BIOSCIENCES BIOTECHNOLOGY RESEARCH ASIA, September 2024. Vol. **21**(3), p. 1227-1239

Probiotic Potential of *Bacillus* Strains Isolated from Traditional Cassava Ferments (*Manihot esculenta Crantz*)

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http://dx.doi.org/10.13005/bbra/3299

(Received: 16 August 2024; accepted: 01 October 2024)

Probiotic potentials of Bacillus strains isolated from traditional cassava ferments, notably their growth at different pH, bile salts, temperatures, NaCl and antibiotics, their inhibition of pathogenic bacteria, non-production of hemolytic enzymes as well as the formation of biofilm were studied. These different probiotic parameters were determined according to the referenced methods. The results obtained showed that all Bacillus strains resisted acidic pH with the highest growths (7.29± 1.40) × 108 CFU/mL and (5.14±0.15) × 108 CFU /mL obtained with Bacillus toyonensis respectively at pH 2 for 24 hours and at pH 3 for 4 hours. All Bacillus strains grow well at 37°C and 44°C, despite their optimum growth temperature of 30°C. They also showed good growth at different bile salt concentrations and were multi-resistance to antibiotics Ciprofloxacin (100%), as was B. subtilis to Rifampicin (100%) but they were multi-sensitive (100%) to Amoxicillin, Imipenem, Gentamycin, Penicillin, Vancomycin, Chloramphenicol and Rifampicin except B. pumilus and B. methylotrophicus which were sensitive to Rifampicin (77%). Bacillus strains inhibited more than half of the pathogens (80.27% with S. aureus and 65.09% with E. coli). No strain showed hemolytic activity but rather a good capacity to form a biofilm (optical density ranging from 0.663 ± 00 to 3.15 ± 02 nm).

Keywords: Bacillus; Cassava, Probiotic, Traditional ferment.

Introduced to Africa by the Portuguese around the middle of the 15th century, cassava, its scientific name *Manihot esculenta* Crantz, is a plant of the *Euphorbiaceae* family with tuberous roots. It is a plant that is an integral part of the diet of more than half a billion human beings around the world with an estimated global production of 302.7 million tonnes during the year 2020 where Africa produces on average half of this production[1].

In Côte d'Ivoire, cassava occupies second place among food crops with 6.4 million tonnes after yam. It is a commodity used for the preparation of several local dishes, most of which require the use of a traditional leaven cassava. The preparation of most of these dishes requires the use of traditional sourdough or cassava ferment. The preparation of most of these dishes requires the use of traditional sourdough or cassava ferment.

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For thousands of years, humans have used the fermentation process for food processing and preservation techniques. It is a process that is generally implemented to diversify different types of foods, make otherwise inedible foods edible, improve nutritional value and energy requirements, decrease toxicity, preserve foods, and reduce cooking times[2]. The African continent has a wide variety of fermented foods that generally have a significant impact on the nutritional quality and socio-economic status of the population[3]. In Côte d'Ivoire, local food fermentation processes are spontaneous and the microorganisms involved are lactic acid bacteria, *Bacillus*, yeasts and molds[4]. These microorganisms not only reduce the growth of pathogens through the production of various organic compounds but also sometimes have probiotic potential.

Nowadays, studies are turning to the search for probiotics of microbial origin. These are mainly bacteria and yeasts present in certain fermented foods[5]. Their use, initially based on empirical observations, is currently more rational and supported by numerous studies[6]. Bacteria of the genera *Lactobacillus* and *Bifidobacterium*, qualified as « beneficial bacteria » and residents of the intestinal tract have been mainly studied and used[7]. Numerous studies show that these probiotic microorganisms are capable of interacting with the intestinal immune system leading to a regulatory immune response.

The concept of probiotic has been extended to the use of other strains absent from the endogenous flora of the intestinal tract. Among them, bacteria of the *Bacillus* genus capable of resisting in the form of spores to extreme pH conditions during passage through the stomach and possess antimicrobial activities against pathogens and immunomodulatory properties. The ingestion of probiotic *Bacillus* spores does not lead to the definitive establishment of the strain within the microbiota. Indeed, probiotics of the *Bacillus* genus are considered transient residents of the intestinal flora and their potential development within this environment depends on the nutritional conditions prevailing there. Among these bacteria of the *Bacillus* genus, those isolated during cassava fermentation have shown their ability to play a role in tissue degradation and detoxification of cassava[8] and to produce a diversity of enzymes[9,10]. However, in Côte d'Ivoire, their role has not yet been elucidated in terms of their probiotic activity.

In this context, many discussions have suggested the use of microbial starters as the best approach to improve not only the quality of cassava ferment and the resulting dishes but also the health of the consumer. It is in this context that this study was initiated in order to evaluate some probiotic activities of *Bacillus* strains involved in cassava fermentation.

MATERIALS AND METHODS

Study material

The study material consists of six strains of *Bacillus* each with its code, namely *Bacillus subtilis* (E7-B4), *B. toyonensis* (AB2-3), *B. pumilus* (AB3-5), *B. methylotrophicus* (AB4-6), *B. vallismortis* (E3-B7) and *B. amyloliquefaciens* (E8-B2), isolated from traditional cassava ferments, identified genetically and coming from microbial heritage of the Laboratory of Biotechnology and Microbiology of Foods of NANGUI ABROGOUA University, Côte d'Ivoire.

Conservation and purification of *Bacillus* strains

Bacillus strains were stored at -80°C in Plate Count Agar (PCA) medium supplemented with 1% starch. The cultures were revived in brain heart broth then incubated at 37°C for 24 hours. After incubation, the strains were subcultured on Moselle agar then incubated at 37°C for 24 hours.

