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Molecular Characterization and Technological Properties of Lactic Acid Bacteria, *Bacillus* and Yeast of Probiotic Interest Isolated from Fermented Porridges

Boureima Kagambèga[1*], Namwin Siourimè Somda[2], Hama Cissé[1], Oumarou Zongo[1,3], Yves Traoré[1], Aly Savadogo[1]

[1]Laboratoire de Biochimie et Immunologie Appliquées (LABIA), Université Joseph KI-ZERBO, Ouagadougou, Burkina Faso
[2]Centre National de la Recherche Scientifique et Technologique (CNRST)/IRSAT/Département Technologie Alimentaire (DTA)/Direction Régionale de l'Ouest, Bobo-Dioulasso, Burkina Faso
[3]Unité de Formation et de Recherche en Sciences et Techniques (UFR/ST), Université Thomas SANKARA, Ouagadougou, Burkina Faso
Email: *kagambou@gmail.com

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Abstract

Cereal-based porridges are among fermented foods with a composite microbiota. The objective of this work is to characterize the microbiota of porridge. Samples of porridge were collected in Ouagadougou and analyzed according to standard methods in microbiology. The presumed strains obtained were characterized by polymerase chain reaction (PCR). Technological abilities were estimated by tests for resistance to acid pH, bile, antibiotics and antimicrobial, proteolytic and lipolytic activities. All presumptive *Bacillus*, lactic acid bacteria and yeast were characterized by PCR. Four (BC1a; BC9b; BC2a and BC1b) strains were confirmed to *Bacillus* by PCR, 6 strains to Lactobacillus and only to Saccharomyces cerevisiae. All strains were sensitive to two antibiotics gentamycin and imipenem. In contrast, all strains were resistant to oxacillin, amoxicillin-clavulanic acid, streptomycin, penicillin and ticarcillin. Strains tested were resistant to bile but in terms of pH this resistance was relative. Potential probiotic strains have been shown to be effective in inhibiting pathogens. Proteolytic and lipolytic activities were positive on all strains. The characterization of strains, although concerned with a non-exhaustive list of primers, has made it possible to confirm the strains that may be of good quality probiotics if a quantitative study is carried out on technological aptitudes.

Keywords

Bacillus, Lactic Bacteria, Yeast, Porridge, Probiotic

1. Introduction

In West Africa, cereals are the most used for the preparation of many pastas, drinks, fermented porridges [1] [2]. Porridge is very important especially in the diet of children of weaning age, although many studies confirm their inability to provide energy and micronutrient satisfaction [3] [4]. Proportions of improvement in the quality of porridge have been proposed through formulations based on combination with various ingredients [5] and germination or malting techniques, but the results are unsatisfactory [6] [7].

Fermentation is a means by which the digestibility and bioavailability of nutrients [8] and consequently the nutritional value can be improved through the microbiological approach [1]-[9]. However, the porridges are filled with a composite microbiota consisting of lactic acid bacteria, yeasts and *Bacillus* intervening upstream in fermentative processes whose profile deserves to be known [10]. Thus, research is now focused on the research of strains with technological skills, namely probiotic and amylolytic virtues.

The characterization of probiotics in fermented foods has been the subject of several studies, mainly aimed at identifying technological skills [11] [12]. This is the case, for example, of lactic acid bacteria isolated from starchy and fermented foods or drinks in West Africa in 2009, thus highlighting their current use [10]. The same is true for fermented milk [12]. The objective of this work is to characterize, by the method of molecular biology, the microbiota of technological interest of fermented porridges based on cereals.

2. Materials and Methods

2.1. Isolation and Conservation

The lactic acid bacteria were isolated from the porridge according to [13] modified after seeding on the agar-MRS medium. As for the yeasts, they were isolated on Sabouraud medium (SAB) according to [14]. In addition, *Bacillus* was isolated on Plate Count Agar (PCA) agar after thermal shock on the porridge. All strains presumed to be *Bacillus* positive to lactic acid bacteria and yeasts were subcultured respectively on PCA, Man, Rogosa, Sharpe (MRS), Sabouraud added chloramphenicol Agar and incubated at 37˚C for 24 hours. All positive cultures of lactic acid bacteria, yeasts and *Bacillus* after successive transplanting were selected. Pure isolated were stored at −20˚C in MSR broth for lactic acid bacteria and 20% - 30% glycerol-infused brain heart infusion broth for *Bacillus* and yeast.

