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Molecular characterization of *Bacillus*, lactic acid bacteria and yeast as potential probiotic isolated from fermented food

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ABSTRACT

Spontaneous fermentation or traditional method of preserving indigenous foods using several microorganisms, is frequently practiced in the marginal world. Fermented seeds and milk are mostly consumed in the form of food condiments and desserts in Africa, Asia and others parts of world. Our previous studies deal with the production of Bacteriocin-Like Inhibitory Substances by *Bacillus* from fermented food and quality of fermented milk consumed in Burkina Faso. The rep-PCR and sequencing were used to characterize thirty eights strains isolated various fermented foods from Burkina Faso. Phylogenetic tree were constructed by the neighbour-joining method based on 16S or 26S rRNA genes sequences using MEGA X. Based on colonies characteristics and cells morphology, biochemical tests and gene sequencing, the isolates were identified as *Bacillus cereus sensu lato* (13), *Bacillus pumilus* group (03) with one strain (LCG1) presumed LAB was identified as *Bacillus australimaris* or *Bacillus pumilus* by 16S rRNA sequencing, *Enterococcus durans* (03), *Lactobacillus paracasei* (03), *Lactobacillus plantarum* (04), *Leuconostoc pseudomesenteroides* (01), *Saccharomyces cerevisiae* (04), *Kluyveromyces marxianus* (01), *Candida tropicalis* (01), *Pichia kudriavzevii* (01), *Clavispora lusitaniae* (02), *Rhodotorula mucilaginosa* (01) and, *Cyberlindnera fabianii* (01). Several microorganisms with potential technological interest are housed in fermented foods from Burkina Faso. These microorganisms are responsible for the fermentation of food through their enzymatic activity, leading to production of fermented food with desirable organoleptic characteristics, improved food safety, the enrichment of nutrients and the promotion of health of consumers.

Introduction

Fermentation is an old method used by the nomadic peoples of sahelian countries for the processing and preservation of milk and other food products. This fermentation vary considerably among regions in South East Asia to Africa, ethnics

Abbreviations: LAB, Lactic acid bacteria; *B.*, *Bacillus*; *s.l.*, *sensu lato*; rep-PCR, repetitive sequence based polymerase chain reaction.

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and cultural knowledge [1]. Fermented foods have been used since Pharaonic Egypt to identify the diets and many tribes in the world. Thus, theses fermented foods have a long history in the developing world, particularly in Burkina Faso. Several fermented foods and beverages like fermented condiments (*Bikalga, Maarri, Soumbala*), fermented milk (traditional yogurt), fermented cereal (*Dolo*, Porridges), fermented fruit products, fermented roots products (*Attiéké*) and others are produced and consumed daily in many rural zones in Burkina Faso. Thus, the characteristics of these foods are due to a microbial diversity including namely bacteria, yeasts and moulds, which originate from the raw materials or often inoculated by starters [2].

Several studies already carried out showed the composition and the importance of *Attiéké, Bikalga*, fermented milk, *Maarri* and *Soumbala* in the human consumption to resolve the major health issues of malnutrition [3,4]. The organoleptic and sanitary qualities of these products are due to the consortium of microorganisms including as well lactic acid bacteria (LAB), *Bacillus*, yeasts and moulds, which, by their metabolites, give a diversity of products finished after fermentation highly appreciated by the consumers [5]. These fermented foods have been used empirically in the past in medicine, but currently their properties for medical purposes have been confirmed by scientific studies [6]. *Lactobacilli* and *Akkermansia* are the most bacteria used as "*probiotic*". Nowadays, LAB are the microorganisms the most studied and used in the agro-alimentary, pharmaceutical and cosmetic industries [7]. The microbiota of spontaneous fermentation of the food matrices origin African is dominated by a consortium of microorganisms including *Bacillus*, LAB and yeasts.

Several potential probiotic are hosted in fermented foods and used in areas of life [8]. In the process of selection of the starters, one of the first stages is the identification of strain, followed to evaluate the technological aptitudes, the harmlessness of the strain and its stability. This identification has recourse to the techniques of molecular biology, because the traditional bacterial farming techniques are not enough to study the complex microbial populations. These tools have largely contributed to identification, classification and reclassification of certain bacterial groups by molecular methods as sequencing, ribotyping, Amplified Fragment Length Polymorphism and rep-PCR [9]. Rep-PCR is a potential tool that uses different primers (REP, ERIC, NGREP, BOX, DRREP, MBOREP, GTG_5) set to analyse the phylogenetic relationship and also to know the genetic variability between the strains [10]. Initial genotyping concentrated on developing assays to differentiate microbial populations. This is a progression from phenotypic characterisation of isolates and afforded more robust methods for monitoring microbial strains of interest or investigating the diversity and dynamics of the cultivable component beyond species level. In general, such genetic fingerprinting provides subspecies discrimination, although certain assays also enable species identification. Thus, several sequences of rRNA or rDNA genes are available to scientists and researchers on Internet network, in general databases as GenBank (http://www.ncbi.nlm.nih.gov/), ABIS online for bacterial identification (http://www.tgw1916.net/bacteria_logare_desktop.html), specialized databases as ribosomal project (http://rdp.cme.msu.edu/), European ribosomal RNA (http://www.psb.ugent.be/rRNA/index.html/), and EZbiocloud (https://www.ezbiocloud.net/) which is a database containing only the sequences of ARN 16S references strains, and the YeastIP databases (http://genome.jouy.inra.fr/yeastip/). In this work, the objectives were to group and to identify the strains respectively by rep-PCR fingerprinting and the sequencing of the rRNA 16S bacteria and rRNA 26S yeasts isolated from fermented indigenous food of Burkina Faso, as well as their diversity to the research of new starters as potential probiotic.

Material and methods

Sampling

Sampling were realized in six (06) cities of Burkina Faso. Fermented condiments and food samples were purchased in markets from Ouagadougou. Fermented milk samples were purchased in markets from *Bobo-Dioulasso, Djibo, Dori, Gorom-Gorom* and *Sebba*. This choice of cities was justified by in fact that Ouagadougou is a commercial center, while the others cities are the major centers of milk production in Burkina Faso. The samples were transported to the laboratory at 4–5 °C using icebox for the different analysis.

Isolation and conservation

Bacillus were isolated in our precedent study and conserved at +4 °C in Brain Heart Infusion with 15% (v/v) glycerol after 24 h incubation at 37 °C [11]. LAB were isolated on Man Rogosa Sharp agar (MRS) added the Nystatin (100 mg/L) and conserved in MRS broth with 30% (v/v) glycerol at −20 °C. Yeasts were isolated on Sabouraud CAF agar with chloramphenicol and conserved at +4 °C in nutrient broth with 15% (v/v) glycerol.

Characterization of isolates

Purified microorganisms were grown for 48 h on appropriate media at 37 °C for bacteria and at 30 °C for yeasts then characterized. Cell morphology, Gram stain (only bacteria), catalase test, oxidase reaction, spore forming and cell motility were determined for all isolates.

Molecular analysis

A total of thirty eight isolates including fifteen Bacillus, twelve LAB and eleven yeasts, representing different groups according to morphological, biochemical characteristics were chosen for molecular typing.

DNA extraction

Each isolate was streaked on the appropriate agar and incubated at 30 °C for 48 h under anaerobic conditions (Anaero-Gen) for LAB and aerobic conditions for *Bacillus* and yeasts. The InstaGene Matrix Kit (Biorad 732-6030, Hemel Hempstead UK) was used for the DNA extraction according to the manufacturer's instructions. DNA purity was verified via a spectrophotometer after extraction and stored at −20 °C according to Ouoba et al. [12].

