Potential Spore-Forming Probiotics Isolated from Ntoba mbodi, Alkaline Fermented Leaves of Cassava from the Republic of the Congo

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Abstract: This work aimed to characterize strains of Bacillus with fermenting properties for the production of α-amylases, proteases and pectinases and to evaluate their probiotic abilities. The detection of the fermentative enzymes of the different strains was carried out on solid media containing a specific substrate. Stress resistance of gastrointestinal transit was evaluated in vitro and the diffusion method in agar medium and antibiotic susceptibility of targeted strains was used to assess antibacterial activity. As results, evaluation of the fermentative potential revealed good proteolytic (100%), pectinolytic (68.42%) and amyloytic (57.90%) activities. The evaluation of the probiotic potential also revealed promising results: resistance to gastric acid from two strains with a survival rate above 70% at pH 2.5; a growth in the presence of 0.3 % biliary salt and an inhibitory capacity against Staphylococcus aureus, Serratia odorifera and Salmonella spp. Intrinsic resistance of the strains was observed with respect to cefoperazone and amoxicillin.

Keywords: Potential fermenting, Bacillus, Probiotics, Ntoba mbodi

1. Introduction

Ntoba mbodi is an alkaline-fermented food derived from the spontaneous fermentation of the leaves of cassava (Manihot esculenta Crantz). The biotransformation of cassava leaves is characterized mainly by the alkalization of the fermentation medium, the softening and browning of the leaves and the detoxification of the fermented product [21]. Bacteria of the genus Bacillus are the main microorganisms associated with such fermentation and can develop spore-forming bacteria to resist the stresses generated by nutritional deficiency and unfavorable physicochemical conditions. The production processes of ntoba mbodi are of the artisanal type making with non-sterile material leading to a great variability in the quality of the final product.

Moreover, since the discovery of antibiotics, by the time of drafting this paper anti-biotherapy and anti-bioprevention are the only way to face human and animal health [4]. Bacterial world has been developed mechanisms of multi resistance to antibiotics [16]. This situation led to search for new strategies to find new products for innovative alternatives. In the majority of cases, studies are focused on probiotics basing on living microorganisms administered in an adequate amount. Probiotics can confer a beneficial effect on the host. Since probiotics bacteria associated in food have been also used [11]. They are carried in the majority of cases by fermented products such as yogurts, fermented milks and cheeses [9]. However, most known probiotics do not survive the technological treatments applied in the product manufacture of the various foods and the storage conditions. In addition, some probiotic bacteria do not reach the intestine due to stress problems generated during the transit inside gastro-digestive tract and competition with pathogenic and indigenous germs [6, 12].

Several studies have been proposed to study spore-forming bacteria by using them as probiotics in order to better resist the environmental stresses and the gastrointestinal transit [26]. Ntoba mbodi constitutes a potential source for the isolation of probiotics spore-forming bacteria.

The aim of this work is to select strains of Bacillus with interesting fermentation and probiotic properties, with a view to the production of ferment for controlled fermentation during the production of ntoba mbodi.

2. Material and Methods

2.1 Strains

Nineteen (19) spore-forming bacteria belonging to eight (8) species of Bacillus were selected for this study. Strains were isolated from ntoba mbodi and then identified by phenotyping and sequencing of the 16S rDNA, gyr A, gyr B and rpo B genes, as reported by [37], (submitted paper). Strains and the corresponding codes were assigned in Table I.

<table>
<thead>
<tr>
<th>Code</th>
<th>Identification</th>
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<tbody>
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<td>B. pumilus</td>
<td>NM 52</td>
<td>B. altitudinis</td>
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<td>NM 29</td>
<td>B. pumilus</td>
<td>NM 56</td>
<td>B. megaterium</td>
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</tr>
<tr>
<td>NM 1</td>
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<td>NM 76</td>
<td>B. amylopecticaciens</td>
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<td>B. licheniformis</td>
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<tr>
<td>NM 16</td>
<td>B. altitudinis</td>
<td>NM 73</td>
<td>Lysinibacillus sp</td>
</tr>
<tr>
<td>NM 24</td>
<td>B. altitudinis</td>
<td>NM 21</td>
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2.2 Evaluation of fermentative potential spore-forming bacteria

The ability to produce three enzymes (α-amylase, protease and pectinase) involved in the biotransformation of cassava leaves has been investigated in different bacteria.