2.2. Molecular Characterization

DNA Extraction

Presumed strains of *Bacillus*, lactic acid bacteria and yeasts were subcultured onto specific media for Deoxyribonucleic acid (DNA) extraction. The extraction of DNA from the strains was carried out by thermolysis using the method used by [15] modified. Samples were taken aseptically from two or three 24-hour incubation colonies and added to an Eppendorf tube (Hamburg, Germany) containing 300 µl of PCR water or sterile distilled water. The whole was thoroughly homogenized by the same pipette until completely dissolved. All the tubes were placed in a boiling water bath at 100˚C for 10 minutes followed by a freeze storage for 5 minutes. After 5 minutes of freezing, the tubes containing the bacterial inoculum were centrifuged at 12,000 rpm for 15 minutes. The supernatant of each tube was recovered in the order of 200 µl and stored at −20˚C until it was used for PCR reaction. The primers, sequences used and expected sizes are as follows: *Lactobacillus* sp (LbF-GGAATCTTCCACAATGGACG, LbR-CGCTTTACGCCCAATAAATCCGG: 230 pb) [16]; *Candida krusei* (CkFKSfor359-CATTGGCCGTTTCCATTGTGTTC, CkFKSrev359-CATCAAACCAAGCGTGATTCTTGC; 359pb) [17]; *Saccharomyces cerevisiae* (SC-5fw-AGGAGTGCGGTTCTTTCTAAAG, SC-3bw-TGAAATGCGAGATTCCCCCA; 215pb) [18]; *Bacillus* sp (B-K1/5F-TCACCAAGGCRACGATGCG, B-K1/5F-TCACCAAGGCRACGATGCG; 1000 - 1200 pb) [19].

Preparation of the Reaction Mixture

The reaction mixture was prepared with a total volume of 20 µL per reaction. It is composed of Mater mix (One Taq® quick-laod®) (5X), Primer F (10 µM), Primer R (10 µM), H_2O PCR (nulease-free water), DNA (50 ng mL -1) with respective volumes of 4 µL, 0.5 µL, 0.5 µL, 12.5 µL and 2.5 µL.

Amplification

The amplification was performed with the Mastercycler Nexus Gradient Thermal Cycler (Eppendorf). The PCR program for each pair of primers used is shown in Table 1.

Migration

A comma five gram (1.5 g) agarose was dissolved in 100 ml of TBE (EDTA Tri

Table 1. PCR program of the primers used.

Primers	Initial denaturation	30 Cycles			Final denaturation
		Denaturation	Hybridization	Elongation	
	Temperature and time of operations				
CkFKSf/CkFKSr	95˚C 5 min^{-1}	94˚C 45 s^{-1}	50˚C 45 s^{-1}	72˚C 1min^{-1}	72˚C 10 min^{-1}
SC-5fw/SC-3bw	95˚C 5 min^{-1}	94˚C 45 s^{-1}	50˚C 45 s^{-1}	72˚C 1min^{-1}	72˚C 10 min^{-1}
LbF/LbR	95˚C 5 min^{-1}	94˚C 45 s^{-1}	60˚C 45 s^{-1}	72˚C 1min^{-1}	72˚C 10 min^{-1}
LcF/LcR	95˚C 5 min^{-1}	94˚C 45 s^{-1}	55˚C 45 s^{-1}	72˚C 1min^{-1}	72˚C 10 min^{-1}
B-K1/5F, B-K1/3R	95˚C 5 min^{-1}	95˚C 30 s^{-1}	55˚C 30 s^{-1}	72˚C 1min^{-1}	72˚C 7 min^{-1}

Sources: [17] [18] [19].

Borate) at a concentration of 1X and heated until dissolved. After cooling the mixture to 40˚C, a few drops of BET (Ethidium bromide) were added and then homogenized. The gel thus formed was poured into a tank containing a comb for producing the wells. The amplicons were deposited in the wells made on the agarose gel. The migration was carried out with an electrophoresis (Midi Horizontal Electrophoresis) tank containing 1X TBE for 60 min at a voltage of 100 mV with a current of 100 mA.

Revelation and Interpretation of the Bands

After the migration, the revelation of the DNA profiles was made on a Transilluninator electrophoresis gel reader coupled with a dark chamber of a digital camera. After the revelation the tapes were interpreted with positive control.