Repetitive sequence-based PCR

Bacillus, LAB and yeasts were differentiated by rep-PCR (repetitive sequence based polymerase chain reaction). The rDNA of selected strains was amplified by PCR procedure described by Ouoba et al. [12] using the GTG_5 (5′-GTGGTGGTGGTGGTG-3′). DNA molecular marker (12,000 pb, Promega, USA) was included as a standard. The migration of the gel was carried out in water during 16 h and 30 min at 47 V in 1X TAE (Tris, Acetate EDTA buffer) and photographed using an UV transilluminator. The strains were clustered according to their DNA profiles obtained.

Sequencing of 16S rDNA of bacteria and 26S rDNA of yeasts

One representing of each group were selected for sequencing. All PCR products were purified using kit GFX ™ PCR DNA and Gel Band Purification Kit (GE Healthcare) and sequenced by Source Bioscience Sequencing (Cambridge, UK). For the bacteria (*Bacillus* and LAB), the 16S rDNA were sequenced as described by Ouoba et al. [12]. A 540 bp portion of conserved regions of 16S rRNA gene was amplified using primers pA (5′-AGAGTTTGATCCTGGCTCAG-3′) and pE (5′-CCGTCAATTCCTTTGAGTTT-3′) and sequenced with primer pD (5′-GTATTACCGCGG CTGCTG-3′) corresponding the position 536-518 bp of 16S rRNA gene of the *E. coli*. Yeasts were identified by sequencing of 26S rDNA to D1/D2 region. NL1 (5′-GCATATCAATAAGCG GAGGAAAAG-3′) and NL4 (5′-GGTCCGTGTTTCAAGACG G-3′) were used for amplification and sequencing [13]. All primers of sequencing genering approximatively 550 bp.

Identification of isolates

The search in database of 16S or 26S rRNA genes sequences was performed in GenBank NCBI (http://www.ncbi.nlm.nih.gov/) using the BLAST program, a base of given general practitioner. A second database search for 16S rRNA gene sequences was performed using EZbiocloud (https://www.ezbiocloud.net/) server, which is a database containing only 16S rRNA sequences of references bacteria. The YeastIP is a database (http://genome.jouy.inra.fr/yeastip/) for 26 rRNA sequences of references yeasts according to Weiss et al. [14].

Phylogenetic trees realization

The alignment of the obtained sequences was checked manually and corrected, and similarity values were determined by Chromas software (version 2.6.5). MEGA X was also used for alignment (Muscle algorithm) and to construct a consensus Neighbour-Joining analysis to assess the phylogenetic relationship of species of the *Fusobacterium gastrosuis* (LN906797). Gaps were excluded. The robustness of tree branches was assessed with 1000 replicates. Phylogenetic and molecular evolutionary analyses were conducted according to Felsenstein [15], Tamura and Nei [16] and Kumar et al. [17].

Sequence accession numbers

The sequences determined in the present study have been deposited under Genbank NCBI (http://www.ncbi.nlm.nih.gov/) with accession numbers (BB4: MK652774.1; BB9: MK652775.1; AB7: MK652776.1; AB6: MK652777.1; MB9: MK652778.1; SY7: MK652779.1; LCG1: MK652780.1; LCJ8: MK652783.1; LGS2: MK652784.1; LGD8: MK652785.1; LGD9: MK652786.1; LBS9: MK652787.1; LBB6: MK652788.1; YCG7: MK656261.1; YCJ10: MK656262.1; YCD3: MK656263.1; YCS1: MK656264.1; YGS5: MK656265.1; YBD6: MK656266.1; YBB1: MK656267.1).

Results

Characteristics of isolates

A total of 38 microorganisms including 15 *Bacillus*, 12 LAB and 11 yeasts isolated from fermented food produced in different areas of Burkina Faso were studied (Table 1). These fermented foods come from different sources of vegetable seeds of *Adansonia digitata* (*Maarri*), *Parkia biglobosa* (*Soumbala* of *Néré*), *Hibiscus sabdariffa* (*Bikalga*), root of *Manihot esculenta* (*Attiéké*), *Glycine* max (*Soumbala* of *Soya*) and fermented milk of animal as *Camelus dromedarius* (camels), *Bos Taurus* (cows) and *Capra hircus* (goats). Macroscopic observation of presumed *Bacillus* revealed round colonies with variable margins (regular,

Table 1
Characteristics of strains used in this study.

Isolates	Fermented food and locality	Cell morphology	Gram	Catalase	Oxidase	Spore	Mobility
AB1	*Attiéké*, Ouagadougou	Rod-shaped, alone, in pairs and small chain	+	+	–	+	+
AB5	*Attiéké*, Ouagadougou	Rod-shaped, alone, in pairs and small chain	+	+	–	+	+
AB6	*Attiéké*, Ouagadougou	Rod-shaped, alone, in pairs and small chain	+	+	–	+	+
AB7	*Attiéké*, Ouagadougou	Rod-shaped, alone, in pairs and small chain	+	+	–	+	+
BB4	*Bikalga*, Ouagadougou	Rod-shaped, alone, in pairs and in chain	+	+	–	+	+
BB9	*Bikalga*, Ouagadougou	Rod-shaped, alone, in pairs and in chain	+	+	–	+	+
BB11	*Bikalga*, Ouagadougou	Rod-shaped, alone, in pairs and in chain	+	+	–	+	+
MB7	*Maarri*, Ouagadougou	Rod-shaped, in pairs and in chain	+	+	–	+	+
MB9	*Maarri*, Ouagadougou	Rod-shaped, in pairs and small chain	+	+	–	+	+
SY3	*Soumbala néré*, Ouagadougou	Rod-shaped, alone, and small chain	+	+	–	+	+
SY6	*Soumbala of néré*, Ouagadougou	Rod-shaped, alone, pairs and small chain	+	+	–	+	+
SY7	*Soumbala of néré*, Ouagadougou	Rod-shaped, alone and small chain	+	+	–	+	+
SY9	*Soumbala of néré*, Ouagadougou	Rod-shaped, in pairs and chain	+	+	–	+	+
SS1	*Soumbala of soya*, Ouagadougou	Rod-shaped, in pairs and small chain	+	+	–	+	+
SS4	*Soumbala of soya*, Ouagadougou	Rod-shaped, in pairs and small chain	+	+	–	+	+
LBB6	Fermented milk cow, Bobo-Dioulasso	Rod-shaped, small, alone and small chain	+	–	–	–	–
LBD1	Fermented milk cow, Dori	Rod-shaped, small, alone and small chain	+	–	–	–	–
LBG12	Fermented milk cow, Gorom-Gorom	Rod-shaped, small, alone and small chain	+	–	–	–	–
LBS9	Fermented milk cow, Sebba	Rod-shaped, small, alone and small chain	+	–	–	–	–
LCG1	Fermented milk camel, Gorom-Gorom	Rod-shaped, alone, pairs and small chain	+	+	+	–	+
LCD3	Fermented milk camel, Dori	Cocci, small, alone, pairs and small chain	+	–	–	–	–
LCJ8	Fermented milk camel, Djibo	Cocci, small, alone, pairs and small chain	+	–	–	–	–
LCG4	Fermented milk camel, Gorom-Gorom	Rod-shaped, small, alone and small chain	+	–	–	–	–
LGD8	Fermented milk goat, Dori	Rod-shaped, small, alone and small chain	+	–	–	–	–
LGD9	Fermented milk goat, Dori	Cocci, oval, pairs and small chain	+	–	–	–	–
LGJ1	Fermented milk goat, Djibo	Cocci, small, alone, pairs and small chain	+	–	–	–	+
LGS2	Fermented milk goat, Sebba	Rod-shaped, small, alone and small chain	+	–	–	–	–
YBB1	Fermented milk cow, Bobo-Dioulasso	Ovoid, spherical, elongated, alone, pairs	NT	+/-	+	Asc+	–
YBD6	Fermented milk cow, Dori	Ovoid, shape, alone, pairs small chain and heap	NT	+	+	Asc+	–
YBG3	Fermented milk cow, Gorom-Gorom	Ovoid, spherical, alone, pairs and heap	NT	+	+	Asc+	–
YBS15	Fermented milk camel, Sebba	Ovoid, shape, alone and heap	NT	+/-	+	Asc+	–
YCD3	Fermented milk camel, Dori	Ovoid, spherical, alone and pairs	NT	+	+	Asc+	–
YCG7	Fermented milk camel, Gorom-Gorom	Ovoid, elongated and alone	NT	+	+	Asc+	–
YCJ10	Fermented milk camel, Djibo	Ovoid, elongated, alone, pairs and heap	NT	+	+	Asc+	–
YCS1	Fermented milk camel, Sebba	Ovoid, shape, alone and heap	NT	+/-	+	Asc+	–
YGG4	Fermented milk goat, Gorom-Gorom	Ovoid, spherical, alone, pairs and heap	NT	+	+	Asc+	–
YGJ2	Fermented milk goat, Djibo	Ovoid, spherical, alone, pairs and heap	NT	+	+	Asc+	–
YGS5	Fermented milk goat, Sebba	Ovoid, spherical, alone, pairs and heap	NT	+	+	Asc+	–