Growth of *Bacillus* strains at different acidic pH concentrations

The study of the growth of *Bacillus* strains at different pHs was carried out according to the method[11]. Heart-brain broths were prepared at different acid pH (2; 3) and control pH (6.8) by adjustment with 1M HCl. The simple prepared medium served as a control. The medium was divided into different test tubes at the rate of 3 mL per tube. The sterile medium contained in each tube was inoculated at 1% v/v with a pre-culture of *Bacillus* seeded in brain heart broth at 37°C for 24 hours, the load of which was adjusted to 3.10^8 CFU/mL. The seeded media were incubated at 37°C for 24 hours. At each time including 0 hour, 4 hours and 24 hours, a tube is removed and the optical density was read at 600 nm using a spectrophotometer (BK-UV1000).

Growth of *Bacillus* strains at different concentrations of bile salts

The growth of *Bacillus* strains at different concentrations of bile salts was carried out according to the method[11]. Different quantities of Ox-bile were added to the different heart-brain broths, namely 3 g, 6 g, and 9 g to give the concentrations of 0.3%, 0.6% and 0.9% of bile salts respectively. Broth without Ox-bile served as a control. The medium was divided into different test tubes at the rate of 3 mL per tube and autoclaved at 121°C for 15 min. Each medium contained in a tube was inoculated at 1% v/v with a *Bacillus* pre-culture seeded in brain heart broth at 37°C for 24 hours and the load was adjusted to 3.10^8 CFU/mL. Then, the media were incubated at 37°C for 24 hours. A tube is removed at each time (0 hour, 4 hours and 24 hours) and the optical density is read at 600 nm.

Resistance of *Bacillus* strains to antibiotics

Resistance of *Bacillus* strains to antibiotics was carried out using the disk diffusion method described by[12]. First, a *Bacillus* pre-culture was carried out in brain heart broth and incubated at 37°C for 24 hours. For each strain, the optical density was read at 600 nm and adjusted to 0.2. Then, 100 µL of each of the strains were placed in empty Petri dishes. The Mueller Hinton medium, sterilized in an autoclave at 121°C for 15 min, was poured into the petri dishes containing the inoculum then homogenized. After solidification of the medium, discs of eight antibiotics from seven families of antibiotics in particular Beta-lactams (Penicillin : 10 µg and Amoxicillin : 30 µg), Carbapenems (Imipenem : 10 µg), Aminosides (Gentamycin : 10 µg), Quinolones (Ciprofloxacin : 5 µg), Rifamycin (Rifampicin : 30 µg) Glycopeptides (Vancomycin : 30 µg) and Phenicolates (Chloramphenicol : 30 µg) were placed on the previously prepared Petri dishes and then incubated at 37°C for 24 hours. After incubation, zones of inhibition observed around the discs were measured and the results were expressed as sensitive (S) where the inhibition diameter > 15 mm or as resistant (r) where the inhibition diameter d" 15 mm[13]. Bacterial strains of *E. coli*, *S. aureus* and *Salmonella* sp. have been used for antibiotic control.

Antimicrobial activities of *Bacillus* strains

This study was carried out according to the method described by[14]. *Bacillus* strains were cultured in brain heart broths and incubated at 37°C for 24 hours. After incubation, the *Bacillus* cultures were centrifuged at 6000 rpm at 4°C for 20 minutes. Then, 100 µL of the supernatant of each of the strains were deposited in 6 mm diameter wells dug under aseptic conditions with the tip of a Pasteur pipette on the nutrient agar. Then, the boxes were placed at 4°C for 2 hours to allow proper diffusion of the antimicrobial substance then incubated at 37°C for 24 hours. The results were read by measuring the diameter of the inhibition zones formed around the wells[14]. At this level, the result is positive when the inhibition diameter > 15 mm and lower when the inhibition diameter d" 15 mm.

Growth of *Bacillus* strains at different temperatures

The growth of *Bacillus* strains at different temperatures was carried out following the method[11]. Different brain heart broths were prepared and then distributed in different test tubes at a rate of 3 mL per tube. After sterilization by autoclaving at 121°C for 15 minutes, the media contained in the test tubes were then inoculated at 1% (v/v) by a pre-culture of *Bacillus* strains seeded in brain heart broth at 37 °C for 24 hours and whose load was adjusted to 3.108 CFU/mL. The seeded media were incubated at different temperatures including 37°C and 45°C for 24 h. A tube is removed at each time (4 and 24 h) and the optical density is read at 600 nm using the spectrophotometer (BK-UV1000).

Determination of hemolytic activities of *Bacillus* strains

The *Bacillus* strains revivified on YPDA agar were tested for their hemolytic activity using blood agar supplemented after autoclaving at 121°C for 15 min with sterile sheep blood (7%, v/v) following the protocol described by[15]. Ten (10) ìL of each *Bacillus* strains suspension were inoculated onto the surface of the culture medium per spot and then the medium was incubated at 37°C for 48 hours in an oven (BJPX-H64II, China). Positive activity is reflected by a zone of lysis around *Bacillus* strains colonies. The non-hemolytic reaction was recorded as one producing no effect on blood agar.

Ability of *Bacillus* strains to form a biofilm

The *Bacillus* strains were cultured in brain heart broths at 37°C for 24 hours and then the optical density was adjusted to 0.5. A volume of 1 mL of each culture was aseptically introduced into Eppendorfs tubes and then incubated at 37°C for 24 hours. Subsequently, the tubes were centrifuged at 4000 rpm for 10 minutes and the pellets were washed 3 times with NaCl solution (0.9%) then dried at 50°C for 30 minutes. *Bacillus* biofilms were stained with 1 mL of 0.1% crystal violet for 20 min and rinsed with NaCl solution (0.9%). The dye was then eluted with ethanol (96%) and the contents of the Eppendorfs tubes were transferred into reading tubes and quantified by measuring the absorbance at a wavelength of 595 nm. Thus, the capacity of a *Bacillus* strain to form a biofilm is considered positive for any the optical density e" 0.5[16].