2.3. Probiotic Properties

Antibiotic Resistance

Antibiotic resistance was assessed and interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [20]. Strains were inoculated on Muller-Hinton agar plates. 11 different antimicrobial agents were tested: amoxicillin (AMX), gentamicin (GEN), imipenem (IMI), oxacillin (Ox), ampicillin (AM), erythromycin (E), penicillin (P), streptomycin (STR), kanamycin (K) ticarcillin (TIC), amoxicillin-clavulanic acid (CMA).

Growth at Acid pH

The pH survival test was performed according to the method of [21] using methyl red as a colored indicator. The pH of different tubes was adjusted to 2.5; 4.5 and 7.2. The cultures were incubated at 30˚C during 24 H for lactic acid bacteria and yeasts, 37˚C during 24 H for *Bacillus*. The bend of the colored indicator reflects growth.

Growth at Bile Salts

The different strains were inoculated on a liquid medium specific for the growth of isolated strains containing 0.3% of "Oxagall" bile, which represents the concentration proposed by [22]. To do this, about 3g of "Oxagall" bile was introduced into 100 mL of growth-specific broth of each strain. A few drops of methyl red were added to each tube giving the red color to the contents. Strains were seeded in specific broths. A control was made by sowing each strain on the same broth without addition of bile. After incubation the growth of the strains was noted by the discoloration of the tubes from red to yellow [23].

Antimicrobial Activity

The antimicrobial activity of the strains was demonstrated according to the method used by [24] rehabilitated using Blanck Discs (Liofilchem s.r.l.). The reference pathogenic strains used are: *Escherichia coli* ATCC 29522, *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29911 and *Pseudomonas aeruginosa* ATCC 27853.

Enzymatic Activity

Lipolytic and proteolytic activities were tested according to spot method. Thus

the appearance of transparent areas translates the activity or an absence in the opposite case [23]

Statistical analysis

Frequencies and averages were calculated by the Microsoft Excel 2013 software. The photos and figures were processed by the paint.net software version 4.0.6.

3. Results

3.1. Characteristics of Strains

Macroscopic observation revealed several types of colonies of different size, shape, and color. As for the microscopic observation, it showed cells in the form of *Bacillus*, hull, ovoid, isolated and in chain more or less long. *Bacillus*, all bacilli are 100% gram, catalase and oxidase positive. Of the 13 lactic acid bacteria selected, 76.92% were bacilli against 23.08% shell. They were all gram positive colonies but totally devoid of catalase and cytochrome oxidase. The yeasts were of variable size and shape, all provided with catalase and oxidase. Thus, 7 *Bacillus* 5 were retained for identification. For lactic acid bacteria 7 out of 13 were chosen. For yeasts, all 13 have been molecularly identified.

3.2. Molecular Characterization of Strains

Identification of *Bacillus* and lactic acid bacteria by PCR respectively with primer pair BK1F/BK1R and LbF/LbR: **Figure 1(a)** shows the profile of the *Bacillus* strains amplified by the primer pair (BK1F/BK1R). This figure reveals the presence of a band specific to the genus *Bacillus* whose size is between 1000 - 1200 bp for strains BC1a; BC9b: BC2a and BC1b. The profile of isolated lactic acid bacteria strains amplified by the LbF and LbR primer pair specific to the *Lactobacillus*

