds (Jeon et al. 2017).

Protease helps in improved protein digestion. It is also involved in defense against pathogens through the cleaving of their receptor sites in intestinal epithelial cells (Patel et al. 2009). *Bacillus* species produce proteases (example, subtilisin) which help digestion and reduce allergenicity. According to Patel et al. (2009), the ability to produce protease could have been the reason why probiotic strain DET6 from food waste showed the best antimicrobial activity of all the isolates they studied. However, this was not observed in the current study. Amylase production is an extra benefit of probiotics given their ability to improve the digestion of starch-rich foods in humans and animals to simpler sugars and oligosaccharides, necessary for generating energy molecules for the microbes. The hydrolytic by-products of these enzymes also engage in biological and chemical reactions to produce flavor compounds which give the fermented food its characteristic properties.

Hemolysis

Both strains showed no hemolysis on sheep blood agar. This shows that the *Bacillus* strains as potential

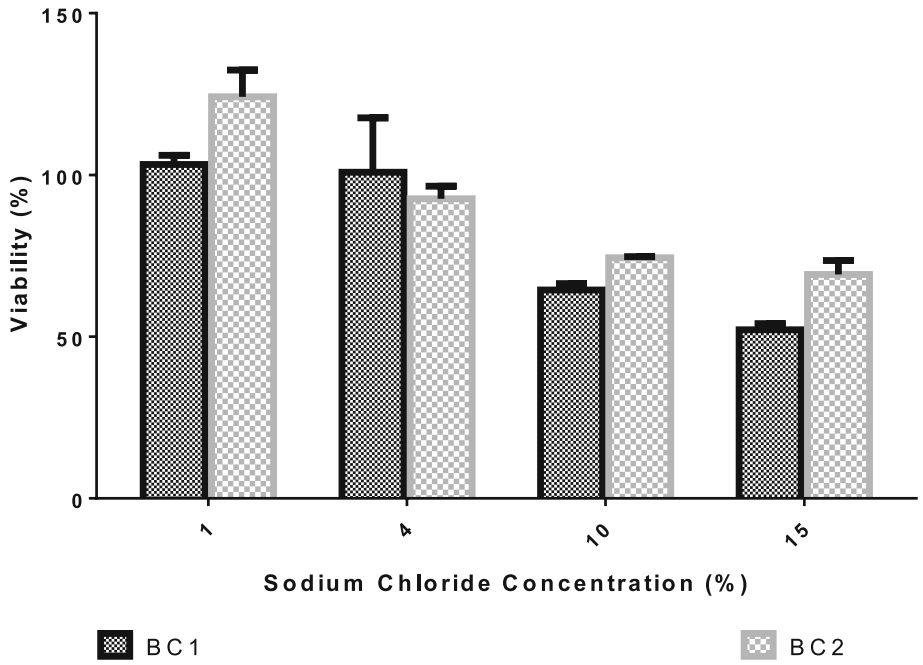

Fig. 5 Sodium chloride tolerance of *Bacillus* Isolates

probiotics satisfy one critical safety parameter. On sheep blood agar, *Bacillus* probiotic strains were reported to be non-hemolytic by Sorokulova et al. (2008). Also, probiotic *Bacillus* strains MKSK-M1, MKSK-E1, and MKSK-J1 showed no hemolysis on sheep blood agar (Lee et al. 2016). Inability of the *Bacillus* strains to lyse blood cells of the host once ingested is an added advantage required for a probiotic strain.

Phenol tolerance

After 24 h, both strains showed high viability in MRS broth containing 0.2% phenol (Fig. 6). Strain BC1 was more viable (245.20%) than BC2 (152.03%). In the broth containing 0.5% phenol, the strains also demonstrated high viability after 24 h of incubation. As can be seen from Fig. 6, viability percentages 190.86% and 160.78% were recorded for strains BC2 and BC1, respectively. It can therefore be said that the two tested bacterial strains are tolerant to both concentrations of phenol with relatively lower stability observed in higher phenol concentration (0.5%). Phenols are toxic metabolites which are released during digestion, by endogenous proteins and some aromatic amino acids. Therefore, a potential probiotic strain should tolerate the limited amounts of phenols in the gastrointestinal tract (Susković et al. 1997). In a similar vein, potential probiotic *Lactobacillus* species investigated by Tallapragada et al. (2018) were tolerant to 0.2% and 0.5% phenol.

Selection of strain for in vivo testing

Statistical analysis indicated that both *Bacillus* strains possess comparable probiotic attributes. However, BC1 was chosen for in vivo anti-inflammatory testing due to its higher antibacterial activity compared to BC2.

