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Molecular identification and safety assessment of *Bacillus* strains isolated from Burkinabe traditional condiment "*soumbala*"

Yérobessor Dabiré[1,2*], Namwin Siourimè Somda[3], Marius K. Somda[1,4], Iliassou Mogmenga[4,2], Abdoulaye K. Traoré[5,6], Lewis I. Ezeogu[2], Alfred S. Traoré[1,4], Jerry O. Ugwuanyi[2] and Mamoudou H. Dicko[1]

Abstract

Purpose: Alkaline-fermented foods (AFFs) play an essential role in the diet of millions of Africans particularly in the fight against hidden hunger. Among AFFs, *soumbala* is a very popular condiment in Burkina Faso, available and affordable, rich in macronutrients (proteins, lipids, carbohydrates, essential amino acids, and fatty acids), micronutriments (minerals, B group vitamins), and fibers. *Bacillus* spp. are known to be the predominant microbial species in AFFs and thus have elicited enhanced interest as starter cultures or probiotics. However, few data exist on identification and safety attributes of relevant *Bacillus* species from African AFFs, particularly from Burkinabe *soumbala*.

Methods: This study aimed to genotypically characterize 20 *Bacillus* strains previously isolated from *soumbala*, using PCR and sequencing of the 16S rRNA genes, and to evaluate their safety attributes.

Results: Phylogenetic analysis revealed that the strains were most closely related by decreasing numbers to *B. cereus*, *B. subtilis*, *Bacillus* sp., *B. tropicus*, *B. toyonensis*, *B. nealsonii*, *B. amyloliquefaciens*, *Brevibacillus parabrevis*, and *B. altitudinis*. Among the isolates, 10 were β-hemolytic and 6 were γ-hemolytic while 4 were of indeterminate hemolysis. The 6 γ-hemolytic (presumptively non-pathogenic) strains were susceptible to all tested antibiotics except bacitracin. Strains F20, and F21 were the most sensitive to imipenem (38.04 ± 1.73 mm and 38.80 ± 1.57 mm, respectively) while strain B54 showed the weakest sensitivity to bacitracin (11.00 ± 0.63 mm) with high significant differences ($p < 0.0001$).

Conclusion: The findings highlight identification and safety quality of *Bacillus* strains which could be further characterized as probiotic-starter cultures for high-quality *soumbala* production.

Keywords: *Bacillus* spp, Genotypic identification, Safety assessment, *Soumbala*, Burkina Faso

Background

Alkaline-fermented foods (AFFs) have a long history and form a significant part of the diet of many indigenous communities in Africa with special reference to nutritional values, safety, and commercial potential (Parkouda et al. 2009; El Sheikha and Montet 2015; Ouoba 2017). They are among the most established of the numerous fermented foods of Africa and often are the most domineering components of the foods in which they are used. They are produced from the seeds and leaves of various plants. Mainly used as food condiments, they are deemed to improve texture, flavor, increase shelf-life, enhanced bioavailability of micronutrients, and reduced

*Correspondence: yerobesordabire@gmail.com; narcisseda@gmail.com
[2] Department of Microbiology, Faculty of Biological Sciences, University of Nigeria, Nsukka, Enugu State 410001, Nigeria
Full list of author information is available at the end of the article

content (or complete elimination) of anti-nutritional factors and toxic compounds among other benefits (Ouoba 2017). Some of these foods have been found to provide useful probiotic effects when they are directly consumed (Farnworth 2005; Franz et al. 2014). Consequently, global interest in them continues to rise for their benefits as health-promoting functional foods (Adebo et al. 2017). Among various AFFs, *soumbala* is a fermented food condiment manufactured from the cotyledons of the seeds of *Parkia biglobosa* ("néré") which is highly appreciated in Burkina Faso. Different variants and names of this condiment exist among many indigenous communities in Sub-Saharan Africa countries (Abdou-Souley et al. 2020; Dabiré et al. 2020; Owusu-Kwarteng et al. 2020; Kambire et al. 2021). It has a pungent ammoniac aroma but fortunately a pleasant taste and is used as a food flavoring. It is produced from spontaneous mixed fermentation of *Parkia biglobosa* seeds, in a process which involves a complex and significant microbial biodiversity responsible for its inherent desirable characteristics.

Several studies on micro-biodiversity of *soumbala* using culture-dependent and culture-independent methods have reported the predominance of *Bacillus* species (Ouoba 2017; Adjoumani et al. 2019; Dabiré et al. 2021). Presently, studies are more focused on the identification, characterization, and classification of technologically relevant microorganisms from legumes-based fermented foods (El Sheikha and Hu 2018; El Sheikha et al. 2018; Akpi et al. 2020; Compaoré et al. 2020; Owusu-Kwarteng et al. 2020). This is aimed at being able to develop appropriate starter cultures for use in pilot and industrial processes for GMP-driven production of these condiments. Studies in this area strive to deploy different accurate and reliable methods including variant PCR techniques (ITS-PCR, qPCR, repPCR, species-species PCR, RT-PCR, etc.) combined with sequencing of 16S rRNA genes (Sarkar et al. 2002; Ouoba et al. 2004; El Sheikha et al. 2018; Adjoumani et al. 2019) and whole-genome sequencing (WGS) to secure definitive characterization of candidate isolates. These tools had helped to clarify the nomenclatural confusion and generalization caused by classical (phenotypic) taxonomic methods. Thereby, molecular technologies were present as an alternative, offering advantages such as accuracy, specificity, sensitivity, and speed (El Sheikha and Hu 2018). Through these recent identification tools, *B. subtilis* have been reported as major *Bacillus* species involved in the fermentation process for *soumbala* production (Ouoba 2017; Adjoumani et al. 2019). Indeed, based on complete genomic data from over 30 different *Bacillus* species, two clearly differentiated groupings, a "*B. subtilis* clade" and a "*B. cereus* clade" were identified (Bhandari et al. 2013). Thus the genus "*Bacillus stricto-sensu*" was proposed for only the monophyletic *subtilis* clade with *B. subtilis* as its type species" (Bhandari et al. 2013). However, members of genus *Bacillus* are heterogenous, some are lacking a common evolutionary history, and have become associated with regular re-identification and re-classification with continual appearance of new species (Logan 2004). Consequently, it is clear that the taxonomical history of this genus is far from complete.