Proteolytic activity

The proteolytic activity of the bacteria has been determined by depositing Wattman paper disks on nutrient agar supplemented with 5 % skim milk. 10 μL was added to each sterile disk and then associated with an overnight culture performed with appropriated strains. After incubation at 37 °C for 24 h under aerobic conditions, the proteolysis is revealed by clear halos around the disks as described by [38].

Pectinolytic activity

To perform pectinolytic activity strains was carried out according to the procedure described by [5, 19]. Briefly, strains have been seeded in a test tube containing nutrient agar, 0.6 % calcium chloride and 1 mL of 1 % pectin. Activity was assessed after 96 h of incubation at 37 °C under aerobic conditions. Pectinolytic activity is viewing by the liquefaction of the agar.

Amylolytic activity

The amylolytic activity was demonstrated on nutrient agar with 1 % of soluble starch. Sterile Wattman paper disks were deposited on the surface of agar medium. 10 μL of an overnight culture was added to each sterile disk and deposit on nutrient agar supplemented with starch. After incubation at 37 °C for 24 h under aerobic conditions, the hydrolysis of the starch is revealed by a translucent zone around the disks [14].

2.3 Evaluation of potential spore-forming probiotics

Four (4) screening criteria for probiotics were evaluated. These include resistance to acid pH, tolerance to biliary salt, antibacterial activity and antibiotic resistances.

Evaluation of acid pH resistance

This test was carried out according to the method described by [17] in a medium simulating gastric barrier. Pure cultures of strains to be tested were cultured aerobically for 6 h in three test tubes containing 10 mL of the nutrient broth (pH 7.2). The bacterial cells were then recovered after centrifugation (13000 rpm / 4 min). The pellet was washed twice with sterile saline buffer phosphate (PBS), and then suspended again in three test tubes containing 10 mL of the nutrient broth adjusted respectively to pH 2.0, pH 2.5 and pH 4.0 with 1N HCl solution. At time 0 and after 4 h of incubation at 37 °C under aerobic conditions, 1 mL of the different bacterial suspensions was taken and serially diluted in peptone water for enumeration on nutrient agar.

The survival percent of the different strains was calculated by the following equation:
Survival rate (%) = \[\frac{\log \text{UFC at } t_{4 \text{h}}}{\log \text{UFC at } t_{0 \text{h}}}\] x 100

Three independent experiments were performed and each test was performed three times.

Assessment of Biliary salt tolerance

Strain growth in the presence of biliary salt was evaluated according to the method described by [36]. Briefly each strain (0.2 mL of concentrated culture) was inoculated into three test tubes containing 10 mL of the nutrient broth containing 0.3 %, 1 % and 2 % (w/v) bovine bile (Oxgall, Sigma). A time zero and after 4 h of incubation at 37 °C under aerobic conditions, 1 mL of the various suspensions was taken and diluted with peptone water for counting on nutrient agar.

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Antibiotics sensitivity

The diffusion method in agar medium was applied as standardized by the Laboratory and Clinical Standards Institution [7]. Each strain was inoculated onto trypticase soya agar. After an overnight incubation at 37 °C, 4 to 5 well-isolated pure colonies were transferred into 5 mL of trypticase soy broth. After baking at 37 °C for 3 h, this bacterial suspension was then adjusted to turbidity close to 0.5 of McFarland range, which corresponds to an inoculum of about 1 to 2. 10^8 CFU/ml. Inoculum was then uniformly spread on the surface of the Mueller-Hinton agar. Petri dishes were dried for 10 min at room temperature. Finally, the antibiotic discs were deposited on the agar. Four (4) antibiotic discs chloramphenicol (30 μg); Erythromycin (15 μg); Amoxicillin (25 μg) and cefoperazone (30 μg) were plated incubated at 35 ± 1 °C under aerobic conditions for 18 h, the diameter of each zone of growth inhibition around each disc was measured. The results were interpreted according to the recommendations of the “Committee of the French Society of Antibiogram (CASFA, 2015). The results are expressed as (S) sensitive, (I) intermediate and (R) resistant.