Legend: (+): Positive reaction, (-): Negative reaction, +-: weak reaction, NT: Not Tested.

irregular) flat or curved, whitish or beige in colour with an opaque appearance. Colonies size's varies between 0.5 mm to 6 mm in diameter, spore forming, Gram-positive and, motile. Morphology colonies of presumed LAB was differed, colour varied from white to pale creamy, the shape was circular and the size varied from 0.5 to 3 mm in diameter. Only representative bacteria Gram-positive, catalase negative and not spore forming isolates were identified at species level by sequencing 16S rRNA. As for yeasts, several morphotypes i.e. white, creamy, red, smooth, rough colonies with varying edges, not motile, ovoid, spherical and sizes (1 to 7 mm) were observed. These isolates were presumptively identified as *Bacillus*, LAB and yeasts according to their morphology and biochemical characteristics, according to the literature.

Grouping of isolates

The strains were classified into different groups according to characteristic bands contained in their profiles genomic fingerprinting (Table 2). The rep-PCR allowed the discrimination at species level for the isolates studied according to Figs. 1–3.

For the presumed *Bacillus*, five groups were observed according to Fig. 1. Group 1 was characterized by two constant DNA bands and comprised SY3, MB9, AB5, SS1 and SS4. Group 2 was characterized by three bands with SY7 and BB11 as representing. Group 3 was characterized by two bands (BB9, SY9, AB1 and MB7). Group 4 was characterized three bands with three isolates AB7, AB6 and SY6. Group 5 (BB4) was characterized by five bands.

Based fingerprinting of Fig. 2, the presumed LAB were classed in seven groups according to the characteristic of DNA bands (Table 2). Group 1 (LCJ8, LCD3, LGJ1), group 2 (LBB6, LBG12, LBD1) and group 4 (LCG1) have two specific bands (Table 2). Group 3 (LGD8) and group 5 (LGS2, LCG4) have three specific bands. Group 6 (LBS9) and group 7 (LGD9) have four bands with only one representative per group. The profile of isolate LCG1 is clearly different of other profiles obtained, having a resemblance between them.

Table 2
Grouping of isolates according to characteristic bands.

Groups and isolates	Constants bands	Approximate size of bands (pb)
Bacillus		
G1 (SY3, MB9*, AB5, SS1, SS4)	Two bands	2200, 1100
G2 (SY7*, BB11)	Three bands	5500, 4500, 2100
G3 (BB9*, SY9, AB1, MB7)	Two bands	3800, 750
G4 (AB7*, AB6*, SY6)	Three bands	1700, 1200, 550
G5 (BB4*)	Five bands	5000, 2500, 1250, 1000, 850
Lactic acid bacteria		
G1 (LCJ8*, LCD3, LGJ1)	Two bands	4500, 1100
G2 (LBB6*, LBG12, LBD1)	Two bands	5000, 760
G3 (LGD8*)	Three bands	3500, 2500, 700
G4 (LCG1*)	Two bands	1500, 1450
G5 (LGS2*, LCG4)	Two bands	950, 1000
G6 (LBS9*)	Four bands	2600, 2100, 1600, 900
G7 (LGD9*)	Four bands	2000, 900, 750, 550
Yeasts		
G1 (YGS5*, YGJ2, YGG4, YBG3)	Six bands	3600, 2500, 1700, 1600, 1100, 750
G2 (YBD6*)	One band	1000
G3 (YCG7*)	Two bands	1200, 700
G4 (YCJ10*)	One band	2700
G5 (YCS1*, YBS15)	Six bands	2600, 2400, 2000, 1800, 1150, 1050
G6 (YCD3*)	Two bands	1300, 900
G7 (YBB1*)	One band	800

Legend: G1 to G7: Group of isolates;.
* : Isolate sequenced per group.