Statistical analysis

Statistical processing of the results was carried out using R software version 4.1.1. The means of the different parameters studied were compared using a one-way analysis of variance (ANOVA 1) associated with the Tukey's Test at the threshold of 5%.

RESULTS

Growth capacity of *Bacillus* strains at different acidic pH

Table 1 presents the growth capacity of *Bacillus* strains at different acidic pH. The growth of *Bacillus* strains in an acidic environment was observed after 0 hours, 4 hours and 24 hours of incubation at pH 2, pH 3 and pH 6.8. Some strains of *Bacillus* show good growth. At 0 hour of incubation, all strains have practically the same growth at pH 2, pH 3 and pH 6.8. After 4 hours of incubation, strains AB2-3 and E7-B4 showed the strongest growth respectively (9±1.06) × 10^7 CFU/mL and (7.29±1.40) × 10^8 CFU/mL at pH 2. At pH 3, strain AB2-3 showed the strongest growth at 4 hours and 24 hours respectively (5.14±0.15) × 10^8 CFU/mL and (1.14±0.22) × 10^9 CFU/mL. On the other hand, at pH 2 and pH 3, strain E8-B2 showed the lowest growth at 4 hours and 24 hours of incubation. All strains have practically the same growth at pH 6.8 apart from the E8-B2 strain whose growth amounts to (2.20±0.2) × 10^9 CFU/mL.

Growth capacity of *Bacillus* strains at different concentrations of bile salts

Bacillus strains showed good growth ability in the presence of different bile salt concentrations from 0.3% to 0.9% during all incubation hours. After 4 hours of incubation with the concentration of 0.3%, the results obtained showed significant differences ($P<0.05$) between all strains. Strain E8-B2 showed the highest growth at 0.3% (1.54±0.21) × 10^9 CFU/mL bile salts. Also, strain AB2-3 had the strongest growth (3.15±0.70) × 10^9 CFU/mL at 24 hours of incubation. As for strains E3-B7 and E7-B4, they showed stronger growth (1.69±0.70) × 10^9 CFU/mL and (3.11± 0.63) × 10^9 CFU/mL respectively at 4 hours and 24 hours of incubation at a concentration of 0.6%. A significant difference ($P<0.05$) was observed between the strains at the concentration of 0.9% bile salts. However, strains E8-B2 and E3-B7 showed similar growth (1.24±0.45) × 10^9 CFU/mL at 4 hours. At 24 hours, strains E8-B2 and E3-B7 similarly showed the highest growth (2.11±0.9) × 10^9 CFU/mL. However, no significant difference (PÃ0.05) was observed between the strains at 0 hour, 4 hours and 24 hours at the control concentration (0%) in bile salts where all the strains had the same growth (Table 2).

Resistance of *Bacillus* strains to antibiotics

The antibiotics that were used in this part of the study were Amoxicillin, Ciprofaxin, Imipenem, Gentamycin, Penicillin, Oxacillin and Rifampicin. All 6 *Bacillus* strains tested (*B. toyonensis, B. pumilus, B. subtilis, B. methylotrophicus, B. vallismortis* and *B. amyloliquefaciens*) demonstrated multi-resistance with regard to Ciprofaxin (100%). All strains showed their multi-sensitivity (100%) towards 7 antibiotics (Amoxicillin, Imipenem, Vancomycin, Gentamycin, Penicillin, Chloramphenicol and Rifampicin) except *B. pumilus* and *B. methylotrophicus* which were sensitive to Rifampicin (77%) and *B. subtilis* which was multi-resistant to Rifampicin (100%) (Table 3). The sensitive (A) and resistant (B) inhibition diameters of antibiotic disks against the *Bacillus* strains tested are presented in Fig. 1.

Antibacterial activities of *Bacillus* strains

The antibacterial activity of *Bacillus* species was evaluated on three pathogenic bacteria in particular *E. coli*, *S. aureus* and *Salmonella* sp. The vast majority of *Bacillus* species inhibited

Table 1. Growth of *Bacillus* strains at acidic pH 2, pH 3 and pH 6.8 at 0 hour, 4 hours and 24 hours of incubation