In vitro antagonistic capability

Four (4) pathogenic strains provided by the medical-surgical clinic COGEMO (Republic of Congo) were used. These include S. aureus, Serratia odorifera, Salmonella sp, and Escherichia coli. All strains were isolated from patients.

The solid-state diffusion method described by [33] has been used to demonstrate the inhibitory effect of selected strains. 100 μL with D.O 0.8 of the pathogenic strain were uniformly spread on the surface of the Mueller-Hinton agar. After incubation for 30 min at 37 °C, sterile discs previously impregnated with 3 μL of an overnight culture of strain supposed to inhibit were deposited on the surface of the agar. Plates were dried at room temperature for 15 min. Petri dishes have been placed at 4 °C for 4 h to allow diffusion of the substances responsible for the interaction. Finally an overnight incubation was done at 37 °C. The appearance of a zone of inhibition with a diameter greater than 2 mm reveals an inhibition of the growth of the pathogen by the strain tested [10].
3. Results

3.1 Evaluation of fermentative potential spor-forming bacteria

The results of the enzymatic activities of the different strains are illustrated in figure 1. Five strains were positive for the three enzymatic activities, NM 16, NM 52, NM 35, NM 39 and NM 74 strains. Two strains presented a significant activity with 3-fold (NM 39 and NM 78). In contrast, all strains exhibited at least two enzymatic activities. The latter was most pronounced at 42.11 % of the strains (NM 39, NM 78, NM 74, NM 65, NM 52, NM 16, NM 67 and NM 33). About 68.42 % of the bacteria revealed pectolytic activity (NM 33, NM 1, NM 39 and NM 74). Only strains NM 33, NM 1, NM 39 and NM 74, corresponding respectively to Bacillus pumilus, B. safensis, B. subtilis and B. licheniformis showed a strong pectolytic activity. Finally 57.90 % of the bacteria showed amylolytic activity. The latter was more important in the strains NM 39, NM 56, NM 78, NM 76 and NM 74 (B. subtilis, B. megaterium, B. amyloliquefaciens and B. licheniformis).

3.2 Evaluation of probiotic potential

The results of the behavior of the various strains subjected to the action of acid pH (2.0, 2.5 and 4.0) are shown in figure 2 and show that almost all the strains survived after 4 hours of acid stress. In general, the resistance to acid conditions decreases with the lowering of the pH of the medium until cancellation (absence of viability) for the NM 73 strain (Lysinibacillus sp) at pH 2.0. The strains of B. subtilis (NM 35 and NM 39) and B. amyloliquefaciens (NM 76 and NM 78) showed better resistance to low pH, respectively with a survival rate of 81 %, 83 %, 73 % and 72 % at pH 2.0 and 95 %, 97 %, 83 % and 86 % at pH 2.5. All strains could be grown at pH 4.0.

The study of resistance to biliary juice revealed that all the strains tested tolerated the bile "Oxgall". Indeed, the strains were affected by the presence of 3 % bile juice, but most strains survived. Moreover, a tolerance to the exposure of the biliary juice of almost all strains was observed at concentrations 0.3 % and 1 % (Figure 3). The best tolerance profiles were shown by B. safensis (NM 5), B. subtilis (NM 39), B. amyloliquefaciens (NM 75) and B. megaterium (NM 56).

Table II presents the class of each strain deduced from the diameter of the zone of inhibition of their growth. The results obtained show a high resistance of the strains to the various antibiotics tested. Indeed, all strains were resistant to amoxicillin and cefoperazone, with the exception of strain NM 21, which is sensitive to cefoperazone. Ten (10) strains resisted the inhibitory effect of chloramphenicol. In contrast, all strains were susceptible to erythromycin. No strain was either sensitive or resistant to all four antibiotics. The breakdown of the percentage categories gives 57.50 % resistance, 34.17 % sensitive and 8.33 % intermediate category.

Table II: Antibiotic sensitivities of different strains.