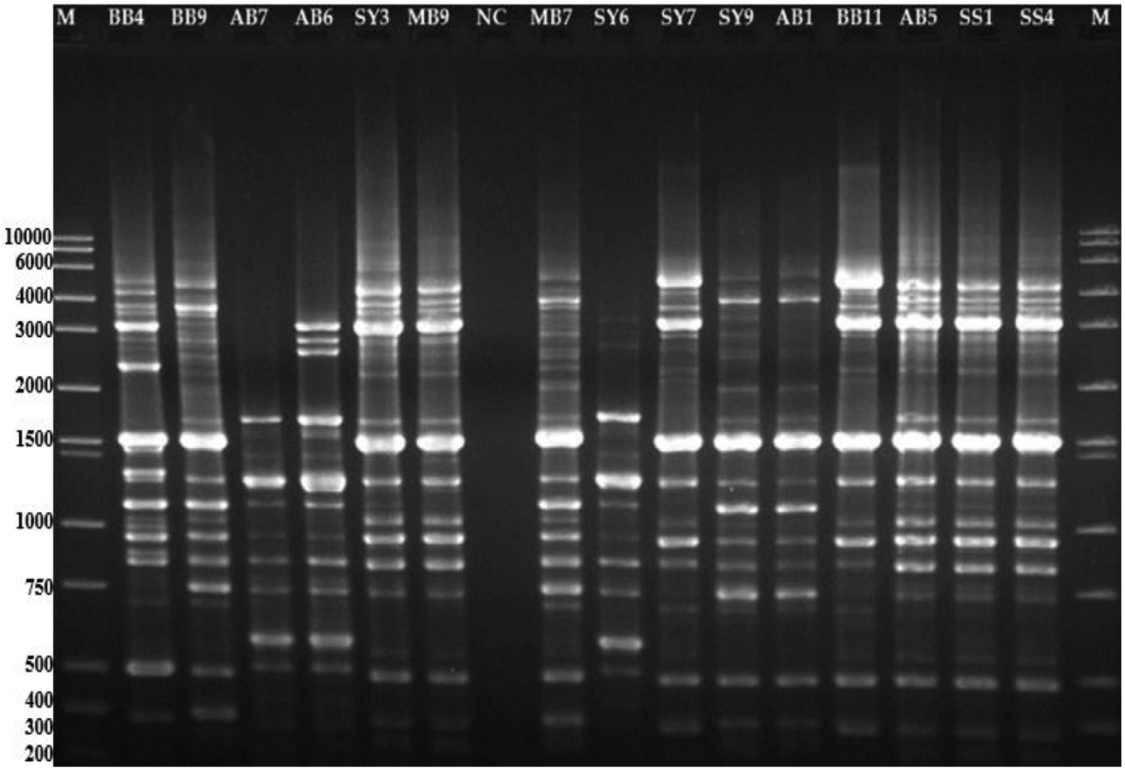

Fig. 1. Profile of fingerprinting of *Bacillus* isolated from *Attiéké, Bikalga, Maarri and Soumbala* by rep-PCR.
Legend: M: DNA molecular marker, NC: Negative control, BB4 to SS4: Isolates.

Yeasts were classed in seven groups according to Fig. 3. The different groups according to the characteristic of DNA bands are consigned in Table 2. Group 1 (YGS5, YGJ2, YGG4 and YBG3) and group 5 (YCS1 and YBS15) are characterized by six specifics bands. Group 2 (YBD6), group 4 (YCJ10) and group 7 (YBB1) are characterized by one specific band and one representative per group. Group 3 (YCG7) and group 6 (YCD3) are characterized by one representative per group with two specifics bands.

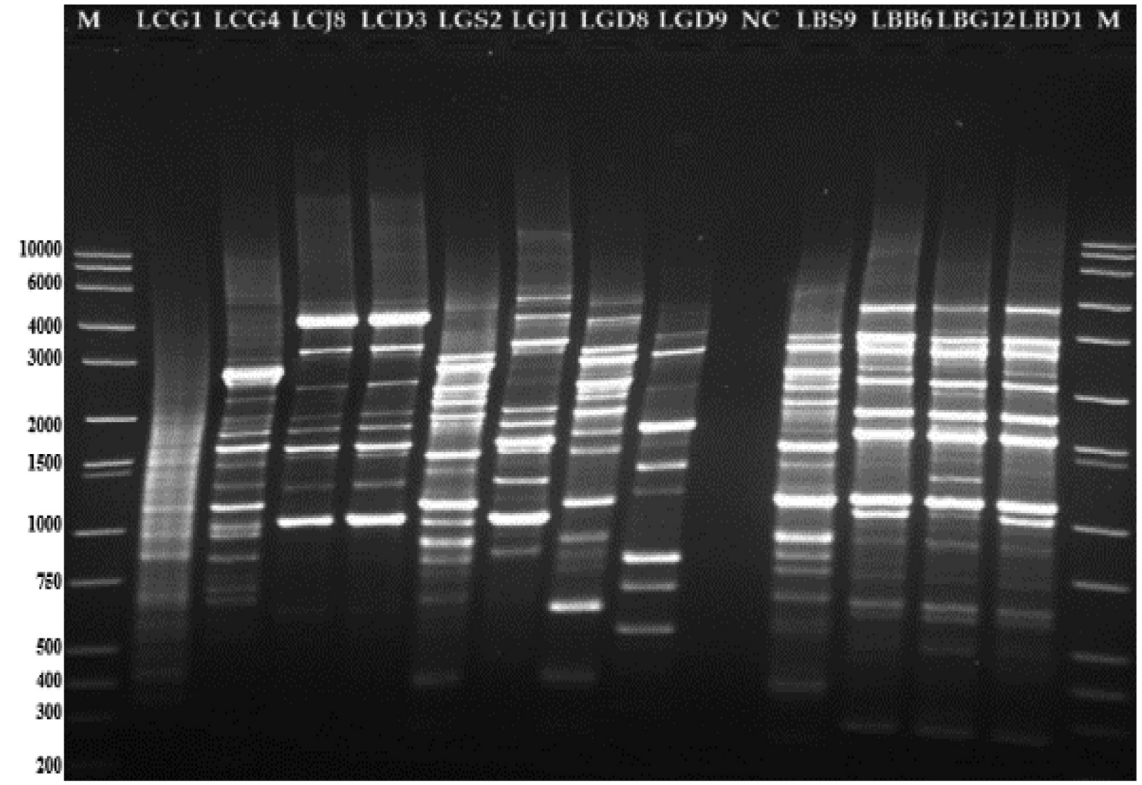

Fig. 2. Profile of fingerprinting of LAB isolated from fermented milk by rep-PCR.
Legend: M: DNA molecular marker, NC: Negative control, LCG1 to LBD1: Isolates.

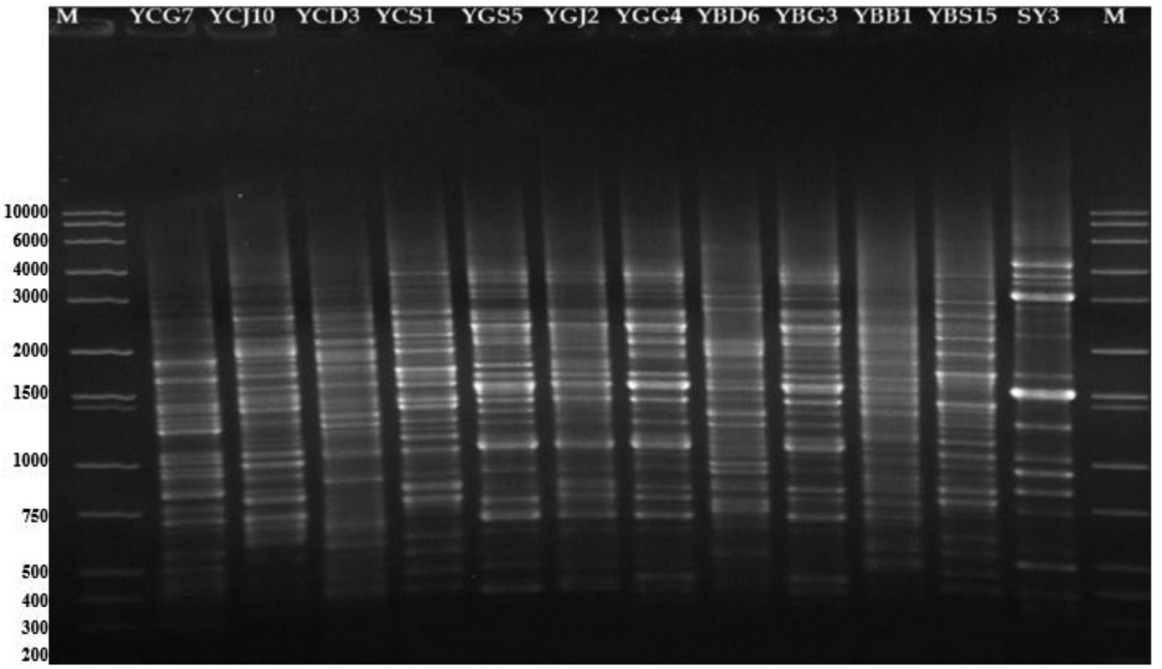

Fig. 3. Profile of fingerprinting of yeasts isolated from fermented milk by rep-PCR.
Legend: M: DNA molecular marker, SY3: positive control, YCG7 to YBS15: isolates.

