Molecular identification of *Bacillus* species during spontaneous fermentation of Lima bean flour (*Phaseolus lunatus*)

ABSTRACT

Aims: *Bacillus* species is used as starter culture to improve quality of the fermented product. Thus, the purpose of this study is to identify *Bacillus* species during the spontaneous fermentation of *Phaseolus lunatus* with prospective selection as starter cultures.
Study design: Spontaneous fermentation of *Phaseolus lunatus* flour was allowed to proceed at ambient temperature (29±2°C) for three days under anaerobic condition.
Methodology: The *Bacillus* counts were determined and 100 isolates were identified by PCR and the sequencing of 16S rDNA domain.
Results: In unfermented sample the *Bacillus* count was 3.14 log CFU/mL. During fermentation the count being between 2.68 and 2.88 log CFU/mL. Based on PCR and the sequencing of 16S rDNA domain, *Bacillus* isolates were assigned to four species *Bacillus sp, Bacillus subtilis*, *Brevibacillus agri* and *Bacillus xiamensisis. Bacillus spp, Bacillus subtillis and Bacillus cereus* were detected at all the fermentation times. Their frequencies were between 14.29 and 45.83%, 25 and 35.71%, 25 and 50% respectively.
Conclusion: Among these species *Bacillus subtillis* could be used as starter culture to improve quality of the fermented product.

Keywords: Bacillus, PCR, fermentation, Phaseolus lunatus, starter

1. INTRODUCTION

The consumption of legumes poses a problem of digestion and bioavailability of certain nutrients due to the high content of anti-nutritional factors [1]. Fermentation is recognized as a natural way to preserve and safeguard foods and beverages, enhancing the nutritional value, improving the digestibility, destroying undesirable components, and inhibiting undesirable microorganisms [2]. Several experiments have demonstrated that fermentation of legumes enhances their nutritive value and antioxidant properties; reduces some anti-nutritional endogenous compounds such as phytic acid, and exerts beneficial effects on protein digestibility and biological value of legumes [3,4]. Spontaneous fermentation is the oldest form of fermentation and it is the form of fermentation in most small scale fermentations in the developing countries.
However, in artisanal fermented products, biological risks such as pathogenic microorganisms, as well as chemical contaminants and toxic molecules of microbial origin, including mycotoxins, biogenic amines, and cyanogenic glycosides can be found [5]. Thus, inoculated fermentation is now commonly used in the food industry because of the increased control it affords. According to [6], starter cultures have been found to reduce fermentation time as well as guarantee product quality of fermented product.
Most authors now agree that there is a predominant development of *Bacillus* species during the various legume fermentation processes. Technologically relevant *Bacillus* spp., mainly

Bacillus subtilis, are the predominant fermentative bacteria responsible for the natural fermentation of condiments across West Africa [7]. Other species of Bacillus including *B. amyloliquefaciens*, *B. licheniformis*, *B. pumilus*, *B. megaterium*, *B. sphaericus*, *B. cereus*, *B. badius* and *B. fusiformis* are also frequently involved in the fermentation process. These bacterial species are responsible for flavor development, bio-conversion of complex food molecules, and production of antimicrobial compounds [8]. *Bacillus subtilis* is used as the potential starter cultures in soybean dawadawa fermentation and finally obtained a good organoleptic quality by sensory evaluation [9]. *Bacillus licheniformis* generally shows good ability of producing the most abundant metabolites that shaping the aroma of fermented food [10]. In Asian countries, several traditional foods are produced from fermented soybeans with *Bacillus subtillis* used as a starter crop. Studies have shown that these foods have a health benefit such as antihypertensive, anti-diabetic, antioxidant and anti-cancer properties [11, 12]. In addition, bioactive peptides have been produced by the use of *B. subtillis* in soybean fermentation [13]. In addition, *Bacillus* species have been isolated and identified from fermented legumes such as soybeans for the production of Thua-nao [14]. The objective of this study is to identify *Bacillus* species isolated during fermentation of lima bean, with prospective selection as starter cultures.

2. MATERIAL AND METHODS

2.1 Materials for fermentation process

The biological material used for this study consists of the black cultivar spotted with red *Phaseolus lunatus* (L.) at stage 4 (52 days) of maturity, harvested in the villages of Assoumoukro (M'batto) and N'guessankro (Bongouanou), two villages located in Côte d'Ivoire. Bean samples were packaged into polythene bags and were transported to the laboratory for cleaning, processing and fermentation.