<table>
<thead>
<tr>
<th>Bacterial strains</th>
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<th>I</th>
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<tbody>
<tr>
<td>Antibiotics</td>
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<tr>
<td>Chloramphenicol</td>
<td>R</td>
<td>R</td>
<td>S</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>R</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Cefoperazone</td>
<td>S</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>S</td>
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</table>

The results showed a variation in the inhibitory effect depending on Bacillus strain and of the pathogenic bacterium (Table III). Five (5) strains have an inhibitory effect against all pathogens used. They belong to the species B. pumilus (NM 21 and NM 33), B. altitudinis (NM 16 and NM 24) and B. megaterium (NM 56). On the other hand, most strains (13) were able to inhibit the growth of S. aureus, Serratia odorifera and Salmonella sp. The growth of E. coli was inhibited by eight strains (NM 21, NM 33, NM 5, NM 16, NM 24, NM 52, NM 56 and NM 73), but the areas of inhibition were generally smaller (diameter ≤ 12 mm) than those obtained with Three other pathogens. Fifteen (15) strains showed inhibitory activity against S. aureus with zones of inhibition of diameter between 9 and 16 mm. The proportion of antimalonalinic strains was highest (89.47 % of the strains). Finally, the antibacterial activity of the strains was more pronounced with Serratia odorifera, strains11 produced zones of inhibition with a diameter ≥ 16 mm.

Table III: Diameter of inhibition zones of growing

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>E. coli</th>
<th>S. aureus</th>
<th>Salmonella sp.</th>
<th>Serratia odorifera</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pathogens</td>
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<td>NM 29</td>
<td>NM 33</td>
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<td>NM 67</td>
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4. Discussion

The different strains of Bacillus showed intrinsic enzymatic properties during ntoba mbodi fermentation. Indeed, the proteolytic activity manifested by all the strains indicates that most strains are able to ensure the degradation of the peptides and proteins of the cassava leaves resulting in the release of the amino acids and deamination to produce an alkalization fermentation food [20, 27].

This alkalization of the fermentation medium acts as a selective factor for alkaline-tolerant bacteria such as Bacillus spp [28]. The demonstration of the pectinolytic activity of strains suggests that spore-forming strains are also capable of degrading the skeletal structures of the cassava leaves cell wall and as consequence contributing to the softening of food. Previous works of [22] on Bacillus spp isolated from ntoba mbodi also reveal both pectinolytic and proteolytic activities (64 %), and only pectinolytic activity (36 %). Finally, the amylolytic strains would be able to hydrolyse the endogenous starch of cassava leaves into fermentable sugars. The small proportion of α-amylase producing bacteria could be explained by catabolic repression, since in the presence of glucose the biosynthesis of amylases is generally suppressed in Bacillus spp. [23]. This work opens a way to constitute a biological ferment consisting of a consortium of spore-forming bacteria even with different activities.

In general, it was established that although there has been a loss of growth of all the bacteria in pH 2.0 and 2.5, the number of viable cells is still important. The average survival rate of the most resistant strains (B. subtilis and B. amyloliquefaciens) at pH 2.0 is 77.25 %. A survival rate above 92 % at this pH value for 3 hours was reported by [38] basing on strains B. licheniformis, B. subtilis and B. amyloliquefaciens isolated from a traditional fermented food of Mongolia. According to the study described by [30], the ideal pH value for the isolation of strains very resisting to acid pH is 2.5. In this study, 63.15 % of strains, B. megaterium (NM 56 and NM 65), B. amyloliquefaciens (NM 76 and NM 78), B. safensis (NM 3 and NM 5), B. altitudinis (NM 24 and NM 52), B. pumilus (NM 21 and NM 29) and B. subtilis (NM 35 and NM 39) showed a survival rate greater than 70 % after 4 hours of acid stress. This result indicates that these bacteria would be able to resist in vivo conditions of gastric acidity. Survival values greater than 83 % were recorded for B. subtilis and B. amyloliquefaciens strains. This result differs from study described [17] stating that only Bacillus laevoatacticus (DSM6475) could survive at pH 2.5 for 3 hours with the rate of 86 %.

The biliary concentrations used to assess the ability of bacteria to survive biliary stress vary from 0.01 % to 7.5 % [34]. According to [12, 13], 0.3 % would be the appropriate concentration for selecting probiotics. In our study most strains showed an excellent growth at this concentration. This could be supposing that they would also be able to tolerate biliary stress in vivo. At the molecular level chloramphenicol and erythromycin act on the target bacteria by inhibiting protein synthesis, whereas the inhibition of peptidoglycan synthesis constitutes the mode of action of amoxicillin and cephalexin [40]. The observation of bacterial resistance to antibiotics can be explained by various mechanisms: production of bacterial enzymes capable of inactivating them, impermeability of bacterial cells or changes in structure [18].