pH	Incubation time	Loads of *Bacillus* strains (UFC/mL) E8-B2	AB2-3	E3-B7	AB4-6	E7-B4	AB3-5
pH 2	0 hour	(4,13±5,3) × 10^{6a}	(4,43± 1,80) × 10^{6a}	(4,58±0,53) × 10^{6a}	(5,25± 3,18) × 106^{a}	(5,03± 1,38) × 10^{6a}	(5,78± 3,50) × 10^{6a}
	4 hours	(2,63±1,17) × 10^{7b}	(9,00± 1,06) × 10^{7a}	(5,4± 2,12) × 10^{7ab}	(4,95± 2,12) × 10^{7ab}	(7,29± 1,40) × 10^{7a}	(5,90± 1,61) × 10^{7ab}
	24 hours	(2,07± 0,13) × 10^{9c}	(1,34± 0,84) × 10^{8c}	(8,05±2,26) × 10^{7a}	(6,97± 1,58) × 10^{7b}	(1,10± 0,78) × 10^{8b}	(8,78± 2,44) × 10^{7a}
pH 3	0 hour	(5,25±0,53) × 10^{6a}	(4,43± 1,80) × 10^{6a}	(5,03±0,53) × 10^{6a}	(4,13± 1,38) × 10^{6a}	(4,58± 1,38) × 10^{6a}	(5,78± 3,50) × 10^{6a}
	4 hours	(5,72±0,21) × 10^{7b}	(5,14± 0,15) × 10^{8b}	(7,80±0,21) × 10^{7a}	(1,47± 1,29) × 10^{8c}	(1,23± 0,62) × 10^{8c}	(8,48± 0,53) × 10^{7a}
	24 hours	(2,32±0,78) × 10^{8c}	(1,14± 0,22) × 10^{9c}	(4,07±0,2) × 10^{8a}	(5,38± 0,75) × 10^{8a}	(4,40± 1,46) × 10^{8a}	(3,98± 0,42) × 10^{8a}
pH 6,8	0 hour	(4,58±0,53) × 10^{6a}	(5,25± 1,80) × 10^{6a}	(4,43±0,53) × 10^{6a}	(4,13± 1,38) × 10^{6a}	(5,78± 1,38) × 10^{6a}	(5,03± 3,50) × 10^{6a}
	4 hours	(4,93±1,18) × 10^{8b}	(1,15± 0,75) ×10^{9c}	(9,57±0,70) × 10^{8a}	(1,12± 0,28) × 10^{9c}	(1,03± 0,28) × 10^{9c}	(1,21± 0,72) × 10^{9c}
	24 hours	(2,20±0,2) × 10^{9a}	(1,67± 0,70) × 10^{9a}	(1,73±0,70) × 10^{9a}	(1,64± 5,66) × 10^{9a}	(1,67± 0,70) × 10^{9a}	(1,60± 0,70) × 10^{9a}

AB2-3 : *Bacillus toyonensis* ; AB3-5 : *B. pumilus* ; E7-B4 : *B. subtilis* ; AB4-6 : *B. methylotrophicus* ; E3-B7 : *B. vallismortis* ; E8-B2 : *B. amyloliquefaciens* ; Average values of 3 repetitions ; In the same line, values followed by the same alphabetical letter are not statistically different (P >0.05) (Tukey, HSD).

Table 2. Growth of *Bacillus* strains at different concentrations of bile salts at 0 hour, 4 hours and 24 hours

Concentrations of bile salts	Incubation time	Loads of *Bacillus* strains (UFC/mL) E8-B2	AB2-3	E7-B4	AB4-6	E3-B7	AB3-5
0%	0 hour	(2,29± 0.31) × 10^{8a}	(4,73± 1,38) × 10^{6a}	(5,25± 1,06) × 10^{6a}	(3,78± 0 ,56) × 10^{8a}	(5,33± 1,59) × 10^{6a}	(5,03± 2,44) × 10^{6a}
	4 hours	(2,55± 0,84) × 10^{8b}	(1,07± 0,23) × 10^{9b}	(1,34± 0,20) × 10^{9a}	(1,12± 0,21) × 10^{9b}	(6,72± 2,55) × 10^{8a}	(1,06± 0,12) × 10^{9b}
	24 hours	(2,07± 0,13) × 10^{9b}	(3,14± 0,12) × 10^{9a}	(2,62± 0,15) × 10^{9ab}	(1,78± 0,42) × 10^{9c}	(2,99± 0,90) × 10^{9b}	(2,40± 0,22) × 10^{9ab}
0,3%	0 hour	(2,29± 0,13) × 10^{8b}	(4,73± 1,38) × 10^{6a}	(5,25± 1,06) × 10^{6a}	(3,78± 0,52) × 10^{8ab}	(5,33± 1,59) × 10^{6a}	(5,03± 2,44) × 10^{6a}
	4 hours	(1,54± 0,21) × 10^{9c}	(6,21± 0,12) × 10^{8b}	(1,29± 0,12) × 10^{9c}	(8,71± 1,47) × 10^{8a}	(6,69± 0,75) × 10^{8b}	(8,97± 0,80) × 10^{8a}
	24 hours	(3,07±0,70) × 10^{9a}	(3,15± 0,70) × 10^{9a}	(2,72± 0,70) × 10^{9b}	(2,10± 0,70) × 10^{9b}	(1,86± 0,70) × 10^{9c}	(2,12± 0,70) × 10^{9b}
0,6%	0 hour	(2,29± 0,13) × 10^{8c}	(4,73± 1,38) × 10^{6b}	(2,28± 0,31) × 10^{8c}	(3,78± 0,52) × 10^{8ab}	(5,33± 1,59) × 10^{6a}	(5,03± 2,44) × 10^{6a}
	4 hours	(1,41± 0,84) × 10^{9b}	(8,58± 0,84) ×10^{8a}	(1,30± 0,63) × 10^{9b}	(1,02± 0,13) × 10^{9b}	(1,69± 0,70) × 10^{9b}	(8,03± 0,85) × 10^{8a}
	24 hours	(2,17± 0,12) × 10^{9a}	(1,83± 0,17) × 10^{9b}	(3,11± 0,63) × 10^{9a}	(1,97± 0,21) × 10^{9b}	(1,61± 0,42) × 10^{9b}	(1,80± 0,17) × 10^{9b}
0,9%	0 hour	(5,33± 0,31) × 10^{6a}	(4,73± 1,38) × 10^{6a}	(2,28± 3,14) × 10^{8b}	(3,78± 0,52). × 10^{8b}	(5,33± 1,59) × 10^{6a}	(5,03±2,44) × 10^{6a}
	4 hours	(1,24± 0,45) × 10^{9c}	(8,30± 1,06) × 10^{8a}	(5,56± 1,29) × 10^{8b}	(1,11± 0,84) × 10^{9c}	(1,24± 0,28) × 10^{9c}	(8,25± 0,84) × 10^{8a}
	24 hours	(2,21± 0,90) × 10^{9a}	(1,98± 0,10) × 10^{9ab}	(1,68± 0,11) × 10^{9b}	(1,80± 0,15) × 10^{9b}	(2,21± 0,49) × 10^{9a}	(1,89± 0,28) × 10^{9b}