Bacillus species are very significant in various biotechnological applications and certain strains such as *B. clausii*, *B. coagulans*, *B. licheniformis* and *B. subtilis* have worldwide record of safe use with humans and animals. These strains have been classified as "generally recognized as safe" (GRAS) and have been found useful as human probiotics or in animal feed supplements. They have been established as safely present as part of human and animal gut microbiota and have been associated with stimulation of the immune system (Cutting 2011; Ilinskaya et al. 2017). Furthermore, several of such *Bacillus* species are involved in the preparation of traditional fermented dishes in Africa and Asia, foods that for millennia have been safely consumed, and identified as possible candidates for commercial production of starter cultures for food fermentation (Anal 2019; Akpi et al. 2020; Nwagu et al. 2020).

Antibiotic resistance among commercially available probiotic *Bacillus* spp. or starter cultures has been reported (Hoa et al. 2000; Adimpong et al. 2012; Lee et al. 2017). However, very limited data exist on the antimicrobial susceptibility profiles of technologically relevant *Bacillus* species from AFFs, particularly from *soumbala*. Taking into account this limited data, genotyping of microbial species and their safety evaluation are important in the microbiological risk assessment process prior to their use as probiotic-starter cultures in the food industry. Thus, the present study aimed to genotypically characterize 20 *Bacillus* spp. isolated from *soumbala* and further evaluate their safety characteristics as food condiment.

Results

16S rRNA gene sequencing

The comparison of the 16S rRNA gene sequences (Fig. 1) of the putative *Bacillus* strains obtained gave similarity rates ranging from 90.01 to 100% and 91.58 to 100%, with type strains available at NCBI and EzBiocloud, respectively (Table 1). Strains F24, F21, O34 and F44 showed similarity levels of 90.01%, 90.08%, 94.74%, and 96.65% with *B. subtilis*, *B. benzoevorans*, *B. nealsonii* and *B. subtilis,* respectively. Strains B54, F20, F32, G23 and O52 showed 99.14%, 99.14%, 99.79%, 99.56%, and 100% similarity to *B. nealsonii*, *B. tropicus*, *B. amyloliquefaciens, Brevibacillus parabrevis* and

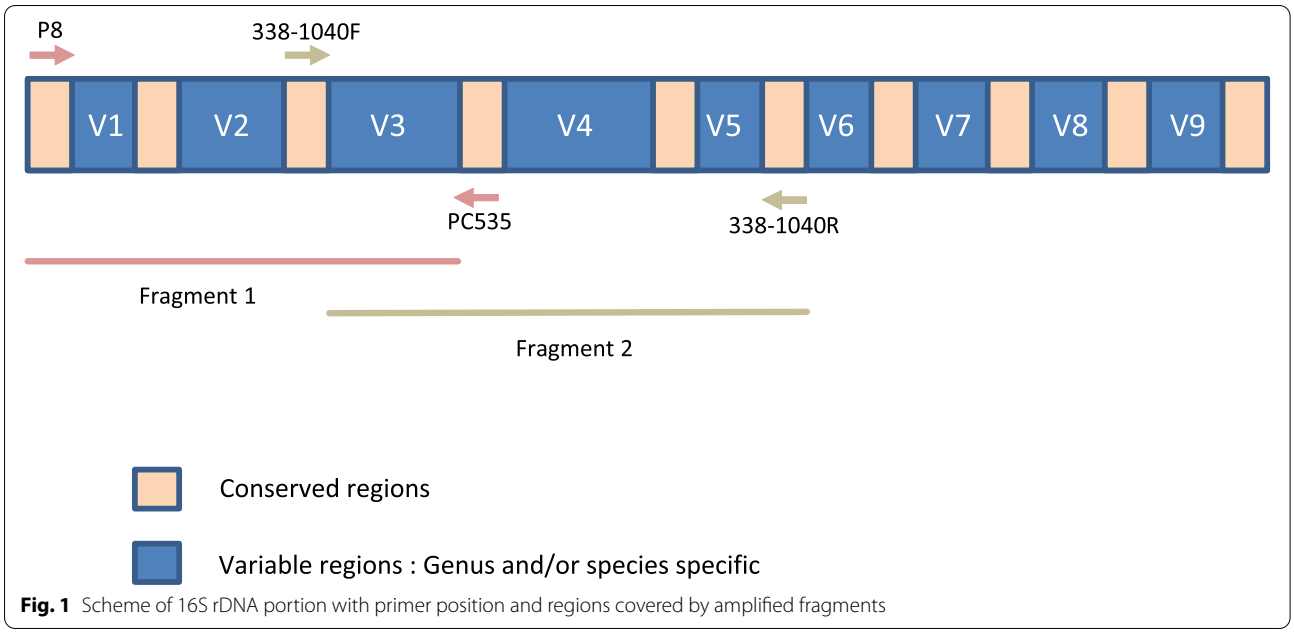

Fig. 1 Scheme of 16S rDNA portion with primer position and regions covered by amplified fragments

Table 1 Comparative taxonomic identification of *Bacillus species* by 16S rRNA sequencing