Very little information are available on antimicrobial susceptibility patterns of Bacillus spp. let alone the patterns of antimicrobial susceptibility of Bacillus spp isolated from African fermented foods [2]. The (intermediate) response of B. licheniformis to chloramphenicol correlates with the results of [8], who found low sensitivity to chloramphenicol in B. licheniformis isolated from the bikalga representing the fermented seeds of Hibiscus sabdariffa. This result also corroborates the report by [32], which reports the chloramphenicol resistance of probiotic B. licheniformis strain 31 from Eastern Europe. The sensitive responses of B. subtilis and B. amyloliquefaciens to chloramphenicol and erythromycin are also consistent with the results of [2], which suggest the absence of resistance genes for these two antibiotics in strains of B. subtilis and B. amyloliquefaciens isolated from bikalga. On the other hand, resistance of Bacillus subtilis to chloramphenicol has been reported by [31]. Similarly, resistance of Bacillus licheniformis to erythromycin has been well understood [3]. Almost all strains are resistant to amoxicillin and cefoperazone. All strains belonging to the same species are resistant to the aforementioned two antibiotics. This interesting result supposes that it could be intrinsic resistance. Contrary to the acquired resistance, the latter represents only a negligible risk of propagation [35].

The observation of an inhibitory effect on the growth of pathogenic bacteria indicates that these strains are likely to play an essential role in the biopreservation of ntoba mbodi. This inhibitory action could be due to the production of antibacterial substances. Indeed, during the logarithmic and / or stationary growth phase, Bacillus spp. are able to produce antimicrobial compounds, such as subtilisin, bacitracin, amicoumacin, gramicidin, coagulin, subtilin, Iturin, polymyxin, colistin and surfactin [1, 25]. These bacteriocins act by forming pores in the cytoplasmic membrane of the target cells, resulting in release of the intracellular content and death of the bacterium [39]. All the strains have the same spectrum of action with respect to the pathogens tested. However, some intra spectral variations have been observed: B. pumilus (NM 21) and B. pumilus (NM 33) inhibit the growth of E. coli strains, whereas the strains NM 29, NM 34 and NM 67 of the same species do not exert any antagonism towards this pathogen. The antagonistic effect against S. aureus of B. licheniformis, B. subtilis and B. amyloliquefaciens strains was reported [38]. However, contrary to our results, the strains belonging to the same species have an inhibitory effect on the growth of E. coli. The inhibition of growth of S. aureus, E. coli and Salmonella typhimurium by B. pumilus strain isolated from soumbala (fermented seeds of Parkia biglobosa) has also been reported [29]. In contrast, B. subtilis strains isolated from the same food inhibit the growth of these three pathogens. Similarly, [15] have shown that B. subtilis strains isolated from alkaline-fermented feeds produce metabolites that inhibit the growth of S. aureus, E. coli, and S. typhimurium. These different pieces of information prove...
that the antibacterial activity is specific to each strain and cannot be extrapolated from one strain to another, even within the same species.

5. Conclusion

The spore-forming strains studied have in vitro interesting fermentation and probiotic profiles such as the α-amylases, proteases and pectinases production, antibacterial activity and resistance to stress in the gastrointestinal transit. The best profiles are showing by NM 39, NM 76, NM 78, NM 1, NM 5, NM 16, NM 33 and NM 56 corresponding to Bacillus subtilis, B. amyloliquefaciens, B. safensis, B. altitudinis, B. pumilus and B. megaterium. Basing on results, the eight strains of genus Bacillus strains studied possess good fermentation potential could be used as a leaven and can also be considered separately as potential probiotic candidates. The results of our study are an essential first step in the development of a probiotic ferment to control production and to valorize ntobambodi as probiotic food.

In order to better elucidate the beneficial effect observed in these strains, other studies deserve to be developed, such as, evaluation of their capacity for adhesion to the epithelial cells of the intestine, evaluation of linamarasic activity and evaluation of the impact of physicochemical factors on the production of enzymes and other interesting metabolites.

6. Acknowledgements

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References


Figure 1: Enzymatic activities of bacteria

Figure 2: Survival rate of strains depending on acidic pH

Figure 3: Survival rate of strains depending biliary concentration