AB2-3 : *Bacillus toyonensis* ; AB3-5 : *B. pumilus* ; E7-B4 : *B. subtilis* ; AB4-6 : *B. methylotrophicus* ; E3-B7 : *B. vallismortis* ; E8-B2 : *B. amyloliquefaciens* ; Average values of 3 repetitions ; In the same line, values followed by the same alphabetical letter are not statistically different (P >0.05) (Tukey, HSD).

more than half of the pathogenic bacteria. The highest inhibition rate (96.7%) was obtained with *B. amyloliquefaciens* on *S. aureus* while the lowest rate (50.15%) was observed with *B. subtilis* on *E. coli* (Table 4). Pathogen inhibition rates are between 65.09% and 80.27%. The most inhibited pathogen was *S. aureus* (80.27%) and the least inhibited was *E. coli* (65.09%) (Fig. 2).

Growth capacity of *Bacillus* strains at different temperatures

The growth capacity of *Bacillus* strains at different temperatures is presented in Table 5. Generally, all *Bacillus* strains showed growth at 37°C and at 44°C after 4 hours and 24 hours. However, growth at 44°C is lower than that at 37°C. After 4 hours of incubation time at a temperature of 37°C and 44°C, a significant difference ($P<0.05$) exists between the growth of the strains studied. At 37°C, the E8-B2 strain exhibited the highest growth (2.27±0.10) × 10^8 CFU/mL at 4 hours while the E3-B7 strain also showed the highest growth (4.51±0.2) × 10^8 CFU/mL at 24 hours. At 44°C, strain AB2-3 showed the highest growth (1.54±0.15) × 10^8 CFU/mL at 4 hours while strain E3-B7 showed high growth (2.23±0.20) × 10^8 CFU/mL at 24 hours.

Hemolytic activities of *Bacillus* strains

Hemolytic activities of Bacillus strains were highlighted. Of all the six *Bacillus* strains tested, none of them secrete enzymes with hemolytic activities into their production environments (Table 6). The illustration reflecting the absence of hemolytic activity is shown in Fig. 3.

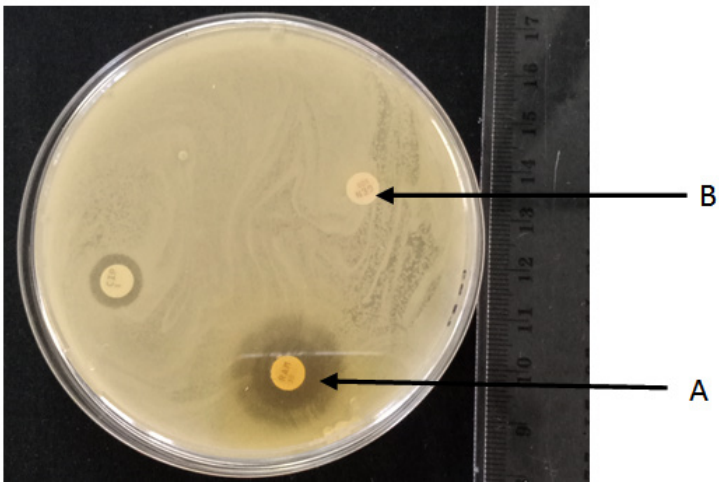

Fig. 1. Petri dish photograph showing the sensitive (A) and resistant (B) inhibition diameters of antibiotic disks against *Bacillus* strains

Table 3. Resistance of *Bacillus* strains to antibiotics

Bacillus strains	Resistance or susceptibility to antibiotics (%)							
	Ciproflo	Rifampi	Amoxic	Imipen	Vanco	Chloram	Penicil	Gentamy
E3-B7	R (100)	S (100)	S (100)	S (100)	S (100)	S (100)	S (100)	S (100)
AB3-5	R (100)	S (77)	S (100)	S (100)	S (100)	S (100)	S (100)	S (100)
AB2-3	R (100)	S (100)	S (100)	S (100)	S (100)	S (100)	S (100)	S (100)
E8-B2	R (100)	S (100)	S (100)	S (100)	S (100)	S (100)	S (100)	S (100)
E7-B4	R (100)	R (100)	S (100)	S (100)	S (100)	S (100)	S (100)	S (100)
AB4-6	R (100)	S (77)	S (100)	S (100)	S (100)	S (100)	S (100)	S (100)

AB2-3 : *Bacillus toyonensis* ; AB3-5 : *B. pumilus* ; E7-B4 : *B. subtilis* ; AB4-6 : *B. methylotrophicus* ; E3-B7 : *B. vallismortis* ; E8-B2 : *B. amyloliquefaciens* ; R : resistant ; S : sensitive ; Ciproflox : Ciprofaxin ; Rifampi : Rifampicin ; Amoxic : Amoxicillin ; Imipen : Imipenem ; Vanco : Vancomycin ; Chloram : Chloramphenicol ; Penicil : Penicillin ; Gentamy : Gentamycin.