Table 3
Comparative taxonomic identification of bacteria isolated in this study by sequencing of 16S rRNA coding gene according to the databases.

Isolates	National Center for Biotechnology Information			EZbiocloud		
	Identity	Similarity	Accession	Identity	Similarity	Accession
MB9*	*Bacillus cereus*	100%	AM397642.1	*Bacillus cereus*	99,35%	AE016877.1
SY7*	*Bacillus cereus*	99%	KY746354.1	*Bacillus nitratireducens*	99,79%	KJ812430.1
BB9*	*Bacillus cereus*	99%	MK066928.1	*Bacillus cereus*	100%	AE016877.1
AB7*	*Bacillus cereus*	99%	KR709243.1	*Bacillus safensis*	99,37%	ASJD01000027.1
AB6*	*Bacillus safensis*	99%	CP010405.1	*Bacillus safensis*	100%	ASJD01000027.1
BB4*	*Bacillus cereus*	99%	MF144543.1	*Bacillus cereus*	99,35%	AE016877.1
LCG1*	*Bacillus pumilus*	99%	GU297609.1	*Bacillus australimaris*	99,14%	JX680098.1
LCJ8*	*Enterococcus durans*	99%	CP022930.1	*Enterococcus durans*	99,79%	BCQB01000108.1
LBB6*	*Lactobacillus paracasei*	99%	HE983621.1	*Lactobacillus paracasei*	100%	D16550.1
LGD8*	*Lactobacillus plantarum*	99%	KR816164.1	*Lactobacillus plantarum*	100%	ACGZ00000000.2
LGS2*	*Lactobacillus plantarum*	99%	MG739433.1	*Lactobacillus plantarum*	100%	ACGZ00000000.2
LBS9*	*Lactobacillus plantarum*	99%	MG739433.1	*Lactobacillus plantarum*	99,80%	ACGZ00000000.2
LGD9*	*Leuconostoc pseudomesenteroides*	99%	LC223100.1	*Leuconostoc pseudomesenteroides*	100%	AB023237.1

Table 4
Comparative taxonomic identification of yeasts isolated in this study by sequencing of 26S rRNA coding gene according to the databases.

Isolates	National Center for Biotechnology Information			YeastIP database		
	Identity	Similarity	Accession	Identity	Similarity	Accession
YGS5*	*Saccharomyces cerevisiae*	99%	MF769605.1	*Saccharomyces cerevisiae*	99%	a143 [NT] (AY048154.1)
YBD6*	*Kluyveromyces marxianus*	99%	MH244202.1	*Kluyveromyces lactis*	99%	a4706 [N] (CR382124.1)
YCG7*	*Candida tropicalis*	100%	HM246692.1	*Candida tropicalis*	99%	a1289 [T] (U45749.1)
YCJ10*	*Pichia kudriavzevii*	100%	MH244203.1	*Pichia kudriavzevii*	99%	a954 [T] (EF550222.1)
YCS1*	*Clavispora lusitaniae*	99%	EF063126.1	*Clavispora lusitaniae*	99%	a2943 [N] (AJ508571.1)
YCD3*	*Rhodotorula mucilaginosa*	99%	JQ965860.1	*Candida ecuadorensis*	96%	a4749 [T] (FR839617.1)
YBB1*	*Cyberlindnera fabianii*	99%	JQ342084.1	*Cyberlindnera fabianii*	99%	a705 [T] (EF550321.1)

Genetic identification

Similarity analysis was used to study the relationships of bacteria by comparison of 16S rRNA gene sequences with NCBI and Ezbiocloud sequences available in these databases by BLAST program.

The 16S rRNA gene sequences obtained in this study exhibited to similarity 99%−100% and 99.14%−100% of sequences in NCBI and Ezbiocloud database, respectively (Table 3). 16S rRNA sequencing of the selected presumed *Bacillus* clearly showed (MB9) 99.35%−100% similarity to *B. cereus*, (SY7) 99% and 99.79% similarity for *B. cereus* and *B. nitratireducens* respectively, (BB9) 99%−100% similarity to *B. cereus*, (AB7) 99% and 99.37% similarity to *B. cereus* and *B. safensis*, (AB6) 99%−100% similarity to *B. safensis* and (BB4) 99%−99.35% similarity to *B. cereus*. This sequencing of the 16S rRNA gene revealed that the presumed *Bacillus* from fermented food were most phylogenetically related to *B. cereus s.l.* (including *B. cereus* and *B. nitratireducens)* and *B. pumilus group* (including *B. pumilus, B. safensis* and *B. australimaris*).

As for the alleged LAB, they could be affiliated with the following species: *Enterococcus durans* (LCJ8) with 99%−99.79% similarity, *Lactobacillus paracasei* (LBB6) with 99%−100% similarity, *Lactobacillus plantarum* (LGD8, LGS2 and LBS9) with 99%−100% similarity and *Leuconostoc pseudomesenteroides* (LGD9) with 99%−100% similarity with a predominance of *Lactobacillus plantarum*. The identification procedure using molecular test with 16S rRNA revealed that the LCG1 isolate presumed LAB was identified as *B. pumilus* or *B. australimaris* with 99%−99.14% similarity.

For yeasts, similarity analysis was used to study the relationships between our isolates by comparing their 26S rRNA gene sequences with NCBI and YeastIP sequences available in these databases by BLAST program. The 26S rRNA gene sequence of these isolates exhibited to 99%−100% (NCBI) and only 99% (YeastID) similarity to the sequences available in these different databases (Table 4). It came out that 26S rRNA sequencing of the selected yeasts clearly showed (YGS5) 99% similarity to *Saccharomyces cerevisiae*, (YBD6) 99% similarity to *Kluyveromyces marxianus* and *Kluyveromyces lactis*, (YCG7) 99%−100% similarity to *Candida tropicalis*, (YCJ10) 99%−100% similarity to *Pichia kudriavzevii*, (YCS1) 99% similarity to *Clavispora lusitaniae*, (YCD3) 96%−99% similarity to *Candida ecuadorensis* and *Rhodotorula mucilaginosa*, and (YBB1) 99% similarity to *Cyberlindnera fabianii*. Thus, these yeasts could be affiliated as *Saccharomyces cerevisiae* (YGS5), *Kluyveromyces marxianus* (YBD6), *Candida tropicalis* (YCG7), *Pichia kudriavzevii* (YCJ10), *Clavispora lusitaniae* (YCS1), *Rhodotorula mucilaginosa* (YCD3) and *Cyberlindnera fabianii* (YBB1) according to their similarity.

Phylogenetic trees analysis

The phylogenetic trees have been realised to determine the taxonomic affiliation the species found in this study with reference strains. They were built by the method of distances neighbour-Joining by Blast program at 1000 bootstrap. Fig. 4–6 show the relationships between *Bacillus*, LAB and yeasts, respectively.