In vivo anti-inflammatory activity of spores of *Bacillus* BC1

The paws of category one rats (comprised of four groups) were injected with carrageenan 30 min after administering oral treatments to them. The thickness of the resulting edema was measured and recorded at various intervals, immediately after injection (0 h), 4 h, and 24 h. The progression of the paw thickness for rats in groups 2 or 200S (200 μl spores) and group 3 or 500S (500 μl spores) were compared to the progression observed in the control experiments, group 1 or NegC (Negative control) and group 4 or PosC (150 mg/kg diclofenac sodium) in Fig. 7. From our observations, the mean paw thickness of the NegC rats almost doubled immediately after the injection (from 0.373 cm before injection to 0.713 cm) and slightly increased to 0.718 cm after 4 h. This was followed by a decrease to 0.568 cm after 24 h, while for PosC rats, paw thickness was only observed to increase from 0.410 cm to 0.673 cm, immediately after injection (0 h). Afterwards, there was a progressive drop in thickness to 0.618 cm and to 0.440 cm after 4 h and 24 h, respectively. From an initial 0.383

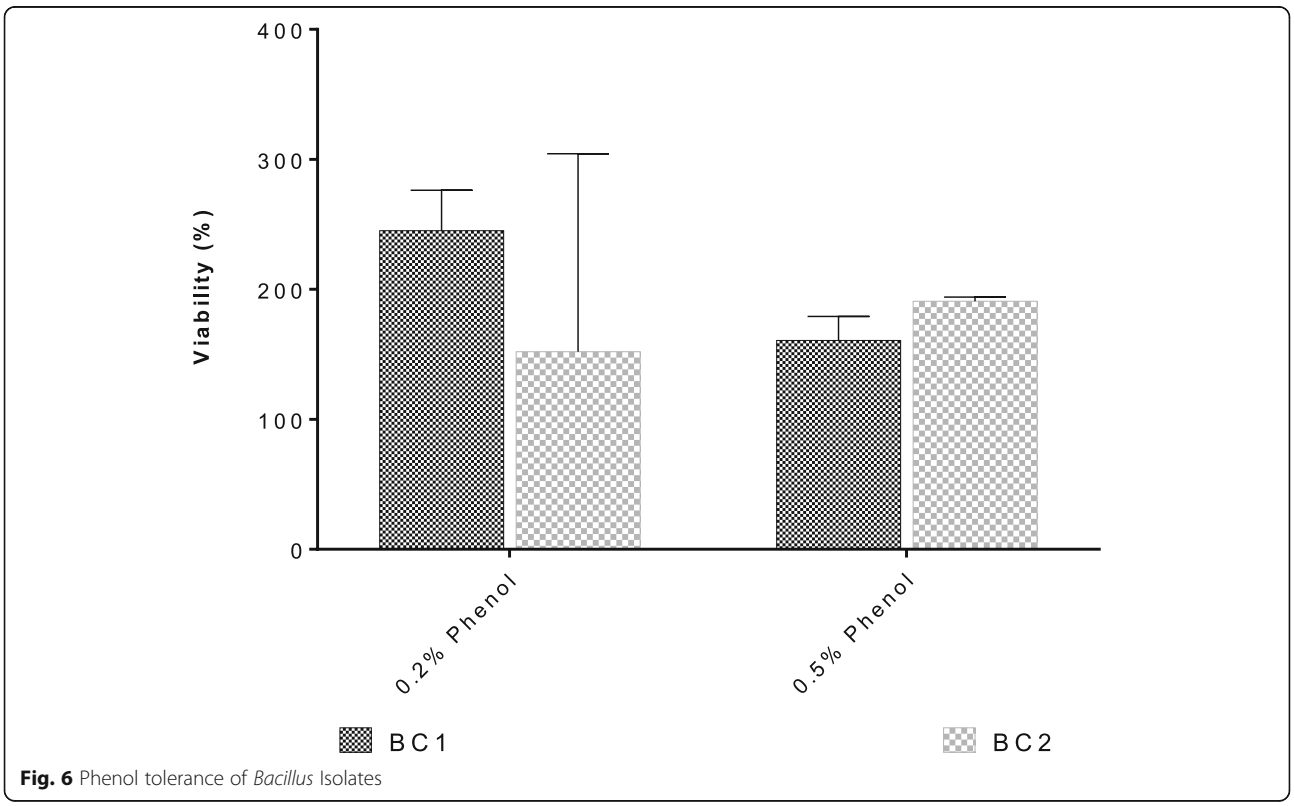

Fig. 6 Phenol tolerance of *Bacillus* Isolates

cm, the paw thickness of the animals in group 2 or 200S (200 μl spores) increased to 0.650 cm and to 0.675 cm at 0 h and 4 h respectively, then dropped to 0.473 cm at 24 h. From 0.380 cm before injection, the paw thickness of 500S rats progressively increased to 0.598 cm at 0 h and to 0.675 cm at 4 h before dropping to 0.500 cm at 24 h. Figure 8 shows edema inhibition (%) of the various treatments for all the groups in category one after 24 h, as evaluated from Fig. 7. When compared to 89.150 % inhibition obtained for PosC (treated with the control drug), 200S and 500S animals showed 76.335 % and 68.130 % edema inhibition, respectively. To also monitor inflammation inhibition by the various treatments, motility scores (on a scale of 1 to 5) were recorded in Fig. 9. All the rats showed some motility 24 h after injection. NegC, 200S, 500S, and PosC rats had motility ratings of 1.250, 3.00, 3.250, and 2.50, respectively.