Fig. 1. Mature seeds of the black red-spotted cultivar of *Phaseolus lunatus* (L.)

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2.2 Natural fermentation

Bean samples were cleaned by sorting out stones, debris and living or dead insects. 1.5 kilograms of bean samples were finely ground in appropriate analytical mill and sieved through a 0.5 mm mesh screen. A suspension of bean flour was prepared by mixing 1000 ml of sterilized tap water into 300 g of unfermented flour. Fermentation was allowed to proceed at ambient temperature (29±2°C) for three days under anaerobic condition [15].

2.3 Microbiological analysis

10 gram of bean flour fermented at different fermentation times (0, 24, 48, 72 hours) were homogenized in 90 mL sterile diluent and treated at 80 °C for 10 minutes in order to kill the vegetative forms. One hundred microliters from ten-fold dilutions of the samples were plated on PCA medium supplemented with rice starch (1%) [16] and incubated at 37 °C for 18 h. The colonies exhibiting a halo were counted and further purified by successive streaking on PCA medium. After purification, isolates were examined for Gram reaction and catalase production. Gram positive and catalase positive isolates were considered presumptive *Bacillus* species. For long term maintenance of isolates, stock cultures were stored at -80 °C in 20% (v/v) glycerol and 80% (v/v) nutrient broth.

2.4 Genotypic identification of *Bacillus*

2.4.1 Extraction of DNA

DNA of 100 isolates was extracted using the Wizard® Genomic DNA Purification Kit (Promega, USA) following the manufacturer's recommendations. Briefly, each isolate was grown in 10 mL of tryptone soya broth (TSB) (OXOID CM129, Hampshire, England) for 24 h at 37 °C. A volume of 1.5 mL of growth medium was centrifuged (10,000 rpm, 5 min), the supernatant discarded and the pellet resuspended in lysis buffer. The mixture was centrifuged at 5000 g for 5 min and the supernatant was used as DNA template for the PCR reaction.

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2.4.2 PCR conditions

The specific groEL gene of each DNA obtained was amplified by using the primers Ba1F and Ba1R **(Table 1)**. The amplification was carried out in 50 µl of reaction mixture containing 25 µl of PCR Master Mix 2x (Promega, Madison, WI, USA), 1 µM each of forward and reverse primers and 15 µl nuclease free water (Promega). The cycling program was started with an initial denaturation at 94 ° C for 3 min, followed by 30 denaturation cycles at 94 ° C for 1 min, annealing at 43 ° C for 30 min and elongation at 72 ° C for 45 s. The PCR was ended with a final extension at 72 ° C for 10 min [17]. Isolates with positive PCR (533 pb fragment) were assigned to *Bacillus cereus*. Next, for negative PCR to groEL gene, 16S rDNA amplified using the primer couple 341F and 515R **(Table 1)**. The amplification program was carried out as follows: initial denaturation at 94 ° C for 1 min followed by 35 denaturation cycles at 94 ° C for 30 s, hybridization at 60 ° C for 30 s, elongation at 72 ° C for 1 min and a final elongation at 72 ° C for 5 min [18]. The DNA fragments were separated by applying 10 µL of each PCR product with 1 Al of loading buffer to 2% agarose gel containing 0.5 µg/ml ethidium bromide. DNA molecular marker (GeneRuler DNA ladder mix, Fermentas, Vilnius, Lithuania) was included as standard for the calculation of the fragments. The gel was run in 0.5x TBE buffer for 1 h at 100 V and photographed using an UV transilluminator.

Table 1. Primers used during this study

Primers	primer sequence (5' → 3')	Size expected	Kind sought	Reference
Ba1F Ba1R	TGCAACTGTATTAGCACAAGCT TACCACGAAGTTTGTTCACTACT	533 bp	*B. cereus* group (groEL gene)	**[19, 1]**
341F	CCTACGGGAGGCAGCAG		*Bacillus* and	**[18]**

515R	ATTACCGCGGCTGCTGGCA	195 bp	other (16S rDNA gene)	

2.4.3 Sequencing

All the PCR product of 16S rDNA was sequenced by Eurofins MWG Operon (Ebersberg, Germany). The obtained sequences were compared to sequences at NCBI (http://www.ncbi.nlm.nih.gov) using blastn.