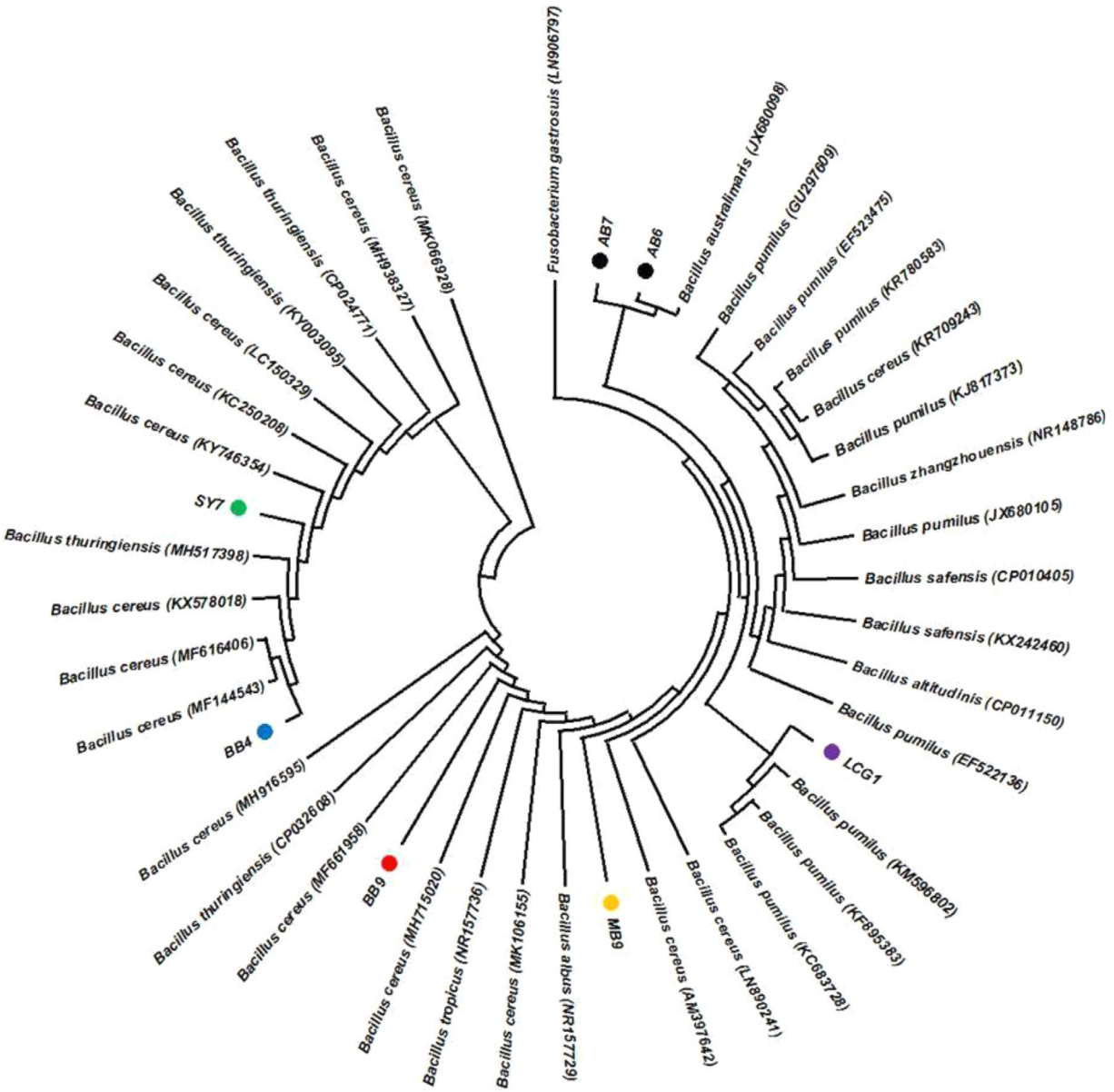

Fig. 4. Phylogenetic tree constructed by the neighbour-joining method showing the position of isolates and related *Bacillus* species based on 16S rRNA gene sequences, *Fusobacterium gastrosuis* (LN906797) was used as an outgroup.

The phylogenetic tree based on 16S rRNA gene sequence (Fig. 4) revealed that AB7 and AB6 strains were very close to *B. australimaris*. As for the SY7, BB7, BB9 and MB9, they are close to *B. cereus*. LCG1 strain is close to *B. pumilus*.

For the LAB (Fig. 5), the phylogenetic tree based on 16S rRNA gene sequence further revealed that LCJ8 and LGD9 strains were very close to *Enterococcus and Leuconostoc genus,* respectively. This Fig demonstrates the phylogenetic infer relationships derived from neighbour-joining analysis of 16S rRNA gene sequences of the LBB6, LGS2 and LBS9 with highest validated described species of the genus *Lactobacillus*.

According to Fig. 6, the phylogenetic tree based on 26S rRNA gene sequence further revealed that YCG7, YGS5, YBD6, YCJ10, YCD3, YCS1 and YBB1 were very close to *Candida tropicalis, Saccharomyces cerevisiae, Kluyveromyces marxianus, Pichia kudriavzevii, Rhodotorula mucilaginosa, Clavispora lusitaniae* and *Cyberlindnera fabianii*, respectively.

Discussion

Attiéké, Bikalga, Maari, Soumbala and fermented milk manufactures are based on the old empirical knowledge of several *Burkinabè* indigenous tribes, whose traditional methods of preparation have changed little over time. The technology