Ability of *Bacillus* strains to form a biofilm

The ability of *Bacillus* strains to form a biofilm is presented in Fig. 4. In general, all *Bacillus* strains studied showed a good ability to form a biofilm through the optical density values. The different optical density values being between 0.663±00 nm for the strain AB4-6 (*B. methylotrophicus*) and 3.15±02 nm for the strain E3-B7 (*B. vallismortis*) greater than 0.5, which thus shows the ability of all *Bacillus* strains tested to form a biofilm.

DISCUSSION

The observed growth of *Bacillus* strains tested at acidic pH (pH 2 and pH 3) could allow them to pass the gastrointestinal barrier governed by an acidic pH of between 2 and 3. Probiotic strains should therefore survive and grow at these pH. The ability to survive stomach acid varies greatly between genera and species.

All strains tested showed good growth at pH 2 and pH 3 during the different times studied. But at pH 2, *B. toyonensis* and *B. subtilis* showed the highest growth. Our results are in agreement with those[17] who reported good resistance of *Bacillus* strains to acidic. Furthermore[18,19] have reported the tolerance of vegetative *Bacillus* cells to acidic conditions. These results could be explained by the fact that bacterial spores are dormant multilayer cellular structures resulting from sporulation which occurs when certain bacteria are placed in an unfavorable environment. Furthermore, these results could also be explained by the production of bacterial enzymes and metabolites produced by *Bacillus* strains. Indeed, according[20], the production of metabolites including organic acids, sideerophores and enzymes such as ACC

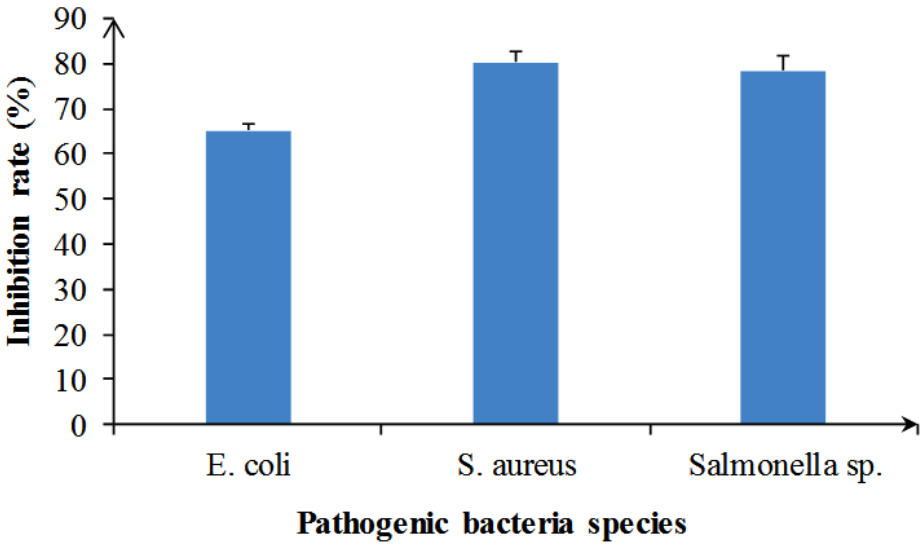

Fig. 2. Rate of pathogens inhibited

Table 4. Inhibition rate (%) of different *Bacillus* species of traditional cassava ferments

Pathogenic bacteria	Inhibition rate (%) of *Bacillus* species					
	AB2-3	AB3-5	E7B4	AB4-6	E3B7	E8B2
E. coli	55,09±0,1^{a}	61,66±0,1^{b}	50,15±0,1^{a}	61,15±0,1^{b}	77,15±0,4^{c}	85,35±0,1^{d}
S. aureus	83,36±0,2^{bc}	69,54±0,1^{ab}	76,9±0,3^{b}	65,71±0,4^{a}	78,8±0,1^{b}	96,7±0,2^{c}
Salmonella sp.	89,56±0,2^{d}	69,15±0,1^{a}	75,53±0,2^{b}	82,45±0,3^{c}	83,08±0,2^{c}	81,83±0,1^{c}

Average values of 3 repetitions; In the same line, values followed by the same alphabetical letter are not statistically different (P >0.05) (Tukey, HSD). AB2-3 : *Bacillus toyonensis* ; AB3-5 : *B. pumilus* ; E7B4 : *B. subtilis* ; AB4-6 : *B. methylotrophicus* ; E3B7 : *B. vallismortis* ; E8B2 : *B. Amyloliquefaciens*

Table 5. Growth of *Bacillus* strains at different temperatures (37°c and 44°c) for 4 hours and 24 hours

Incubation temperatures	Incubation times	Loads of Bacillus strains (UFC/mL)					
		E8-B2	AB2-3	E3-B7	AB4-6	E7-B4	AB3-5
37°C44°C	4 hour	$(2,27\pm0,10) \times 10^{8a}$	$(2,12\pm0,35) \times 10^{8a}$	$(1,85\pm0,45) \times 10^{8b}$	$(1,71\pm0,15) \times 10^{8b}$	$(1,77\pm0,15) \times 10^{8b}$	$(1,92\pm0,75) \times 10^{8b}$
	24 hours	$(3,30\pm0,15) \times 10^{8b}$	$(3,99\pm0,16) \times 10^{8ab}$	$(4,51\pm0,20) \times 10^{8a}$	$(4,43\pm0,65) \times 10^{8a}$	$(3,37\pm0,23) \times 10^{8b}$	$(3,34\pm0,85) \times 10^{8b}$
	4 hours	$(1,34\pm0,90) \times 10^{8b}$	$(1,54\pm0,15) \times 10^{8b}$	$(1,29\pm0,35) \times 10^{8b}$	$(1,29\pm0,25) \times 10^{8b}$	$(9,88\pm0,30) \times 10^{7a}$	$(1,08\pm0,42) \times 10^{8b}$
	24 hours	$(1,77\pm0,10) \times 10^{8ab}$	$(2,00\pm0,45) \times 10^{8a}$	$(2,23\pm0,20) \times 10^{8a}$	$(1,61\pm0,50) \times 10^{8cab}$	$(1,42\pm0,25) \times 10^{8b}$	$(1,54\pm0,55) \times 10^{8b}$