Carrageenan-induced inflammation is one of the most appropriate methods of evaluating the effect of anti-inflammatory agents in animal models (Du et al. 2018). The edema produced by carrageenan injection is characterized by swelling and increase in paw thickness (Cuzzocrea et al. 1998). Commonly used non-steroidal anti-inflammatory agents like aspirin and indomethacin are associated with a lot of adverse effects (Hatt et al. 2018). This has led to increased interest in natural substances with anti-inflammation potential. Just like other probiotic organisms, probiotic *Bacillus* is known to confer a number of health benefits on the host including the alleviation of inflammation (Chen et al. 2010; Schultz et al. 2017). In this study, spores of *Bacillus* strain BC1, instead of the commonly used vegetative cells, were tested for anti-inflammatory potential. The high stability of spores when used as probiotic formulations informed this choice. There are questions over whether spores of *Bacillus* can become active in the gastrointestinal tract for probiotic benefit (Spinosa et al. 2000). Schultz et al. (2017) are of the opinion that non germinated spores could possibly provide immunologic benefit to the host and argues that the spores are likely to germinate and grow in order to become fully active. Spores are able to survive transit through the acidic stomach, after which they can germinate, grow, proliferate, and resporulate before being excreted in the faeces (Le Duc et al. 2004; Hong et al. 2009). It was observed that spores of *Bacillus* BC1 being investigated for probiotic potential produced significant ($p < 0.05$) edema inhibition. Probiotics increase the production of short-chain fatty acids (SCFAs) such as butyrate, acetate, and propionate in the gastrointestinal tract (GIT) of humans: compounds known to stimulate anti-inflammatory effects (Kerry et al. 2018). This is one mechanism by which probiotics cells and spores inhibit inflammation in the treated host.

Since probiotic strains are expected to confer health benefits on the host, this quality makes the strain an excellent probiotic candidate. It was strangely observed that 200 μl spores produced better inflammation inhibition than 500 μl. It is possible that at a relatively high concentration (500 μl), spore germination ratio is reduced, resulting in lower anti-inflammatory effect

