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Molecular identification of biofilm-producing *Bacillus* species and yeasts isolated from food sources and their interaction with *Lysinibacillus louembei* strain

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The microorganisms contained in fermented foods are known to harbor metabolic products, possibly improving human and animal health. However, despite several studies on the functional effects of fermented foods, isolation and identification of the effective *Bacillus* species strains are still in progress. The objective of this study was to molecularly identify biofilm-producing *Bacillus* spp. (BPB) and yeasts from fermented food sources and to study their interactions with the *Lysinibacillus louembei* strain. A total of 133 isolates of *Bacillus* as well as 32 isolates of yeast were obtained for detailed identification and investigation. Based on a combination of phenotypic and molecular characterization using *fibE* polymerase chain reaction (PCR) multiplex and ITS-PCR techniques, species of *Bacillus* were identified as *Bacillus pumilus* (12%), *Bacillus subtilis* (12%), *Bacillus safensis* (6%), *Bacillus amyloliquefaciens* (6%), *Bacillus licheniformis* (6%), and *Saccharomyces cerevisiae* (0.05%). The *yfiQ, epsH, ymcA,* and *tasA* genes involved in the biofilm formation process were amplified by using PCR multiplex in *B. subtilis, B. licheniformis,* and *B. pumilus* have been identified and confirmed. As a phenotypic result, 45% of isolates of BPB by using the Congo Red Agar method (CRA) have been identified. The ability of *Bacillus* and yeasts to produce biosurfactants was tested by using the emulsification index (EI24). 65 and 69% of *Bacillus* and yeast isolates were able to emulsify petrol. 56% of the crude extract of biosurfactants from *Bacillus* isolates demonstrated antimicrobial activity against *Escherichia coli, Staphylococcus aureus,* and *Salmonella* species cultures were done between *Bacillus* spp., *S. cerevisiae,* and *L. louembei.* As a result, commensalism-like interactions were obtained in yeast strain V3 and *B. pumilus* strain VB15 and *L. louembei* and *B. amyloliquefaciens,* competition-like interactions in *S. cerevisiae* strain P3 and *Bacillus* spp. strain VP11, and amensalism-like interactions with *B. pumilus* and *S. cerevisiae* and *Bacillus* spp. strain VP34 and *S. cerevisiae* strain P1. These results illustrate that microorganisms maintain different relationships that occur during fermentation process.

Keys words: *Bacillus, Saccharomyces cerevisiae, Lysinibacillus louembei,* fermented foods, microorganism interactions, biosurfactants, biofilms.

INTRODUCTION

Fermentation of various food stuffs by microorganisms is one of the oldest forms of food biopreservation (Diaz-

Munoz et al., 2022; Mgbodile and Nwagu, 2023), and leads to various products (Mgbodile and Nwagu, 2023). As previously reported, fermented foods are ecological niches abounding in significant biodiversity of microorganisms. It represents an asset for the daily meals of households. Lactic acid bacteria (LAB), *Bacillus* species, and yeasts are the most commonly found in fermented foods (Kayath et al., 2020). In the same ecosystem, *Bacillus subtillis*, *Bacillus pumilus*, *Bacillus mojavensis*, *Bacillus safensis*, *Bacillus amyloliquefaciens*, *Bacillus licheniformis,* and *Bacillus altitudinis* could be isolated (Kimura and Yokoyama, 2019). Microorganisms including *Bacillus*, LAB, and yeasts are able to produce biosurfactant-like molecules that strongly contribute to the biopreservation of fermented foods (Elenga-Wilson et al., 2021; Marchut-Mikolajczyk et al., 2021; Stancu, 2020; Wu et al., 2022). Studies showed that *Lysinibacillus louembei* is able to produce various biomolecules, including biosurfactants, proteases, cellulases, and amylases (Kaya-Ongoto et al., 2020). Fermented foods are produced through controlled microbial growth, and the conversion of food components through enzymatic action. The microorganisms are able to produce proteases, amylases, cellulases, and pectinases that are important for a couple of biotechnological interests (Dai et al., 2020; Rajesh and Gummadi, 2022). Many studies have demonbstrated that many bacteria are also able to participate in the formation of molecular complexes such as biofilms (Arnaouteli et al., 2021; Qin et al., 2022).