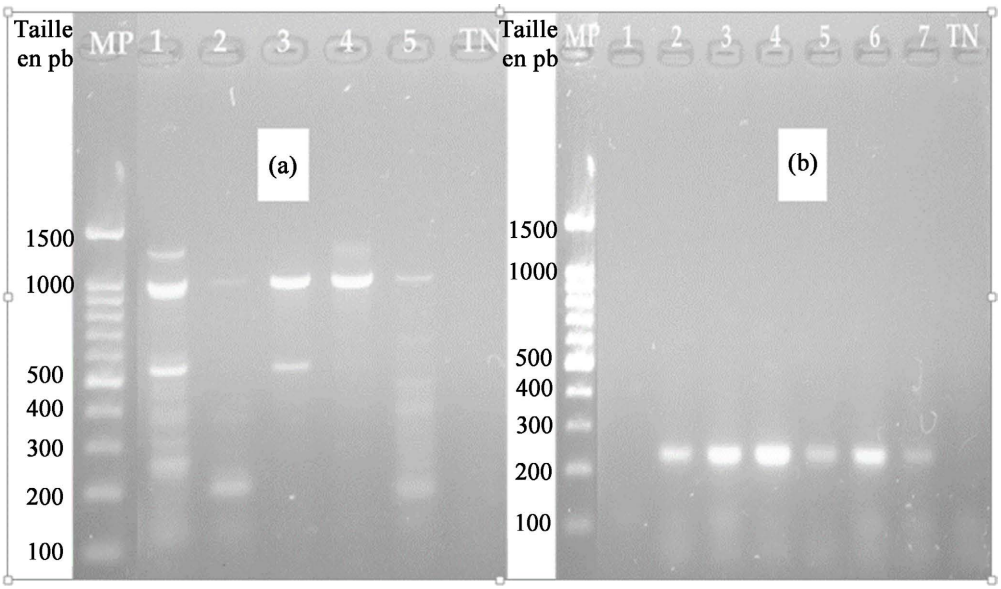

Figure 1. Identification of *Bacillus* and lactic acid bacteria. (a) MP: Molecular weight marker; 1: BC1a; 2: BC9a; 3: BC9b; 4: BC2a; 5: BC1b. (b) 1: BL12a; 2: BL5d; 3: BL12c; 4: BL12g; 5: BL5b; 6: BL5c; 7: BL21a.

genus. Thus, a specific 230pb band is observed for the BL5d strains; BL12c; BL12g; BL5b; BL5c; BL21a corresponding to the genus *Lactobacillus*. While, the amplification of strain BL12a was negative (Figure 1(b)).

Yeast identification by PCR: For yeasts, two pairs of primers specific *to Candida krusei* species (CkFKSfor359/CkFKSrev359) and *Saccharomyces cerevisiae* (SC-fw/SC-rw) were used for molecular identification. Amplification of the strains with the Candida krusei primer pair revealed no specific band for this species (Figure 2(a)). Figure 2(a) shows the profile of the yeasts amplified by the primer pair specific for the *Saccharomyces cerevisiae* species. Note the presence of a specific band of about 215 bp with only the strain Lev5c.

3.3. Probiotic Fitness of Isolated Strains

Susceptibility of antibiotics: the *Bacillus* strains tested were resistant to five antibiotics (Ox, STR, P, E and TIC), total sensitivity to two antibiotics (GEN and IMI) and intermediate resistance to two antibiotics (AMP and K). Thus the susceptible to antibiotics depends on one strain to another. The BL5b strain was sensitive to all antibiotics (Table 2).

Strain resistance to pH and bile salts: Table 3 summarizes the results obtained;