2.5 Statistical analysis

The analysis of variance (ANOVA) was used to determine the differences between treatments. When a difference was observed, the multiple range test Duncan at 5% was performed to separate treatment means. Statistical tests were performed using the STATISTICA software version 7.1

3. RESULTS AND DISCUSSION

3.1 Enumeration of *Bacillus* spp

The variation in *Bacillus* spp. load during fermentation of *Phaseolus lunatus* is shown in Figure1. In unfermented sample the count was 3.14 log CFU/mL. During fermentation variation in *Bacillus* spp. load was not statistically significant (P > .05). The counts being between 2.68 and 2.88 log CFU/mL. In another work the *Bacillus* spp., counts reaching 10 log CFU/g in the final products [20, 21, 22]. Our results could be explained by the production of organic compounds by other microorganisms, thus making unfavorable environmental conditions favorale to the growth of *Bacillus*. According to [23], social interactions can affect the dynamic and function of the microbial community. Furthermore, presence of *Bacillus* spp. during the spontaneous fermentation of *Phaseolus lunatus* could be having a benefit effect. *Bacillus* spp. secrete a wide range of degradative enzymes, such as amylases and proteases [24], and can also produce antimicrobial compounds such as bacilysin, which is able to inhibit molds and bacteria; and iturin and chloromethane, which inhibit bacteria [25], thus playing an important role in the fermented product.

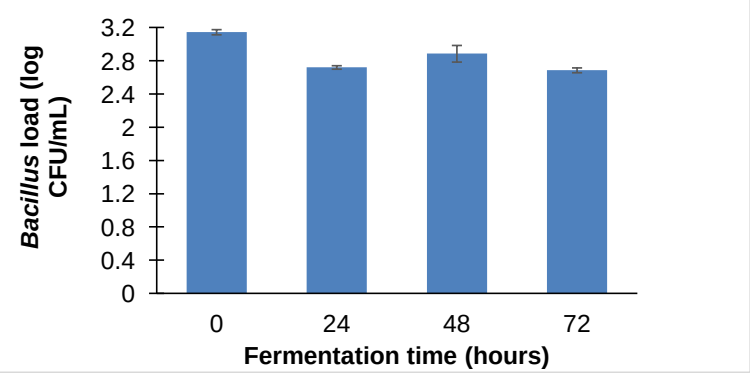

Fig. 2. *Bacillus* spp. load during fermentation of *Phaseolus lunatus*
Mean ± S.E.M = Mean values ± Standard error of means of six experiments

3.2 Identification of *Bacillus* species

A total number of 101 bacterial strains were isolated from fermented *Phaseolus lunatus* flour. All 101 isolates were rods, Gram-positive and catalase positive. These characteristics allowed the preliminary identification of *Bacillus* genus [26, 27]. The amplification of the groEL gene showed bands about 500 pb **(Figure 3)** for 33 isolates. The results allowed us to distinguish *Bacillus cereus* to other *Bacillus* species. In order to find the identity of the others 64 isolates, 16S rDNA was amplified and sequencedx. The PCR fragment was about 200 pb for all the isolates (**Figure 4**). The sequences of 16S rDNA obtained were compared with 16S rDNA sequences of NCBI database. The sequence of 33 isolates showing 100 % identity with *Bacillus* spp. Those of 26 isolate showed 99 % identity to *Bacillus subtillis*. The similarity degree of 3 and 2 isolates reached 96% compared with to *Brevibacillus agri* and *Bacillus cereus* respectively **(Table 2)**. *B. cereus* and *B. subtilis* are some of the main species identified in other African natural fermented foods [28, 20].

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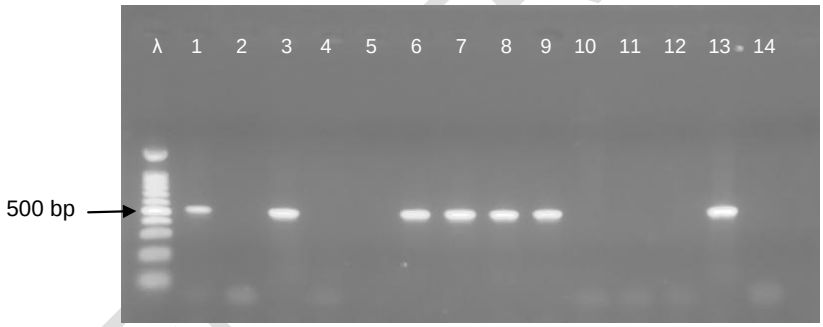
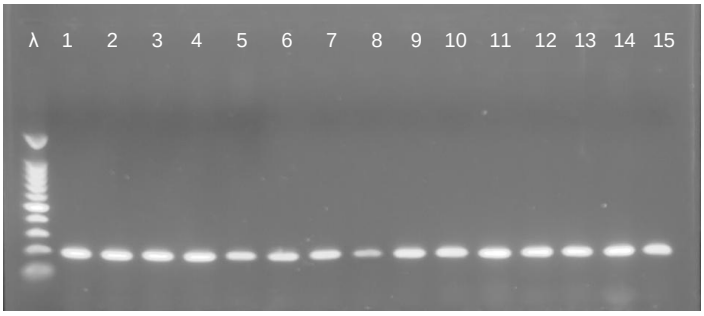