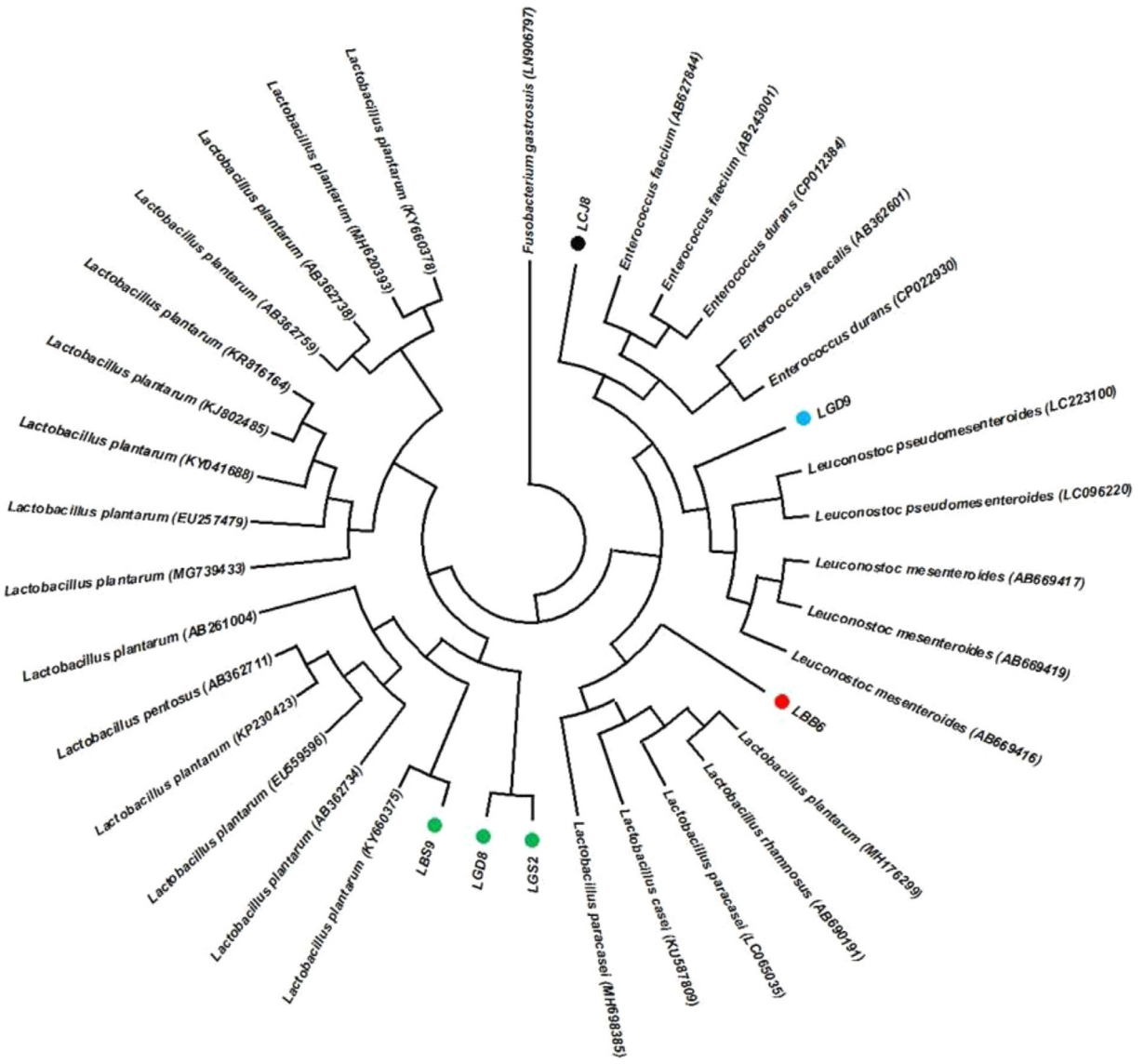

Fig. 5. Phylogenetic tree constructed by the neighbour-joining method showing the position of isolates and related lactic acid bacteria species based on 16S rRNA gene sequences, *Fusobacterium gastrosuis* (LN906797) was used as an outgroup.

of *Attiéké* production was exported from Ivory Coast and presumably adapted locally, resulting in many cassava-fermented products, such as *Gari, fufu, lafun, dawa-dawa, chickwanghe, agbelima, kivunde* and *peujeum* in Africa [18]. These manufactures involve many microorganisms, which are important sources for the production of bioactive substances during the fermentation. Certain bacterial and yeast are used as probiotic or biocatalysts producing compounds of interest (aromas, vitamins, antibiotics) in fermentations. Fermentation is a simple way of processing and preserving food for human consumption [19]. Among these foods, the fermented condiments and dairy products have a prominent place within the functional foods consumed in developing countries [20]. The health benefits of the microbiota of foods are due to LAB, yeasts, moulds, *Bacillus*, Bacteriophage and *Escherichia coli* Nissle 1917, which have already been demonstrated in many applications as probiotic [21]. They have long been used for their beneficial properties, including their antibacterial, anticarcinogenic, wound healing, antiallergenic, immunomodulation, and gastrointestinal immunity activities as *Lactobacillus rhamnosus GG* and *Saccharomyces boulardii* CNCM I-745. Several authors have reported that LAB and Bacillus are the majority bacteria in indigenous fermented food from Africa. These bacteria have good probiotic characteristics in terms of acid tolerance, bile tolerance, antibiotic sensitivity and antibacterial activity against pathogens [22]. The probiotic microbiome plays an important role between the gut microbial metabolism and mental health [23].

Many studies have described LAB's and *Bacillus*'s ability to produce antimicrobials. These Antimicrobial peptides of LAB are the most widely used as food additives for preservation in world [24]. They are involved in both spontaneous

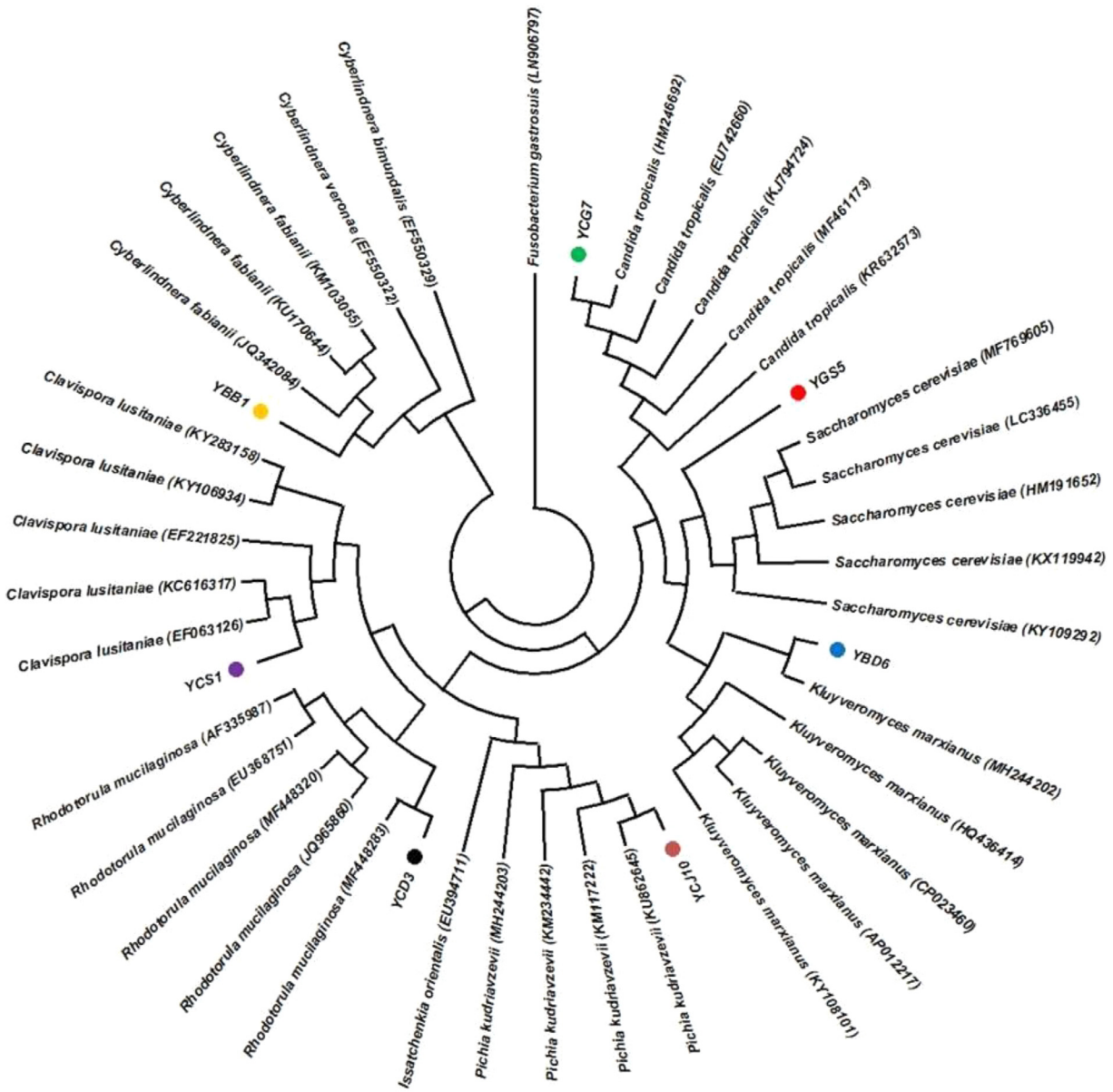

Fig. 6. Phylogenetic tree constructed by the neighbour-joining method showing the position of isolates and related yeasts species based on 26S rRNA gene sequences, *Fusobacterium gastrosuis* (LN906797) was used as an outgroup.

fermentations and large-scale fermentation processes for the preservation and transformation of many raw materials from animals and vegetables.