AB2-3 : *Bacillus toyonensis* ; AB3-5 : *B. pumilus* ; E7B4 : *B. subtilis* ; AB4-6 : *B. methylotrophicus* ; E3B7 : *B. vallismortis* ; E8B2 : *B. Amyloliquefaciens* ; Average values of 3 repetitions ; In the same line, values followed by the same alphabetical letter are not statistically different (P >0.05) (Tukey, HSD).

deaminase by *Bacillus* allows them to tolerate acidic and stressful conditions.

The tolerance of bacteria to bile salt concentrations is a criterion for which condition their survival in the conditions of the Gastro-Intestinal tract and to colonize the intestinal environment. All *Bacillus* strains studied were resistant to bile salt concentrations. Our results corroborate those of[21,22] who reported the survival of *Bacillus* strains at bile concentrations and to adapt to them in a stable manner.

This study also showed that most of the *Bacillus* strains tested are multi-resistant to the antibiotic Ciprofloxacin and the same as *B. subtilis* which is multi-resistant to Rifampicin. These results are consistent with those of the work of[23,24] which reported the resistance of *Bacillus* strains to the antibiotics ciprofloxacin and rifampicin. These results could be due to certain proteins, notably bacitracin, synthesized by *Bacillus* strains. Indeed[21], reported that *Bacillus* strains produce the substance bacitracin for their natural resistance to antibiotics. According to these same authors, this resistance of *Bacillus* strains to antibiotics occurs either through the specific transport protein which takes bacitracin out of the cell or through an undecaprenol kinase. The resistance of *Bacillus* strains to antibiotics could also be explained by intrinsic or acquired factors. Indeed, according to[25,26], the resistance of

Table 6. Production of hemolytic enzymes by *Bacillus* strains

Activity	*Bacillus* strain	
	Positive	Negative
Hemolytic	0	6

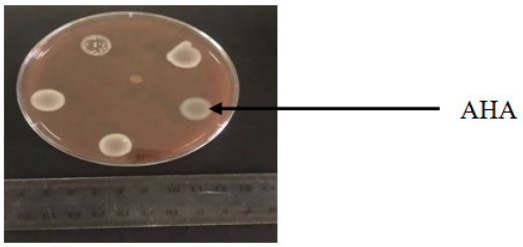

Fig. 3. Photograph reflecting the Absence of Hemolytic Activity
AHA: No Lysis Zone around colonies

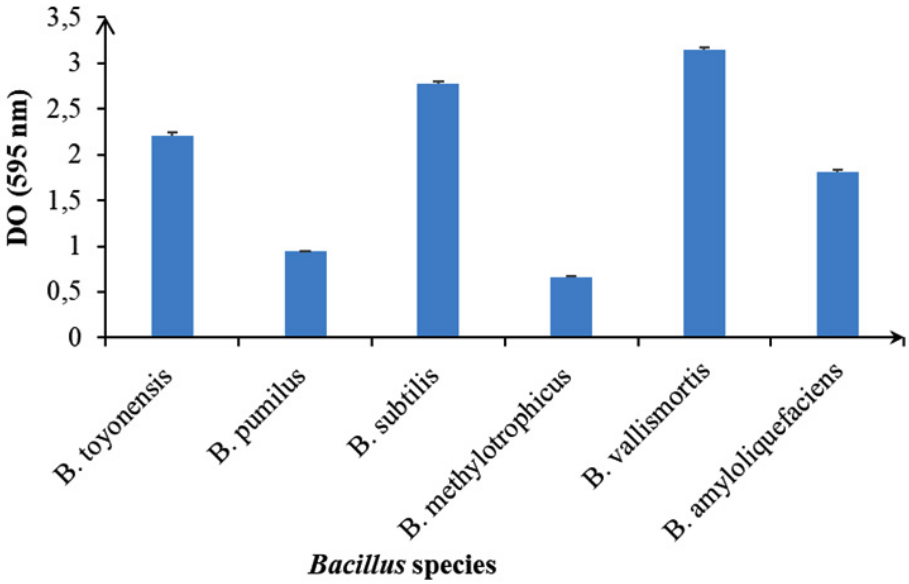

Fig. 4. Ability of *Bacillus* species to form biofilm

Bacillus strains to antibiotics occurs either by the transfer of a gene from a plasmid or by the mutation of a bacterial gene. Furthermore, the majority of *Bacillus* strains studied were sensitive to most of the antibiotics studied. These results corroborate those of[27,28,29] who reported that several strains of *Bacillus* expressed no resistance to several antibiotics including Vancomycin, Gentamicin, Chloramphenicol, Imipenem and Penicillin. This sensitivity of different *Bacillus* strains to antibiotics could be due to their intrinsic gene as reported by[29].