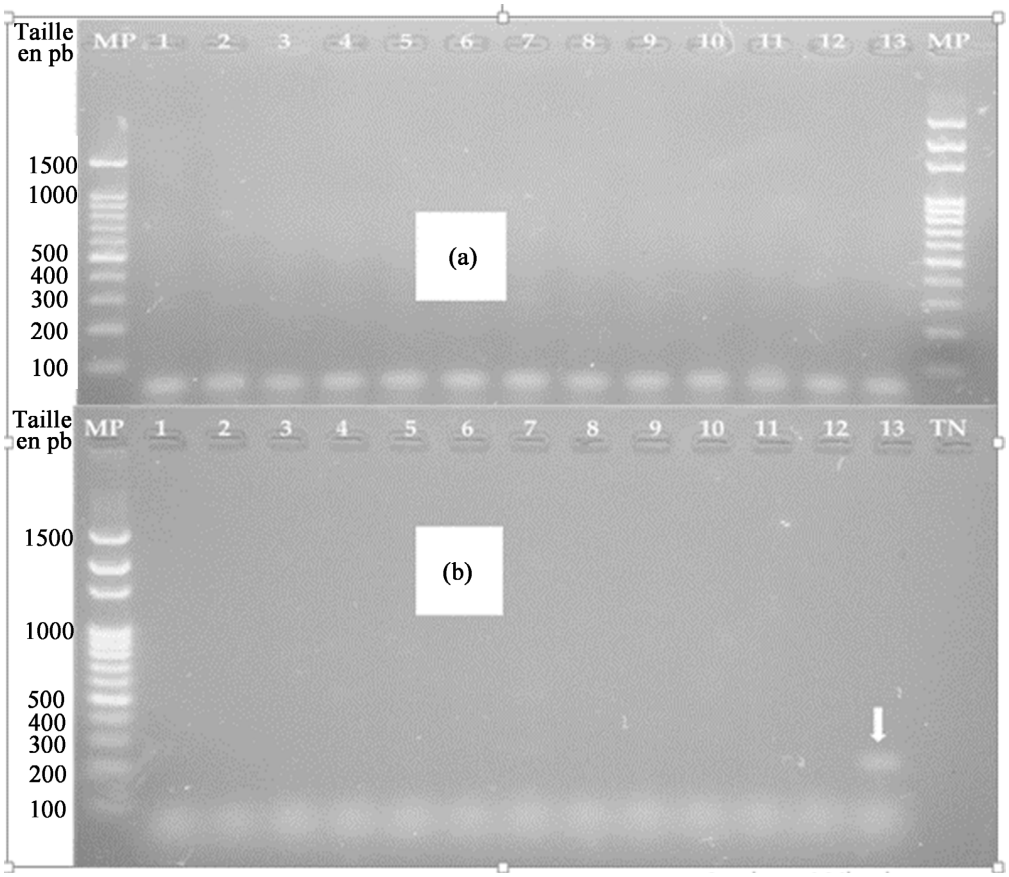

Figure 2. Yeast identification from *Candida krusei* and *Saccharomyces cerevisiae*. 1: Lev3; 2: Lev21b; 3: Lev9; 4: Lev2a; 5: Lev28c; 6: Lev21a; 7: Lev21d; 8: Lev5a; 9: Lev21a; 10: Lev28a; 11: Lev21c; 12: Lev5d; 13: Lev5c.

Table 2. Antibiotic Susceptibility of *Bacillus* Strains and Lactic Acid Bacteria.

Strains/Codes		Antibiotics										
		Ox	AMX	AMP	K	AMC	GEN	IMI	STR	P	E	TIC
Bacillus	BC1a	R	R	S	S	R	S	S	R	R	R	R
	BC1b	R	R	R	S	R	S	S	R	R	R	R
	BC2a	R	S	S	S	S	S	S	R	R	R	R
	BC2b	R	R	I	I	R	S	S	R	R	R	R
	BC9b	R	S	S	R	R	S	S	R	R	R	R
Lactic acid bacteria	BL5b	S	S	S	S	S	S	S	S	S	S	S
	BL5c	S	S	R	S	S	S	S	S	S	S	R
	BL12a	R	I	S	I	S	I	S	R	S	R	S
	BL12c	R	R	S	R	R	R	R	R	R	R	S
	BL12g	R	R	R	R	S	R	S	R	R	R	R

AMX: Amoxicillin; GEN: Gentamicin; IMI: Imipenem; Ox: Oxacillin; AMP: Ampicillin; E: Erythromycin; P: Penicillin; STR: Streptomycin; K: Kanamycin; ICT: Ticarcillin; AMC: Amoxicillin-Clavulanic Acid; R: Resistant; S: Sensitive; I: Intermediate

Table 3. Strain resistance to pH and bile.

Strains/Codes		Bile (0.3%)		pH				
		Resistance	pHf	2.5	4.5	7.2	pHf (2.5)	pH (4.5)
Bacillus	BC1a	++	7.11	−	−	++++	2.49	4.42
	BC1b	++	6.69	−	−	++++	2.60	4.02
	BC2a	+	6.45	−	−	++++	2.51	4.05
	BC2b	++	6.99	−	−	++++	2.59	4.28
	BC9b	++	6.75	−	−	++++	2.53	4.62
Lactic acid bacteria	BL5b	+	6.17	−	−	++++	2.71	2.61
	BL5c	+	6.38	−	+	++++	2.51	2.76
	BL12a	++	6.60	−	+	++++	2.46	2.68
	BL12c	++	6.18	−	+	++++	2.48	2.58
	BL12g	+++	7.14	−	−	++++	2.64	2.62
Yeasts	Lev3	+	6.28	−	+	++++	2.67	4.05
	Lev5c	+	6.34	−	++	++++	2.47	3.96
	Lev5d	++	6.62	−	++	++++	2.64	4.18
	Lev21c	++	6.67	−	+++	++++	2.73	3.89
	Lev28a	+	6.51	−	++	++++	2.76	4.04

−: No growth; +: sensitive turn, ++: partial turn; +++: total turn; ++++: appearance of the tube; pHf: final pH of the solution after incubation; Lev: Levure.

visual observation of the state of fading of the tubes and taking the final pH. All strains survived the presence of bile (0.3%) and acidified culture media (decreased pH). For all strains, no discoloration of the culture media was observed at pH 2.5 and pH 4.5 at all with *Bacillus*. While discoloration of the culture media was observed at pH 4.5 for all yeast strains tested. Discoloration was also observed in three strains of lactic acid bacteria (BL5c, BL12a and BL12c) at pH 4.5 (Table 3).