Bacillus strains	NCBI			EzBioCloud	
	Identified species	Similarity (%)	Accession number	Identified species (accession number)	Similarity (%)
B21	*B. cereus* ATCC 14579	100	NR_074540.1	*B. cereus* ATCC 14579 (AE016877)	100
B54	*B. nealsonii*	99.14	NR_044546.1	*B. dakarensis* (LT707409)	97.51
F6	*B. cereus* ATCC 14579	100	NR_074540.1	*B. cereus* ATCC 14579 (AE016877)	100
F20	*B. tropicus*	99.14	NR_157736.1	*B. cereus* ATCC14579 (AE016877)	100
F21	*B. benzoevorans*	90.08	NR_044828.1	*B. benzoevorans* (D78311)	97.99
F24	*B. subtilis*	90.01	NR_027552.1	*B. subtilis* (ABQL01000001)	91.58
F25	*B. toyonensis*	98.94	NR_121761.1	*B. toyonensis* (CP006863)	99.85
F26	*B. subtilis*	99.88	NR_027552.1	*B. cabrialesii* (MK462260)	100
F32	*B. amyloliquefaciens*	99.79	NR_117946.1	*B. siamensis* (AJVF01000043)	100
F44	*B. subtilis*	96.65	NR_027552.1	*B. tequilensis* (AYTO01000043)	97.86
F48	*B. subtilis*	99.79	NR_027552.1	*B. subtilis* (ABQL01000001)	99.90
G23	*Brevibacillus parabrevis*	99.56	NR_113589.1	*Brevibacillus parabrevis* (RHHV01000040)	100
G37	*B. subtilis*	99.90	NR_112116.2	*B. tequilensis* (AYTO01000043)	100
O28	*B. cereus*	100	NR_115526.1	*B. cereus* ATCC 14579 (AE016877)	100
O34	*B. nealsonii*	94.74	NR_044546.1	*B. nealsonii* (EU656111)	95.88
O44	*B. cereus*	100	NR_115714.1	*B. cereus* ATCC 14579 AE016877	100
O46	*B. cereus*	100	NR_115714.1	*B. cereus* ATCC 14579 (AE016877)	100
O48	*B. subtilis*	99.90	NR_027552.1	*B. tequilensis* (AYTO01000043)	99.90
O49	*B. subtilis*	99.90	NR_112116.2	*B. tequilensis* (AYTO01000043)	100
O52	*B. altitudinis*	100	NR_042337.1	*B. altitudinis* (ASJC01000029)	100

B. altitudinus, respectively. Strains F26, F48, G37, O48 and O49 showed 99.88%, 99.79%, 99.90%, 99.90%, and 99.90% similarity levels with *B. subtilis,* respectively. Strains B21, F6, O28, O44, and O46 all presented 100% similarity with *B. cereus*. According to these varying similarity levels, the presumptive *Bacillus* strains isolated from *soumbala* in this study, with similarity levels ≥ 99%, were phylogenetically very close to *B. subtilis* (F26, F48, G37, O48 and O49), *B. cereus* (B21, F6, O28, O44, and O46),

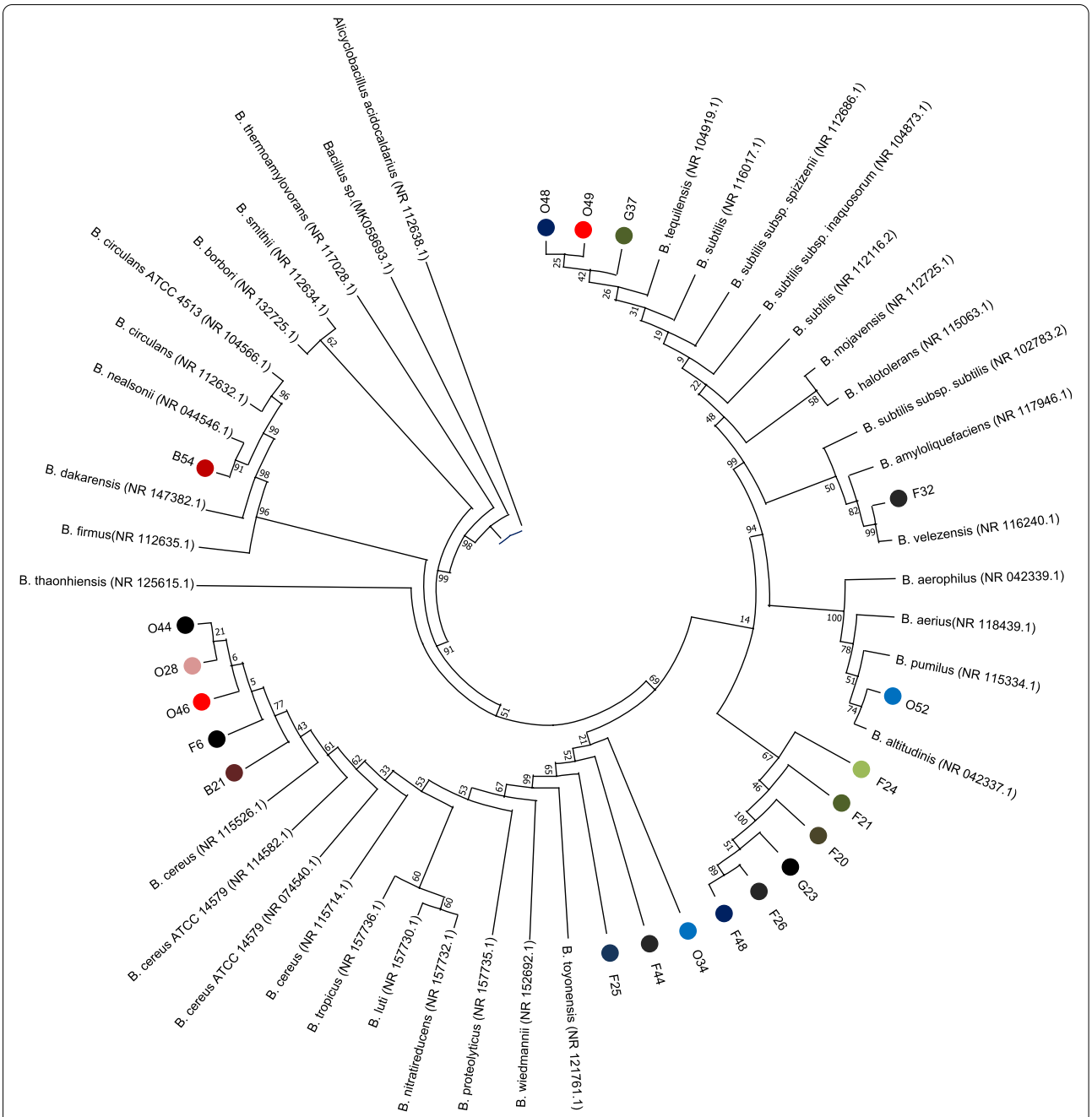

Fig. 2 Phylogenetic tree of *Bacillus* strains based on 16S rRNA constructed by neighbor joined method with *Alicyclobacillus acidocaldarius* as outgroup. Legend: *B.* = *Bacillus*. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. Numbers next to the branches are the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates). This analysis involved 55 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option)

B. nealsonii (B54), *B. tropicus* (F20), *B. toyonensis* (F25), *B. amyloliquefaciens* (F32), *Brevibacillus parabrevis* (G23) and *B. altitudinis* (O52). However, the *Bacillus* strains with a similarity level between 90 and 97% could be affiliated to the genus *Bacillus* (F21, F24, O34, and F44).