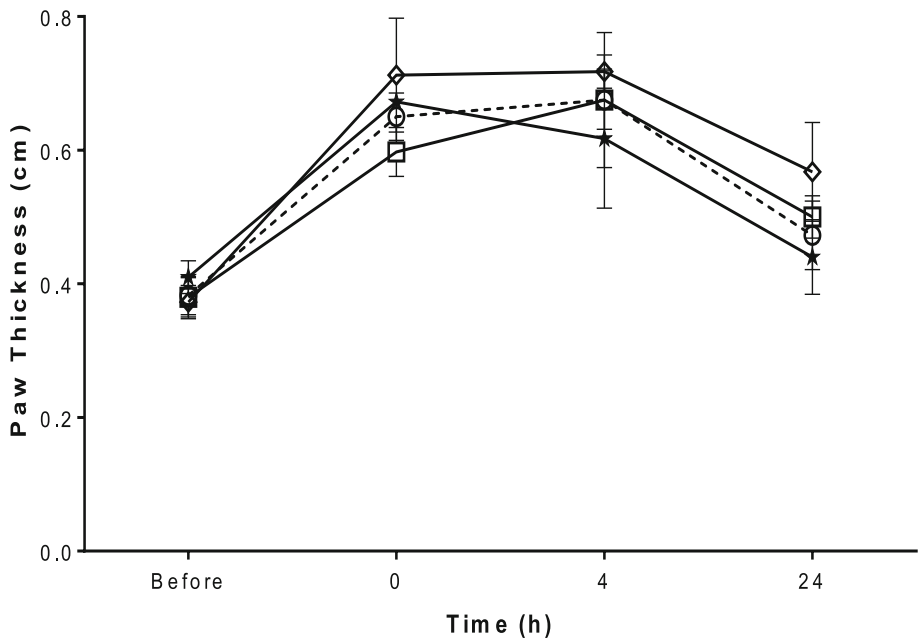

Fig. 7 Changes in paw thickness (cm) of Wistar rats after administering the *Bacillus* spores. NegC (diamond), 500 μl distilled water; 200S (circle), 200 μl Spores; 500S (square), 500 μl Spores; PosC (star), diclofenac sodium (150 mg/kg)

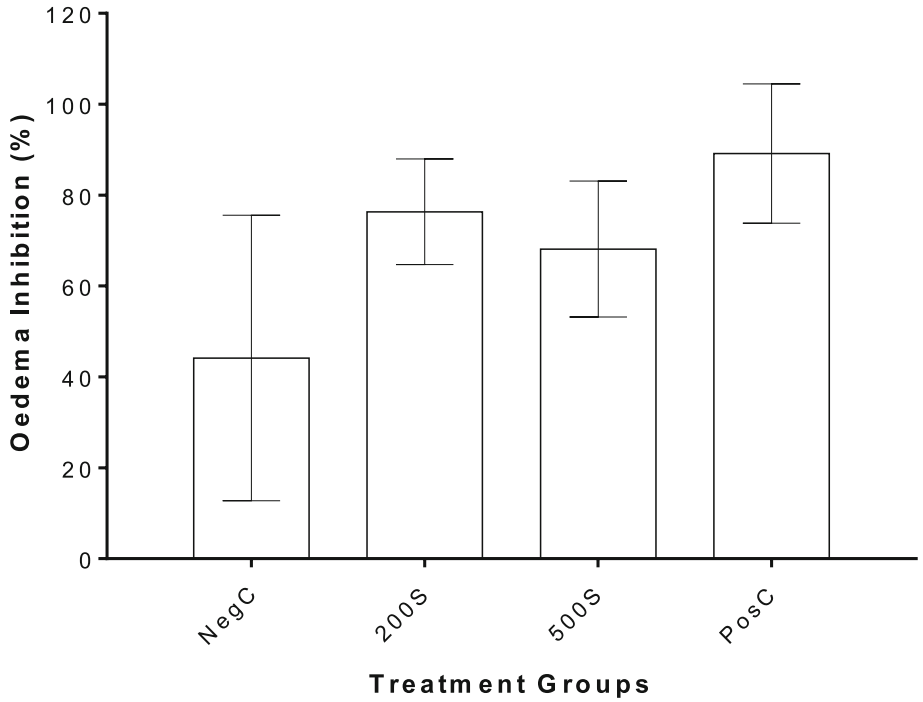

Fig. 8 Edema inhibition after administering the *Bacillus* spores. NegC, 500 μl Distilled water; 200S, 200 μl spores; 500S, 500 μl Spores; PosC, diclofenac sodium (150 mg/kg)