Antimicrobial activity: among *Bacillus*, only strain BC9b showed action of inhibitory actions against pathogens *Enterococcus faecalis* ATCC 29911 and *Pseudomonas aeruginosa* ATCC 27853 with diameters of 10 mm. Similarly, two strains of lactic acid bacteria (BL5c and BL12c) inhibited these two pathogens. The inhibitory action of yeasts was more remarkable because only two strains (Lev3 and Lev21c) were ineffective against *Escherichia coli* ATCC 29522. The other strains were able to inhibit pathogens with diameters ranging from 10 to 21 mm (Figure 3(a)).

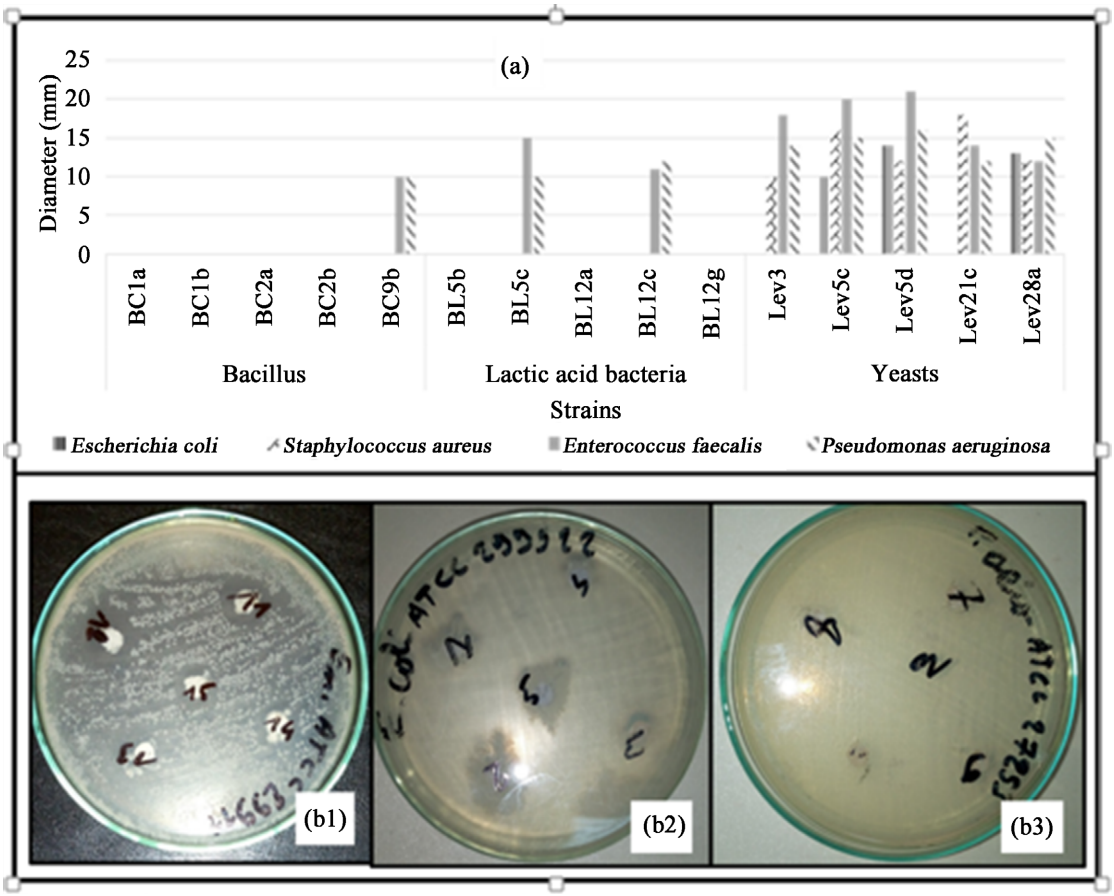

Figure 3. Pathogens Inhibition Diameters by the Strains Tested. (a) *Escherichia coli* ATCC 29522; *Staphylococcus aureus* ATCC 25923; *Enterococcus faecalis* ATCC 29911; *Pseudomonas aeruginosa* ATCC 27853. (b1): Zones of inhibition of *Enterococcus faecalis* ATCC 29911 by yeasts; (b2): Invasion without inhibition of *Bacillus* in the presence of *Escherichia coli* ATCC 29522; (b3): Action of lactic acid bacteria *on Pseudomonas aeruginosa* ATCC 27853; 1: BC1a; 2: BC1b; 3: BC2a; 4: BC2b; 5: BC9b; 6: BL5b; 7: BL5c; 8: BL12a; 9: BL12c; 10: BL12g; 11: Lev3; 12: Lev5c; 13: Lev5d; 14: Lev21c; 15: Lev28a.