Phylogenetic tree analysis

Phylogenetic tree analysis revealed that strains B21, F6, O28, O44 and O46 are closely related to *B. cereus* (Fig. 2). Similarly, strains G37, O48 and O49, F32, O52 and B54 have a very close phylogenetic relationship to *B. subtilis*, *B. velezensis*, *B. altitudinis* and *B. nealsonii,* respectively.

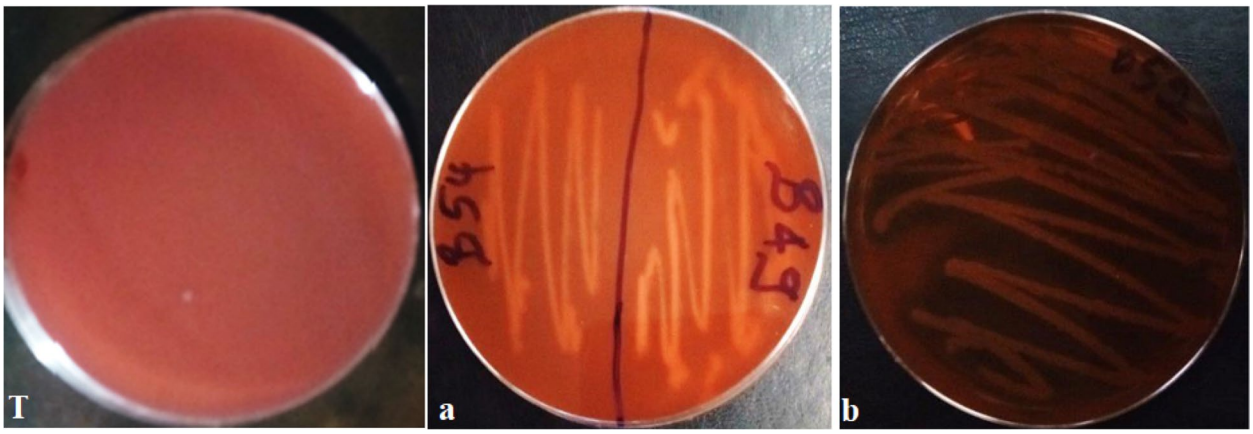

Fig. 3 Hemolytic activity of *Bacillus* strains. Legend: **T** = negative control, **a** = γ-hemolysis, **b** = β-hemolysis

Strains F25, F44 and O34 are related to *B. toyonensis.* However, strains F20, F21, F24, F26, F48 and G23 can be related to any *Bacillus* sp. member of "*B. subtilis* clade" or "*B. cereus* clade."

Safety assessment of Bacillus strains

Hemolysis activity

Out of the 20 identified *Bacillus* strains, ten (G37, F25, F32, B21, O28, O34, O44, O46, O49, and O52) were found to be β-hemolytic (formation of clear zone around the bacterial colonies), four (G23, F6, F48, 048) showed indeterminate hemolytic activity while the remaining six (F20, F21, F24, F26, F44, B54) were γ-hemolytic (absence of clear halo around the bacterial colonies, Fig. 3). These γ-hemolytic *Bacillus* strains were selected as non-pathogenic for antibiogram analysis.

Antibiotic susceptibility

The analysis of antibiogram showed that all tested *Bacillus* strains were found to be susceptible to almost all antibiotics except bacitracin for which they were all resistant (Table 2). Strains F20 and F21 showed the highest vulnerability to imipenem with 38.04 ± 1.57mm and 38.80 ± 1.57 mm sensitivity diameters, respectively, while strain B54 showed the lowest sensitivity to bacitracin (11.00 ± 0.63 mm) with high significant differences ($p < 0.0001$).

Discussion

Soumbala, like other traditional fermented food condiments, has a diverse microbiota dominated by *Bacillus* species responsible for its fermentation process, flavor development and bio-preservation and development of the characteristic organoleptic features (Ouoba 2017; Adjoumani et al. 2019). However, the presence of *B. cereus* and *B. cytotixicus* in this product could be a potential source of food poisoning and therefore a public health concern (Adjoumani et al. 2019). The complexity of micro-biodiversity of indigenous fermented condiments makes it difficult to identify and characterize the relevant functional microorganisms using single conventional methods. However, developed molecular tools have given alternative to conventional methods because genome sequence is independent of phenotypic characteristics and vary among species. Thus, PCR analysis and 16S rRNA gene sequencing have become very useful for the identification of microorganisms from various sources due to their simplicity, speed and reliability (Unban et al. 2020). In the present study, sequencing of the gene encoding 16S rRNA and comparison of the sequences of presumptive *Bacillus* spp. revealed similarity rates ranging from 90.01 to 100% with type species in NCBI and EzBioCloud (Table 1). Based on the identification criteria, BLASTn allowed the entire *Bacillus* spp. studied with a similarity rate between 90 and 97%, and ≥ 99% to be assigned to the corresponding genus and species, respectively. The phylogenic tree analysis showed that 6 strains (F20, F21, F24, F26, F48 and G23) were phylogenetically related to *Bacillus* sp., 5 strains (B21, F6, O28, 044 and O46) to *B. cereus*, 3 strains (G37, O48 and O49) to *B. subtilis*, 2 strains (F25 and F44) to *B. toyonensis* and the others to *B. nealsonni* (B54) and *B. velelensis* (F32). Previous studies on 16S rRNA genes sequencing of *Bacillus* spp. isolated from *soumbala* and other similar food condiments reported the identification of *B. subtilis* group species and *B. cereus sensu lato* group species (Ouoba et al. 2004; Adjoumani et al. 2019). It has been noted that *B. subtilis*, *B. pumilus, B. clausii*, *B. licheniformis*, *B. vallismortis*, *B. mojavensis*, *B. lentus*, *B. coagulans*, *B. fusiformis*, *B. atrophaeus*, and *B. amyloliquefaciens*, "can be