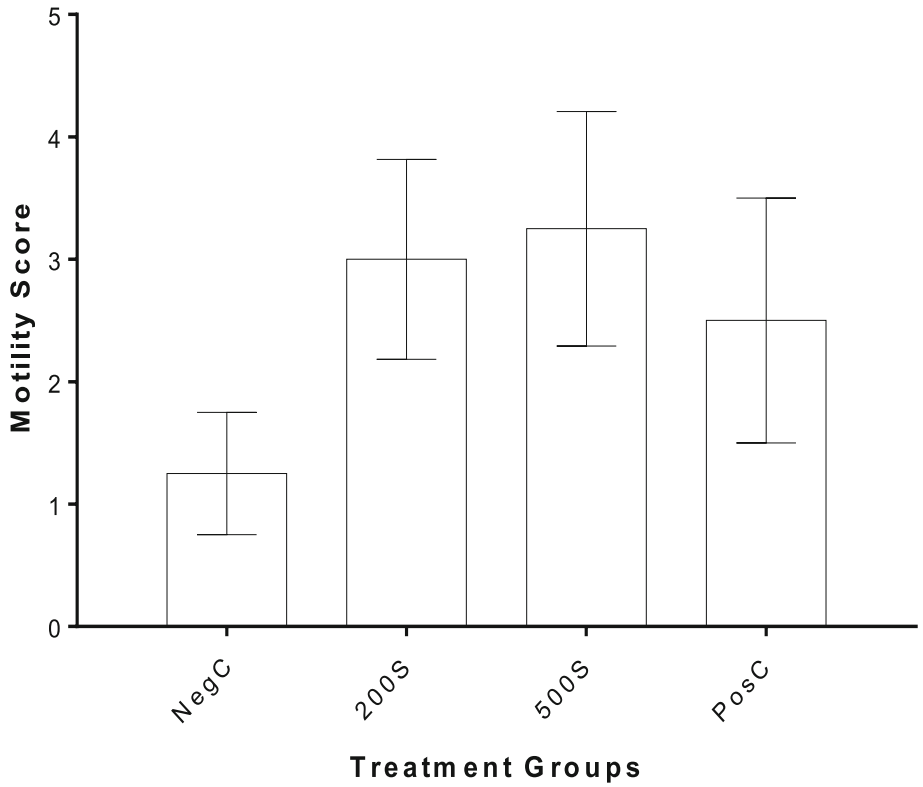

Fig. 9 Effect of the *Bacillus* spores on the mobility of rats after induction of inflammation. NegC, 500 μl distilled water; 200S, 200 μl spores; 500S, 500 μl spores; PosC, diclofenac sodium (150 mg/kg)

compared with 200 μl spore concentration. Elsewhere, spores of potential probiotic strain *B. subtilis* PB6 decreased the serum levels of IL-6 and SAA, important systemic markers of inflammation in mice (Foligné et al. 2012).

As earlier stated, these organisms were isolated based on their ability to produce "daddawa" of accepted qualities and properties. Considering the above findings, it is obvious that the *Bacillus* cereus strains in this study possess probiotic qualities. This has a number of implications; fermented African foods in this case "daddawa" produced from alkaline fermentation harbor probiotic *Bacillus* strains, and these strains are expected to play a large role in conferring the health benefits attributed to consuming fermented foods. It is worthy to note at this point that *B. cereus* strains abound in "daddawa" and its consumption in the fresh or cooked form are regarded as safe, as there is no report of associated intestinal disorder (2010). It is well documented that fermented maize grain popularly known as "ogi/akamu" in Nigeria possesses anti-diarrhoeal properties, though no research has been done to determine which particular organisms are responsible for this property. Having this in mind, the consumption of these indigenous fermented foods should be encouraged and the controlled production given more attention. Fermented Africa locust bean seed is consumed in many Africa countries as seasoning agents for many soups and stews. In some parts of Eastern Nigeria, it is consumed right after fermentation (without further cooking) as seasoning agents and protein supplements in foods such as wet cassava flakes popularly known as "African salad." The benefits of fermenting "daddawa" include detoxification of the seed, removal of anti-nutrients, increasing the availability of plant nutrients as well as essential vitamins, improved taste, food flavor, and consistency in product quality. However, using probiotic cultures during these fermentations will equally ensure that these foods serve as a vehicle for probiotic therapy ensuring multiple health benefits to the host. The *Bacillus* strains have indicated promising probiotic qualities and with further research may be invaluable as probiotic strains for animals and probably humans.

Conclusion

The *Bacillus cereus* strains isolated from traditional fermented "daddawa" possess probiotic attributes. Considering that daddawa/iru is popularly consumed as food condiment by various tribes in West Africa, either in cooked or raw form, it is important that the fermenting cultures used should not only improve its organoleptic quality but also afford a wide array of health benefits, especially considering the poor socio-economic conditions of majority of the consumers. Probiotic strains not only have the potential to confer a lot of health benefits when consumed but also produce antimicrobial substances capable of inhibiting growth of pathogenic strains. The use of these *Bacillus* cultures for "daddawa" production will therefore enhance product taste and quality. Also, this implies that "daddawa/iru" could be used as vehicle for probiotic delivery. The *Bacillus* strains studied have the potential for application in both human and animal food/feed formulations though this is subject to further examination.

Acknowledgements
Not applicable

Authors' contributions
TNT and CJU designed the experiment. AC, UOC, and OI carried out the laboratory experiments, CJU assisted in the laboratory experiments and analyzed the data and participated in writing the manuscript, TNT and COO assisted in data analysis and wrote the manuscript. All authors read and approved the final manuscript.

Funding
None

Availability of data and materials
Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Ethics approval and consent to participate
All experimental protocols were approved by the Ethics Committee, Faculty of Biological Sciences, University of Nigeria.

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

Received: 12 January 2020 Accepted: 31 March 2020
Published online: 30 April 2020

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