Figure 3(b) shows the zones of inhibition of pathogenic strains by isolated strains. All yeasts exhibited an inhibitory action against Enterococcus faecalis ATCC 29911 (Figure 3(b1)). *Bacillus* colony invasion is observed without inhibiting *Escherichia coli* strain ATCC 29522 (Figure 3(b2)), but in lactic acid bacteria the inhibitory action is only visible with strain 7 (BL5c) and 9 (BL12c) (Figure 3(b)).

Enzymatic activity: All the strains tested presented enzymatic activities through the appearance of the transparent zones around the colony spots except the BC9b strain in *Bacillus*. Figure 4(a) shows the lipolytic activity of *Bacillus* strains on red palm oil, Figure 4(b) on lactic acid bacteria and Figure 4(c) on yeast. Figure 4(d), Figure 4(e), and Figure 4(f) show the ability of isolated strains to hydrolyze milk proteins. Transparent areas appear around all the colonies deposited in spots. The activity is stronger in *Bacillus* (Figure 4(d)) than in lactic acid bacteria (Figure 4(e)) and yeasts (Figure 4(f)).

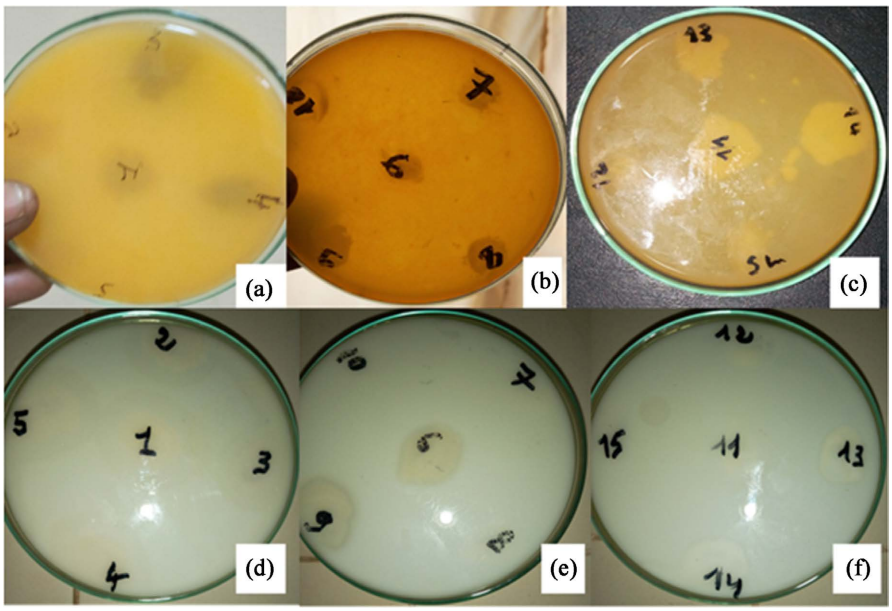

Figure 4. Enzymatic activity of isolated strains. (a) Lipolytic activity of *Bacillus* strains; (b) Lipolytic activity of lactic acid bacteria; (c) Lipolytic activity of the yeasts; (d) Proteolytic activity of *Bacillus* strains; (e) Proteolytic activity of lactic acid bacteria; (f) Proteolytic activity of yeasts.

4. Discussion

The strains characterized in this study come mainly from *Benkida*. This porridge is the most produced and popular by the population unlike other porridge such as *Benkoonré* and rice porridge [25]. Among these strains, *Bacillus* were dominant in the flora of these porridges because of their ability to sporulate. But we also note the presence of lactic acid bacteria and yeasts in these porridge. Thus the identification of these microorganisms has been confirmed by PCR via the use of specific primers.