Table 2 Antibiotic susceptibility profile of *Bacillus* strains

Antibiotic (AB, charge in µg) used	*Bacillus* strains											
	F20		F21		F24		F26		F44		B54	
	Diameter (mm)	S/R	Diameter (mm)	S/R	Diameter (mm)	S/R	Diameter (mm))	S/R	Diameter (mm)	S/R	Diameter (mm)	S/R
Amikacin (ANK, 30)	24.05 ± 0.16^{bc}	S	25.94 ± 0.3^{b}	S	32.28 ± 0.14^{a}	S	21.62 ± 1.05^{bc}	S	23.95 ± 1.40^{bc}	S	25.32 ± 3.07^{b}	S
Amoxycillin-clavulanic acid (AMC, 30)	30.85 ± 3.1^{ab}	S	27.91 ± 0.47^{abc}	S	34.53 ± 0.67^{a}	S	19.16 ± 0.18^{cd}	S	34.62 ± 0.45^{a}	S	23.57 ± 4.13^{bcd}	S
Ampicillin (AMP, 10)	22.41 ± 0.1^{ab}	S	25.18 ± 1.67^{ab}	S	24.95 ± 1.69^{ab}	S	18.66 ± 0.36^{bc}	S	26.80 ± 2.14^{a}	S	26.62 ± 2.79^{a}	S
Bacitracin (BA, 10)	0.000 ± 0^{b}	R	0.000 ± 0^{b}	R	0.000 ± 0^{b}	R	0.000 ± 0^{b}	R	0.000 ± 0^{b}	R	11.00 ± 0.63^{a}	R
Ceftriaxone (CTR, 30)	21.51 ± 1.01^{bcd}	S	23.31 ± 0.33^{abc}	S	25.34 ± 0.95^{ab}	S	21.95 ± 0.91^{bcd}	S	26.895 ± 2.46^{a}	S	19.60 ± 1.48^{cd}	S
Cefuroxime (CXM, 30)	19.01 ± 3.66^{ab}	S	19.05 ± 1.80^{ab}	S	23.75 ± 0.44^{ab}	S	23.98 ± 0.30^{a}	S	21.20 ± 2.19^{ab}	S	22.50 ± 1.41^{ab}	S
Chloramphenicol (C, 30)	27.59 ± 0.78^{bcd}	S	28.34 ± 0.62^{abc}	S	28.97 ± 0.20^{ab}	S	$26{,}61 \pm 0.17^{cde}$	S	29.75 ± 0.34^{a}	S	26.27 ± 0.38^{ce}	S
Ciprofloxacin (CIP, 5)	31.14 ± 1.03^{ab}	S	32.96 ± 0.03^{ab}	S	34.36 ± 0.86^{a}	S	29.08 ± 1.23^{abc}	S	32.66 ± 0.06^{ab}	S	25.55 ± 4.31^{bc}	S
Cotrimoxazole (TS, 25)	26.47 ± 0.24^{c}	S	29.64 ± 0.79^{b}	S	33.39 ± 0.48^{a}	S	27.77 ± 0.36^{bc}	S	33.14 ± 0.26^{a}	S	27.57 ± 1.52^{bc}	S
Doxycyclin (DO, 30)	31.71 ± 0.84^{abc}	S	34.66 ± 3.59^{ab}	S	35.24 ± 2.39^{ab}	S	31.49 ± 0.62^{abc}	S	36.73 ± 0.11^{a}	S	28.60 ± 2.75^{ac}	S
Erythromycin (ERO, 15)	28.80 ± 0.28^{bc}	S	29.27 ± 0.38^{bc}	S	33.67 ± 0.41^{a}	S	28.52 ± 0.69^{bc}	S	31.86 ± 1.04^{ab}	S	26.55 ± 2.75^{c}	S
Gentamicin (CN, 10)	23.53 ± 2.21^{ab}	S	21.85 ± 0.67^{abc}	S	25.76 ± 0.43^{a}	S	19.76 ± 1.41^{bc}	S	22.68 ± 0.33^{abc}	S	25.50 ± 1.41^{a}	S
Imipenem (IMP, 10)	38.04 ± 1.73^{a}	S	38.8 ± 1.57^{a}	S	29.41 ± 0.98^{b}	S	30.36 ± 0.63^{b}	S	30.97 ± 1.34^{b}	S	27.55 ± 2.89^{b}	S
Nitrofurantoin (F, 30)	22.73 ± 1.13^{ab}	S	23.96 ± 0.91^{a}	S	23.69 ± 0.43^{ab}	S	17.40 ± 0.46^{bc}	R	23.86 ± 0.71^{a}	S	21.55 ± 1.34^{ab}	S
Oxacillin (OXA, 5)	33.15 ± 0.77^{a}	S	34.12 ± 1.01^{a}	S	34.39 ± 0.53^{a}	S	30.22 ± 0.04^{b}	S	35.79 ± 0.04^{a}	S	27.30 ± 2.40^{c}	S
Pefloxacin (PEF, 5)	31.40 ± 0.45^{ab}	S	33.66 ± 0.38^{a}	S	32.62 ± 0.60^{ab}	S	20.50 ± 0.70^{cd}	S	34.340 ± 0.55^{a}	S	24.77 ± 1.73^{bc}	S
Penicillin G (PEN, 10)	24.30 ± 0.96^{b}	S	26.09 ± 0.15^{ab}	S	28.83 ± 0.77^{a}	S	19.29 ± 0.97^{c}	R	28.33 ± 2.14^{ab}	S	27.75 ± 1.06^{ab}	S
Piperacillin (PRC, 10)	25.67 ± 0.79^{ab}	S	27.78 ± 0.85^{a}	S	24.19 ± 2.01^{ab}	S	$21{,}90 \pm 0.27^{ab}$	S	26.77 ± 1.31^{a}	S	27.15 ± 3.46^{a}	S
Trimethoprim-sulfa-methoxazole (SXT, 25)	29.69 ± 1.84^{ab}	S	29.62 ± 1.95^{ab}	S	32.83 ± 0.70^{a}	S	26.23 ± 0.89^{b}	S	31.19 ± 0.90^{ab}	S	27.12 ± 2.29^{ab}	S
Vancomycin (VAN, 5)	19.85 ± 1.5^{a}	S	20.72 ± 0.82^{a}	S	19.07 ± 2.72^{a}	S	22.50 ± 0.70^{a}	S	22.35 ± 1.48^{a}	S	21.02 ± 0.67^{a}	S