The *Bacillus* strains tested had an inhibitory power on *E. coli, S. aureus* and *Salmonella*. Our results corroborate those of[30,31] who reported the inhibition of *E. coli, S. aureus* and *Salmonella* bacteria by several *Bacillus* species. Similar observations on the antibacterial activity of *Bacillus* species have been reported on the inhibition of *E. coli* and *S. aureus* by *B. vallismortis*[32]. These results could be due to various antibacterial agents produced by the *Bacillus* strains. Indeed, according to[33], several strains of *Bacillus* produce a wide range of antibacterial agents including peptide and lipopeptide compounds which have an inhibitory effect on Gram-positive and Gram-negative pathogenic bacteria. These results could also be explained by the competitive exclusion mechanism. Indeed, according to[34,35], when probiotics compete with pathogenic bacteria on adhesive and nutrient receptors, they can effectively destroy them with various antimicrobial agents including bacteriocins, hydrogen peroxides, organic acids, peptide and lipopeptide compounds that they secrete. Furthermore[36], indicated that *B. subtilis* produces extracellular compounds including proteases and bacteriocins involved in antagonistic activities against pathogenic bacteria. The inhibition of several pathogenic bacteria of food origin including *E. coli, S. aureus* and *Salmonella* by bacteriocins has been reported[37,38].

In this study, none of the six *Bacillus* strains studied showed hemolytic activity in the blood after 48 hours of incubation, although all were able to grow. Non-hemolytic strains are considered safe for their hosts while hemolytic strains are considered pathogenic. Our results corroborate those of[39,40] who reported the non-hemolytic activity of several *Bacillus* species including *B. amyloliquefaciens, B. pumilus, B. subtilis* and *B. amyloliquefaciens*. These results could be explained by the absence of hemolytic agents which are responsible for the secretion and release of hemoglobin as indicated by[41,42]. According to these same authors, the hemolytic activity is due to the positive regulation of the transcription of the zrt1 gene which codes for a zinc/iron permease, which suggests that iron can be acquired from binding to a host molecule

such heme which is used to transport blood gases to cause hemolysis. These results could also be explained by the fact that the expression of hemolysin, which is an inherent factor, is triggered under specific conditions[43]. Microorganisms exhibiting hemolytic activity can disrupt red blood cells resulting in the release of hemoglobin. This activity is often associated with the production of hemolysins which can have cytolytic effects on host cells and with the reduction in the hemoglobin content available as a source of Iron[44]. In *Bacillus*, hemolytic activity is known to be a virulence factor contributing to pathogenicity and therefore poses a risk to the health of the host.

All *Bacillus* strains tested during this work showed good growth at temperatures of 37°C and 44°C, knowing that 30°C is their optimal growth temperature. The results corroborate those of[45] who reported the growth of *Bacillus* strains at 37°C. Studies carried out by[46] showed that probiotic *Bacillus* spores could germinate in the intestine, particularly under the extremely high conditions of temperature, pH and bile salts favoring their development in the tract in order to exert its beneficial effects. Furthermore[47], reported the growth of *Bacillus* strains at 45°C very close to the temperature studied (44°C).

The ability of potentially probiotic *Bacillus* species to form biofilm remains an important property given, which provides an ability rapid assimilation and metabolism. In our study, all *Bacillus* species tested showed an ability to form a biofilm. The highest biofilm aggregates were seen with *B. toyonensis, B. subtilis* and *B. vallismortis* thus confirming the work of[48,49] who reported biofilm formation by several *Bacillus* species. These results would be due to the synthesis of extracellular matrices of *Bacillus* species which maintain all the constituent cells. Indeed, according to[50,51], the EPS and TasA operon genes which are the two essential components of the *B. subtilis* biofilm matrix contribute to the establishment of a stable biofilm. Furthermore, these same authors also reported that in *B. subtilis*, the extracellular matrix includes components including exopolysaccharide synthesized by the products of the epsA-O operon, amyloid-type fibers encoded by tasA and ã-poly-DL-glutamic acid which can promote the formation of submerged biofilms.

CONCLUSION

At the end of this study, we can conclude that the different strains of *Bacillus*, namely *B. amyloliquefaciens, B. subtilis, B. methylotrophicus, B. toyonensis, B. pumilus* and *B. vallismortis* are resistant to acidic pH (pH 2 and pH 3) with higher growth observed in *B. toyonensis* and *B. subtilis*. In addition, all strains showed good growth at 44°C and 37°C, knowing that their optimal growth temperature is 30°C. All these strains also showed good growth in the presence of bile salts during all hours of incubation (0 hour, 4 hours and 24 hours). All *Bacillus* strains studied demonstrated multi-resistance to Ciprofaxine and were sensitive to Amoxicillin, Imipenem, Gentamycin, Penicillin, Oxacillin and Rifampicin. The vast majority of *Bacillus* strains inhibited more than half inhibited *Salmonella* and *E. coli*. No strain of *Bacillus* studied showed hemolytic activity and these strains showed a good capacity to form a biofilm.

ACKNOWLEDGMENT

All authors would like to thank the producers of traditional cassava ferments and without forgetting the managers of the Biotechnology and Microbiology Laboratory at Nangui Abrogoua University

Funding Sources

The author(s) received no financial support for the research, authorship, and/or publication of this article.

Conflict of Interest

The authors do not have any conflict of interest

Data Availability

Data used to support the findings of this study are included in the article

Ethics Statement

This research did not involve human participants, animal subjects, or any material that requires ethical approval.

Informed Consent Statement

This study did not involve human participants, and therefore, informed consent was not required.

This work was carried out with the collaboration of all the following authors. Authors Zamblé Bi Irié Abel Boli and Yao Serge Junior

N'goran carried out the literature searches, drafted the experimental protocol and participated in writing the final manuscript. Author Abodjo Celah Kakou designed and validated the study. Author Kouassi Roselin Cyrille Goly processed the formal analysis and software. Authors Rose Koffi-Nevry and Marina Koussemon supervised the study and corrected the final manuscript. All authors read and approved the final manuscript.

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