Fig. 3. Results of electrophoretic analysis of PCR products of groEL gene with primers Ba1F and Ba1R
λ –marker "100 bp DNA Ladder" ; *1,3,7,8,9,10,14- Bacillus cereus* isolates ; *2,4,5,6,11,12,13*-other species of *Bacillus*



200 bp ⟶

Fig. 4. Results of electrophoretic analysis of PCR products of 16S rDNA gene with primers 341F and 515R
λ –marker "100 bp DNA Ladder"; 1-15- positives PCR

Table 2. *Bacillus* identified after sequencing of the 16S rRNA gene

GenBank corresponding species	Number of nucleotides	Percent of identity	Number (%) of strain isolated
Bacillus spp	139	100	33 (32,67)
Bacillus subtillis ATCC 6051	140	99	26 (25,74)
Brevibacillus agri NBRC 15538	140	96	3 (2,97)
Bacillus xiamensis MCCC 1A00008	148	96	2 (1,98)

3.3 *Bacillus* diversity during fermentation

The species identified and their frequencies are shown in **Figure 5**. *Bacillus* spp, *Bacillus subtillis* and *Bacillus cereus* were detected at all the stages. Their frequencies were between 14.29 and 45.83%, 25 and 35.71%, 25 and 50% respectively. *Brevibacillus agri* and *Bacillus xiamensis* were isolated at specific times with percentages under 5% of isolates. Among the species found *Bacillus cereus* was identified as the most predominant species in unfermented bean flour and during the two first days of fermentation except for 72 h (Figure 4). [29] also reported that *B. cereus* was dominant among all isolated *Bacillus* species. The occurrence of *B. cereus* in foods at numbers of 10^3–10^5 CFU/g or mL is considered unsafe, due to its ability to cause food poisoning [30]. This species causes food spoilage and two distinct types of food poisoning: the diarrheal type and the emetic type [31]. However, several studies reported that *Bacillus subtilis*, are the predominant fermentative bacteria responsible for the natural fermentation of condiments and bean across West Africa [7, 32, 33, 34]. In this study, *Bacillus subtilis* was regularly detected during the fermentation with frequencies between 25 and 35.71 % of isolates. It could be use as starter culture to reduce fermentation time as well as guarantee product quality of fermented product. *B. subtilis* is known to produce the bacteriocins. Bacteriocin producing strains of *B. subtilis* that exhibit antibacterial activity against foodborne pathogens, including *L. monocytogenes* and *B. cereus* were isolated from maari in Burkina Faso [35, 22].

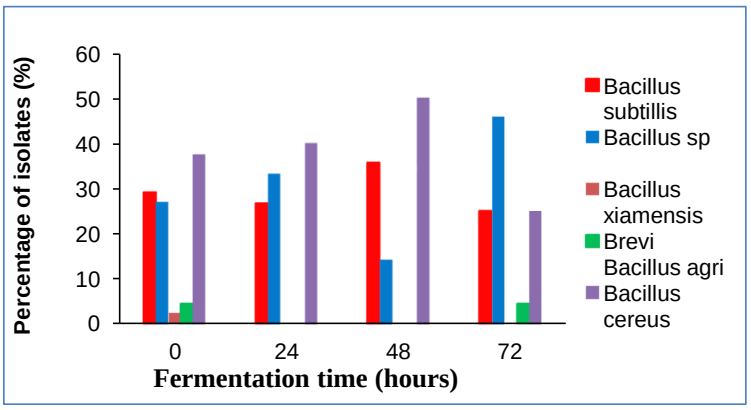


Fig. 5. Dynamic of *Bacillus* species (%) during spontaneous fermentation of *P. lunatus*

4. CONCLUSION

Bacillus spp, *Bacillus subtillis*, *Bacillus cereus, Brevibacillus agri* and *Bacillus xiamensis* were *Bacillus* species identified during fermented of *P. lunatus* bean flour. Among these species *Bacillus subtillis* could be used as starter culture to improve quality of the fermented product.

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