In row and column, the averages affected by different superscript letters are significantly different at the 5% threshold according to the Newmann-Keuls test. Legend: *D* = diameter, *S/R* = sensible/resistant; values are means ± standard error

reliably identified using a 16S rRNA gene sequencing" (EFSA 2007). Although 16S rRNA gene sequencing is considered an accurate identification tool, the majority of strains displayed high similarity to almost two different type species in GenBank databases. Moreover, some strains were assigned as *Bacillus* sp. due to the weakness of similarity level (between 90 and 97%). These observations could be explained by the limit of this technique for microbial identification of organisms in this complex and often nebulous group. Indeed, previous studies have shown that this approach alone does not allow precise difference between *B. subtilis* and *B. amyloliquefaciens* (Porwal et al. 2009; Sumpavapol et al. 2010) and between *B. cereus* and *B. thuringensis* (Chang et al. 2003; Bhandari et al. 2013) due to the high similarity observed in these species. Thus, *gyrA* and *gyrB* genes sequencing have been reported to be more informative and discriminatory for the identification of *Bacillus* species, subspecies and strains (Chun and Bae 2000; Chen and Tsen 2002; Lefevre et al. 2016). Distinct phenotypic traits such as degree of pathogenicity, food spoilage enzyme potential, thermotype and colony morphology are also used as important criteria for the classification of *B. cereus* strains (Tolieng et al. 2018). Presently, identification of microorganisms from various sources is benefiting from techniques based on the combination of 16S rRNA gene sequencing with MALDI-TOF-MS and chemometric or whole-genome sequencing. Thus, the identification of *Bacillus* strains of this study could be more accurately done using this multi-factorial analysis.

Although *Bacillus* strains are recognized as principal bacteria responsible for flavor development and, bio-preservation of fermented oil seed foods, concern for the safety of the resulting foods is an important factor for consideration before their use as starter cultures or for probiotics formulation, given that some strains are known pathogens or are able to transfer antibiotic resistance genes. Hence, hemolytic activity and antibiotic susceptibility are important criteria for assessing the safety of bacteria of food interest (Nwagu et al. 2020). Hemolysis, whether partial (α) or complete (β) indicates virulence. Indeed, β-Hemolysis is an indication that bacteria contain cytotoxic phospholipases (Sorokulova et al. 2008) and the hemolytic factor decreases the amount of hemoglobin available as an iron source for the host (Şeker 2010). Thus, of the 20 *Bacillus* strains tested for hemolytic activity, only 6 strains showed non-hemolysis and were γ-hemolytic on sheep blood agar plates. This is similar to probiotic *Bacillus cereus* strains BC1, and BC2 (Nwagu et al. 2020), *Bacillus* strains (Lee et al. 2017) and *B. polyfermenticus* CJ6 (Jung et al. 2012). They were unable to lyse blood cells (erythrocytes). This inability of *Bacillus* strains to lyse host blood cells once ingested is an additional advantage required for probiotic qualification (Nwagu et al. 2020). These non-hemolytic strains were therefore selected as GRAS to assess their sensitivity to antibiotics commonly used in medicine, as they must not harbor antibiotic resistance genes transferable to other bacteria including pathogens (Danielsen and Wind 2003; Compaoré et al. 2013). Indeed, bacterial antibiotic resistance is due to either (i) intrinsic properties (natural phenotypic traits) or (ii) the acquisition of resistance genes through mobile genetic elements, such as plasmids and transposons, or the mutation of indigenous genes (Sharma et al. 2014). All the six γ-hemolytic *Bacillus* spp. strains were susceptible to almost all antibiotics tested including those recommended by European Food Safety Agency (EFSA 2008), except for bacitracin for which they were all resistant (table 2). Strains F21, and F20 were the most sensitive to imipenem (38.80 ± 1.57 mm and 38.04±1.73 mm, respectively) while strain B54 displayed the weakest sensitivity to bacitracin (11.00 ± 0.63 mm) with a very high significant difference ($p \leq 0.0001$). Similar results were reported for susceptibility of *Bacillus* species to several antibiotics (Compaoré et al. 2013; Thankappan et al. 2015; Kavitha et al. 2018; Nwagu et al. 2020). Furthermore, our findings are in line with that reported by Adimpong et al. (2012) on the resistance of *Bacillus* strains to bacitracin. Indeed, the resistance of some *Bacillus* strains to certain antibiotics could be intrinsic/natural or acquired and linked to the presence of resistance genes involved in the production of resistance enzymes to these antibiotics (Adimpong et al. 2012; Compaoré et al. 2013). However, natural resistance would cause less risk than acquired resistance in transferring resistance genes to other pathogenic bacteria. Indeed, antibiotic resistance has become a major global concern because resistant bacteria can be transmitted from the food chains to humans (Bell et al. 2018). The sensitivity to a wide range of antibiotics suggests that isolated *Bacillus* strains may not carry antibiotic resistance genes that can be transferred to pathogenic microorganisms. The current finding indicates that, genotypically, *Bacillus* spp. isolated from *soumbala* belonged to *B. subtilis* or *B. cereus* clades. Some of these *Bacillus* strains were γ-hemolytic and susceptible to the majority of antibiotics tested and currently used as medicines. These strains could be further investigated as potential probiotic-starter cultures for optimization and valorization of the production of high-quality, therapeutic and functional or health-promoting *soumbala*.