Of the five presumptive *Bacillus* strains selected for identification, four (BC1a,

BC9b: BC2a and BC1b) were confirmed *Bacillus* after molecular characterization by PCR. The presence of *Bacillus*-specific bands around 1000 bp confirms their identity (**Figure 1(a)**). These results are similar to those reported by [26] [27] and [28] who used the same primer pairs for the identification of *Bacillus* isolated from fermented foods respectively from Burkina Faso, Tchad and Gabon.

As for the identification of lactic acid bacteria, the amplification revealed the presence of *Lactobacillus*-specific bands whose size is close to 230 bp (**Figure 1(b)**). Thus, six (6) strains were confirmed as *Lactobacillus* (BL5d, BL12c, BL12g, BL5b, BL5c, and B21a). This same result was reported by [29] and [30] when identifying lactic bacteria isolated from *Attiéké*, a cassava-based fermented food.

For the identification of yeasts via the use of Candida krusei-specific primer, no specific band was revealed (**Figure 2(a)**). The use of *Saccharomyces cerevisiae*-specific primer confirmed the identity of the Lev5c strain, as a specific band belonging to this strain was revealed (**Figure 2(b)**, 215 bp). Similar results have been reported by [29] and [30]. Other strains that did not give specific bands could belong to other genera such as *Cryptococcus*, *Geotrichum*, *Brettanomyces*, *Leucosporidium*, and *Kluveromyces*. Only primers specific to these genera will allow a complete identification of isolated strains.

Some bacteria have developed antibiotic resistance mechanisms. These mechanisms include the production of various antibiotic inactivation enzymes, modifications of sites of attack and antibiotic permeability [31] [32] reported that *Bacillus* has a capacity for resistance to beta-lactams. A strain of lactic acid bacterium showed sensitivity to all antibiotics tested. The work of [33] reported a relative sensitivity of lactic acid bacteria and intrinsic resistance of *Lactobacillus* to the aminoglycoside family.

The discolourations observed in some tubes reflect growth of strains that have probably released metabolites. The latter varied the pH, which resulted in the shift of methyl red. The resistance to bile presented by all the strains tested offers interesting information on the satisfaction of one of the criteria of eligibility as a probiotic. As for the ability to withstand the pH noted especially in yeasts and some strains of lactic acid bacteria, this could be related to the fact that the pH value is within the tolerance limits. *Bacillus* did not survive pH, even at 4.5, but acidification was noted in some cases, which is why pH declines were observed [34].

A single strain of *Bacillus* (BC9) showed efficacy against two pathogens (*Enterococcus faecalis* ATCC 29911 and *Pseudomonas aeruginosa* ATCC 27953), confirming its ability to produce bacteriocins. These results were obtained by [35] who identified *Bacillus* strains producing these substances against pathogens such as Micrococcus luteus. Our results showed that two strains of lactic acid bacteria inhibit the same pathogens as those made with the genus *Bacillus*. [36] obtained inhibitory actions of *Lactobacillus* strains on *E. coli* and *Staphylococcus aureus*, but these strains were isolated from cheeses confirming that the antimicrobial activity of the strains also depends on the food matrix. As for yeasts, their activity was noticed with greater inhibition diameters on reference pa-

thogens compared to those of *Bacillus* and lactic acid bacteria. These results are confirmed by the work of Hatoum who identified in milk mycocine-producing yeasts capable of inhibiting several enterobacteria [37].

All strains tested exhibited proteolytic activities. These results are confirmed by the work of [21]. The proteolytic activity was much more visible for *Bacillus* compared to lactic acid bacteria (moderately) and yeasts (weakly). As for the lipolytic activity, it remains as low in the latter two groups of microorganisms namely lactic acid bacteria and yeasts [38].

5. Conclusion

This study allowed the isolation of three categories of strains namely *Bacillus* lactic bacteria and yeasts. The molecular biology method allowed the identification of strains from *Bacillus*-specific primers, *Lactobacillus* and yeasts belonging to the species Saccharomyces cerevisiae. These results are close to those obtained from the preliminary tests. These strains, mostly non-pathogenic according to literary research, may be of significant technological interest after further tests, namely probiotic efficacy and amylolytic potency. The tested strains resisted a wide range of antibiotics except Gentamicin and Imipenem. This resistance of bacteria to antibiotics is considered a public health problem according to the World Health Organization (WHO). An exhaustive study is necessary for the rigorous selection of non-resistant strains proving necessary. Tests on some enzymatic activities have provided interesting results proving that part of the microbiota because of their properties can be standardized

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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