According to literature, the microbiota of fermented milk mainly includes LAB (*Lactobacillus, Enterococcus, Lactococcus, Leuconostoc, Pediococcus, Oenococcus, Carnobacterium*), yeasts (*Pichia kudriavzevii, Pichia fermentans, Kluyveromyces marxianus, Kzachstania exigua, Candida kefir, Candida pseudotropicalis, Saccharomyces cerevisiae, Saccharomyces exiguous, Torulaspora holmii, Zygotorulaspora florentina, Yarrowia lipolytica*) and *Bacillus* (*B. subtilis, B. cereus, B. pumilus*). Microbiota's of fermented condiments is dominated by *Bacillus* (*B. subtilis, B. licheniformis, B. pumilus, B. megaterium, B. circulans and B. cereus*) [4].

Molecular methods are important for bacterial identification and possibly more accurate for microorganisms than the conventional phenotypic methods. Recently, new molecular tools have been applied for the routine identification of microbes, and had led to an increase in the number of identified bacteria comparatively to phenotypic and biochemical tests for identification of bacteria [25]. In these years, rep-PCR and 16S or 26S-RNA sequencing method has been proven useful for identification and characterization of bacteria and yeasts.

The rep-PCR allows good discrimination of isolates while, the 16S or 26S rRNA sequencing was performed for the molecular identification of isolates. Our findings confirmed that the rep-PCR and 16S or 26S rRNA analysis provides good

discrimination of strain at species level. The alignment of these sequences for identification of microorganisms was considered at a percentage of similarity ≥ 99%. The Rep-PCR is a molecular biology tool recently used for the differentiation of microorganisms at the species level. A visual observation of results and partial sequencing rRNA allowed the differentiation of LAB in four groups (Fig. 5) and yeasts in seven groups (Fig. 6).

For the *B. cereus* group, many publications affiliate *B. cereus* actually as *B. cereus s.l.* because these species are very indistinguishable by 16S rDNA sequencing. This group has the diverse faces according to activity for food-borne intoxications and playing the role of probiotic. *B. cereus s.l.* consists of eight species: *B. anthracis, B. pseudomycoides, B. mycoides, B. thuringiensis, B. weihenstephanensis, B. cytotoxicus, B. toyonensis, B. megaterium* and *B. cereus s. stricto*. Several authors reported *B. cereus-like* enterotoxins from non-*Bacillus cereus* species of the genus, but without providing a conclusive identification of those toxins. Presently, their classification rely mainly on distinctive phenotypic traits, such as pathogenic potential to mammals, enzymatic ability causing food spoilage, thermotypes, as well as colony morphology. Despite their pathogenicity, *B.* cereus might be an excellent candidate for bioremediation, detoxification of Aflatoxin from both field and food matrices, production of L-lactic acid and antibacterial peptides [26,27]. In this study, the presumed LAB were genotypically grouped by GTG_5 based rep-PCR fingerprinting. Only isolate presumed LAB was revealed to *Bacillus* after sequencing 16S rRNA. Satomi et al. [28] reported that *B. pumilus* group have a similarity rate of 99.9% for 16S rRNA gene sequencing in the Planetary Protection archive. In this study, AB7 and LCG1 were high the percentage of similarity to *B. safensis* (99.37%) and *B. australimaris* (99.14%), respectively. *Bacillus* species are the species microbial difficult to differentiate by conventional methodologies and represent the most widespread terrestrial species microbial [29,30]. According to Branquinho et al. [29], the using of MALDI-TOF MS analysis was able to resolve taxonomic identifications of bacteria that are indistinguishable by 16S rRNA sequences. But, the combined of MALDI-TOF-MS and chemometric approach has clearly discriminated *B. pumilus* and *B. safensis* [29,31]

A combination of the results obtained with rep-PCR and sequencing showed that eleven species of LAB of this study was affiliated to genus *Lactobacillus* (Fig. 5). Alegría et al. [32] reported that the rep-PCR allowed a differentiation between sub species of *Lactococcus lactis*. The *Lactobacillus plantarum* group was identified as the predominant flora in samples studied. According to Fig. 5, the genus *Lactobacillus* (66.67%) are the highest, followed by genus *Enterococcus* (16.67%) and *Leuconostoc* (16.66%). Moreover, other genera, namely, *Pediococcus, Weisella*, and *Lactococcus*, were not detected in these isolates. The development of biopreservation technologies using LAB and their metabolites represents an additional hurdle in the protection of food against microbial contamination as these bacteria produce several antimicrobial substances including organic acids, hydrogen peroxide and bacteriocins [33]. However, the use of LAB for this purpose requires confirmation of the safety of strains, as well as their virulent potential, in order to ensure the safety of consumers [34]. LAB are commonly part of the microbiota of fermented foods and beverages due to their important role in the technological aspects of foods maturation and contribution to the sensorial characteristics of these foods. LAB are known to be able to produce many bioactive compounds used in several fields of life. They are considered as biopreservative tool to control the growth of spoilage-related and pathogenic bacteria [22,35].

According to their profile (Fig. 3), yeasts belonged to the genera most frequently highlighted in dairy products: *Candida, Kluyveromyces, Pichia, and Rhodotorula. Pichia kudriavzevii* was once called *Issatchenkia orientalis* [36]. *Cyberlindnera fabianii* is used in wastewater treatment and fermentation of alcoholic beverages. Miao et al. [37] have reported the presence of *Issatchenkia orientalis* in tropical fruit and food sources and traditional African fermented foods. Koutinas et al. [38] have reported than *Issatchenkia orientalis* produce ethanol and have higher thermotolerance, salt tolerance, and acid tolerance than *Saccharomyces cerevisiae*. This later has an ability on the reduction of Aflatoxin M1 in milk [39]. Fermentation, by certain LAB, *Bacillus* and yeasts, removes or reduces the levels of antinutritional factors such as phytic acid, tannins and polyphenols present in foods and releases minerals such as manganese, iron, zinc and calcium. This fermentation reduces also the cyanogenic toxicity and enhances flavour, taste and aroma of the fermented products. This strategy allows native microbes to degrade contaminants and co-substrates [40].

Although identification of microorganisms is currently based on 16S rRNA sequencing, it remains low for discrimination in some genera. The microbiota of fermented food analysed have a very heterogeneous and exploitable microbial diversity in the field to research the new probiotic starters. This microbial diversity and the presence of opportunistic pathogens in the fermented food and milk are due to various sources of contamination (mammary glands, udder skin, raw material, milking means, air quality of farm and the practices of producers).

Conclusion

Traditional fermented foods products have high prebiotics and probiotic activity. The fermented food product based on seed, roots and fermented milk of animals from Burkina Faso was confirmed to be rich in *Bacillus*, LAB and yeast. *B. pumilus, B. cereus, Enterococcus durans, Lactobacillus paracasei, Lactobacillus plantarum, Leuconostoc pseudomesenteroides, Saccharomyces cerevisiae* and *Kluyveromyces marxianus* are the most studied strains and used as probiotics. These microorganisms are involved in both spontaneous fermentations and large-scale fermentation processes for the preservation and transformation of many raw food materials. Their metabolites may contribute to characteristics these fermented foods. Thus, the fermented milk and seeds are important sources of foods contributing to resolve the problems of diseases in world developing countries.

Declaration of Competing Interest

The authors declare that there is no conflict of interest and confirm that this manuscript does not infringe any other copyright or property rights. All authors agreed to publication of the work.

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