Conclusion

This study highlights the identification of *Bacillus* spp. from *soumbala,* fermented seeds of *Parkia biglobosa,* as *B. subtilis* or *B. cereus* group species. The evaluation

of safety quality allowed the selection of non-hemolytic *Bacillus* strains with a wide antibiotic sensitivity spectrum. These presumptively selected safe *Bacillus* strains need more investigation for probiotic properties to further confirm their safety quality as potential probiotic-starter cultures for the development of novel technological processes for production of therapeutic and health-promoting functional fermented foods.

Materials and methods

Bacterial strains and cultivation conditions

Bacterial strains used in this study were previously isolated from *soumbala* and identified as *Bacillus* spp. based on their phenotypic profiles and PCR analysis (Dabiré et al. 2021). Stock-cultures were maintained in brain heart infusion supplemented with 20% glycerol at − 80 °C. Working cultures were made by inoculating 10 mL nutrient broth with frozen-stock culture then incubated at 37 °C overnight in a standard incubator without agitation.

Genotypic characterization

Genomic DNA preparation for PCR and sequencing reactions

Overnight-culture of each *Bacillus* strain was streak-placed on Tryptic Soy Agar (TSA) and incubated at 37 °C under aerobic conditions for 24 h. Genomic DNA was extracted from a single colony of each strain using the thermal chock technique. The DNA extracts were subjected to PCR amplification and sequencing using facilities available at Genoscreen society (Lille, France). This service included a single PCR amplification of rDNA followed by Sanger sequencing of 16S rRNA portion encoding the 30S ribosomal subunit. The variable regions V1 to V5 (Fig. 1), used to discriminate the different bacterial populations by comparison with an international reference database, were targeted.

PCR amplification of 16S rRNA genes

PCR amplification of 16S rRNA genes of all the isolates was performed with primer pairs P8 (5′-AGAGTTTGATCCTGGCTCAG-3′) and P535 (5′-GTATTACCGCGGCTGCTGGCAC-3′), and 338-1040F (5′-CTCCTACGGGAGGCAG-3′) and 338-1040R (5′-GACACGAGCTGACGACA-3′) used to amplify approximately 550 bp and 750 bp, respectively. These primers are specific to the universal conserved regions and allowed the amplification of a fragment of approximately 1500 bp from the genomic DNA of each strain. The reaction mixture consisted for each fragment; 0.3 mM dNTP-mix (Fermentas, Germany), 0.5 mM $MgCl_2$, 0.5 pmol.μL^{-1} each primers, 0.24 µL DreanTaq^{TM}DNA polymerase (1.25 U.μL^{-1}) (Fermentas, Germany) and 2 µL of the extracted genomic DNA. The volume of the PCR mixture was adjusted to 50 µL with sterile MilliQwater. A positive control and a negative one were used in each case. The PCR program was performed as follows: an initial denaturation at 95 °C for 12 min followed by 40 cycles consisting of a denaturation phase at 95 °C for 30 s, a hybridization phase at 58 °C for 30 s, an elongation phase at 72 °C for 1 min, and a final elongation phase at 72 °C for 10 min. The obtained amplicons were stored at 4 °C. In order to check for successful PCR amplification, 10 µL of the PCR products were analyzed by electrophoresis using 1.5% agarose gel in 1XTBE (1 h, 100 V). PCR products were purified using GFX^{TM} PCR DNA and Gel Band Purification Kit (GE Healthcare) by following the manufacturer's instructions. The molecular weights of the amplified DNA fragments were calculated using 50- to 1500-bp size markers (QXDNA size marker, QIAGEN). The efficiency of the PCR amplification was checked by application of 1 µL PCR product on QIAxcel (QIAGEN) with the DNA Fast Analysis parameters. The individual validated PCR products were then sequenced.

16S rRNA gene sequencing

After a purification step of the PCR products on the membrane (Macherey Nagel), the samples were assayed by a fluorimetric method (SYBR Green). They were then sequenced in two directions using the same primers according to an optimized protocol. The resulting sequence reactions were purified on Sephadex-G50 gel (GE Healthcare) loaded on an ABI 3730XL capillary sequencer.

Bioinformatics' analysis and data processing

Sequences analysis

Raw sequences were optimized with Auto Peak Trace 6 RP software (Nucleics) then assembled into contigs using Sequencher V4.9 software (Gene Codes Corporation). Obtained 16S rRNA genes were subjected to BLAST against those of other bacteria available in GenBank at NCBI, a generalist database using the BLASTn option[1] (Zhang et al. 2000) and a specialized one EZbiocloud[2], for bacterial 16S rRNA sequences (Yoon et al. 2017), to define the phylogenetic affiliation of the isolates.

Phylogenetic tree construction

Obtained sequences were checked and manually corrected using BioEdit software version 7.2. The alignment of the sequences was carried out by the MEGAX.10 software using the Muscle algorithm. The phylogenetic relationship between the different species identified in this

[1] http://www.ncbi.nlm.nih.gov/genbank/

[2] https://www.ezbiocloud.net/

study and those existing in the NCBI database was carried out using a nearest neighbor consensus analysis with *Alicyclobacillus acidocaldarius* (NR_112638.1) as outgroup. The robustness of the tree branches was assessed by bootstrap analysis with 1000 replicas as previously described (Felsenstein 1985; Tamura and Nei 1993; Kumar et al. 2018).

Identification criteria for the bacterial strains studied

The identification criteria proposed by Drancourt et al. (2000) were used for the identification of bacterial strains at the genus and species level.

Nucleotide accession numbers

The nucleotides sequences of the *Bacillus* strains determined found in this study have been assigned as GenBank accessions N° MZ773904-MZ773923 (i.e., B21 = MZ773904, B54 = MZ773905, F6 = MZ773906, F20 = MZ773907, F21 = MZ773908, F24 = MZ773909, F25 = MZ773910, F26 = MZ773911, F32 = MZ773912, F44 = MZ773913, F48 = MZ773914, G23 = MZ773915, G37 = MZ773916, O28 = MZ773917, O34 = MZ773918, O44 = MZ773919, O46 = MZ773920, O48 = MZ773921, O49 = MZ773922, and O52 = MZ773923).

Safety assessment

Hemolysis test

Bacillus strains were tested for hemolysis on Columbia agar (OXOID Ltd, PB pH 7.5 ± 0.2, Wesel, Germany) supplemented with 5% (V/V) sheep blood (CA-SB) by streaking fresh culture on the blood agar plates followed by incubation at 37 °C under aerobic conditions for 24–48 h. The sheep blood was obtained aseptically from veterinary animal medicine research laboratory of "Ecole Nationale de santé animale," Ouagadougou, Burkina Faso. Isolates that formed a clear or green halo around bacterial strains were assessed as β-hemolytic or α-hemolytic, respectively. Isolates without any halo around the colonies were denoted γ-hemolytic (Kavitha et al. 2018). Thus, the γ-hemolytic bacteria were considered non-hemolytic and then selected for antibiotic susceptibility.

Antibiotic susceptibility of Bacillus strains

Antibiotic susceptibility of selected non-hemolytic *Bacillus* strains (B54, F20, F21, F24, F26, and F44) was evaluated by using the disk diffusion method according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2019). Commercial antibiotic disks with defined concentrations according to Clinical and Laboratory Standards Institute standards (CLSI, 2012) were used. An aliquot of 1 mL of each *Bacillus* strain at the concentration of 10^6 CFU/mL (0.5 McFarland) was spread-plated with sterile beads on Muller-Hinton (MH) agar (Hi-media, India). Afterwards, the plates were allowed to dry for 1 h. Antibiotic disks were placed on the agar plate inoculated with each *Bacillus* strain.

After incubation for 24 h at 37 °C, the diameters of the inhibition zones around the antibiotic disks were measured with an electronic ruler (Hardened, China). This enabled to indicate whether the strain was susceptible "S," intermediate "I," or resistant "R" to antibiotics according to CLSI standards (2012). The antibiotic disks, of a number of 20 included amikacin (ANK, 30 µg), amoxicillin-clavulanic acid (AMC, 30 µg), ampicillin (AMP, 10 µg), bacitracin (BA, 10 µg), cefuroxime (CXM, 30 µg), chloramphenicol (C, 30 µg), ciprofloxacin (CIP, 5 µg), cotrimoxazole (TS, 25 µg), ceftriaxone (CTR, 30 µg), doxycyclin (DO, 30 µg), erythromycin (E, 15 µg), gentamicin (CN, 10 µg), imipenem (IMP, 10 µg), nitrofurantoin (F, 30 µg), oxacillin (OXA, 5 µg), pefloxacin (PEF, 5 µg), penicillin G (PEN, 10 µg), piperacillin (PRC, 10 µg), trimethoprim-sulfamethoxazole (SXT, 25 µg), and vancomycin (VA, 5 µg).

Statistical analyses

The analysis of variance (one-factor ANOVA) was performed with the XLSAT software version 2016.02.27444 at the significance level ($\alpha = 0.05$). In case of significant difference between the studied parameters, the ranking of the means was done according to the Newman-Keuls test.

Abbreviations

AFFs: Alkaline-fermented foods; GRAS: Generally recognized as safe.

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Authors' contributions

Yérobessor Dabiré: methodology, experiments, data curation, formal analysis, writing-original draft; Namwin Siourimè Somda: data curation, writing-review and editing; Marius K. Somda: visualization, validation; Iliassou Mogmenga: writing-review and editing; Abdoulaye K. Traoré: data curation, formal analysis, and writing-review and editing; Lewis I. Ezeogu: supervision, validation, visualization; Alfred S. Traoré: conceptualization, project management; Jerry O. Ugwuanyi: conceptualization, validation, visualization, writing-review and editing; Mamoudou H. Dicko: conceptualization, project administration, validation, visualization, writing-review and editing. All authors read, commented on, and approved of the final manuscript.

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Availability of data and materials
All data generated and/or analyzed during the current study are included in this article, except the GenBank accession numbers (MZ773904-MZ773923) for the strains' nucleotide sequences which have been deposited at Genbank under Project number SUB10173179 (https://submit.ncbi.nlm.nih.gov/subs/?search=SUB10173179) and are not publicly available as not yet published but are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Competing interests
The authors declare that there are no conflicts of interest regarding the publication of this paper.

Author details
[1]Laboratoire de Biochimie, Biotechnologie, Technologie Alimentaire et Nutrition (LABIOTAN), Département de Biochimie Microbiologie, Ecole Doctorale Sciences et Technologies (EDST), Université Joseph KI-ZERBO, 09 P.B. 848, Ouagadougou 09, West Africa, Burkina Faso. [2]Department of Microbiology, Faculty of Biological Sciences, University of Nigeria, Nsukka, Enugu State 410001, Nigeria. [3]Département Technologie Alimentaire (DTA), Centre National de la Recherche Scientifique et Technologique (CNRST)/IRSAT/Direction Régionale de l'Ouest, 03 B.P.2393, Bobo-Dioulasso 03, West Africa, Burkina Faso. [4]Laboratoire de Microbiologie et de Biotechnologies Microbiennes (LAMBM), Département de Biochimie-Microbiologie, Ecole Doctorale Sciences et Technologies (EDST), Université Joseph KI-ZERBO, 03 P.B. 7031, Ouagadougou 03, West Africa, Burkina Faso. [5]Laboratoire de Biologie Moléculaire, d'Epidémiologie et de Surveillance des Bactéries et Virus Transmissibles par les Aliments (LaBESTA), Département de Biochimie-Microbiologie, Ecole Doctorale Sciences et Technologies (EDST), Université Joseph KI-ZERBO, 03 P.B. 7031, Ouagadougou 03, West Africa, Burkina Faso. [6]Unité de Formation et de Recherche en Sciences et Technologies (UFR ST), Département de Sciences de la Vie et de la Terre (SVT), Université Norbert Zongo, Koudougou, West Africa B.P. 376, Burkina Faso.

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