Biosurfactant-like molecules could accumulate at the interface area between liquid phases and hydrogen bonding. In the food industry, biosurfactants are widely used for their properties in food preservation (Anjum et al., 2016). Microorganisms live in association by building multicellular tissues called biofilms. Biofilm-like structures involve different mechanisms and can harbor one or more species of bacteria and yeasts (Bisht et al., 2023; Zara et al., 2020). *B. subtilis* is widely used as a model organism to study the formation and characteristics of bacterial biofilms (Arnaouteli et al., 2021; Qin et al., 2022). Some genes have been found to be involved in the biosynthesis of biofilm molecules. Loci involved in biofilm formation are generally named *eps* or *cps* in *Bacillus.* Other loci for polysaccharide production could be localised on plasmids in *Lactococcus* and *Bacillus* spp. In *B. subtilis,* exopolysaccharide matrix is under the expression of the *eps*A-O locus (Irnov and Winkler, 2010)*.* One of the genes of this locus, *eps*E*,* encodes a bifunctional protein, which is at the same time a glycosyltransferase involved in the inhibition of mobility by blocking flagellar rotation. Four genes (*eps* H, I, J, and K) of this locus intervene in the production of N-acetyl-galactosamine (Blair et al., 2008).

Microbial interactions have been described many years ago (Schroeckh et al., 2009). Some of them have been qualified as direct and indirect interactions (commensalism, mutualism, competition, amensalism, parasitism, etc.) resulting in the production of compounds (Sieuwerts et al., 2008). During fermentation, microorganisms coexist and interact with each other to lead to a quality end product (Kayath et al., 2020; Kimura and Yokoyama, 2019).

Yeasts have been shown to be involved in increasing polyphenols compounds and may also interact synergistically with *Bacillus* spp. (Kayath et al., 2020). To our knowledge, interaction between bacteria and yeasts remains a big challenge. Many previous studies did not clearly explain the molecular interactions in fermented foods. The objective of this study was to molecularly identify biofilm-producing *Bacillus* spp. (BPB) and yeasts isolated from food sources and to study their interaction with the *L. louembei* strain.

METHODS

The microorganisms tested in this study were collected from fermented foods and beverages, including palm wine (*Nsamba (VP)*), fermented cassava leaves (*Ntoba mbodi (NM)*), banana wine (*Mbamvu (VB)*), and ginger wine (G) (*Tangawiss* as local name). *L. louembei* has been previously isolated in our laboratory (Ouoba et al., 2015).

Isolation and characterization of *Bacillus* and yeasts strains

Ten-fold serial dilutions of each sample including VP, NM, VB, and G were prepared in sterilized distilled water. For *Bacillus* isolation, isolation and characterization have been previously described (Elenga-Wilson et al., 2021). For yeast isolation, decimal dilutions were inoculated on Sabouraud agar medium supplemented with 0.1 mg/L of chloramphenicol. Incubation was done at 30°C for 24 to 48 h. Each colony associated with different phenotypic characteristics was separately isolated. The purification of isolates was carried out as in the case of Bacilli. Purity was estimated by using microscopic observations in terms of morphological characterizations.

Detection of *Bacillus* spp. ability of biofilms formation

Phenotypic characterization of slime-producing ability

Detection of biofilm formation using Congo Red Agar has been evaluated according to a modified and adapted protocol recommended as previously discribed (Freeman et al., 1989). This method consists of the cultivation of isolates on Congo Red Agar. The Congo Red Agar (CRA) medium is composed of 23.5 g/L PCA medium, 50 g/L sucrose, and 0.8 g/L Congo Red dye. Congo Red has been separately prepared from other constituents of the medium in the form of a concentrated aqueous solution and then autoclaved; then it was added when the agar cooled to around

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Table 1. Primers used for PCR amplification of encoding internal spacers transcribed in yeasts.

Primers	Sequence
ITS1	5'-TCCGTAGGTGAACCTGCGG-3'
ITS4	5'- TCCTCCGCTTATTGATATGC-3'

55°C. The different isolates were inoculated by streak seeding. Petri dishes were incubated at 37°C for 24 h. The ability to form biofilm is revealed by the presence of black colonies on a lens of dry consistency.

Production of biofilms by using crystal violet

A qualitative method for evaluation of biofilm formation has been used (Christensen et al., 1982). Each *Bacillus* isolate was cultured in 10 mL of Trypticase Soy Broth (TSB) supplemented with 2% glucose (TTM), the whole being contained in tubes. For 24 to 48 h, the tubes are incubated aerobically at 37°C. The tubes were then gently decanted by tapping them and washed with PBS pH 7.3, and stained with 2% crystal violet (gentian violet). After incubation at 37°C for 30 min, tubes were rinsed with distilled water and dried in the reverse position on the bench. The biofilm formation is signed as positive when a visible film lines the wall and/or the bottom of the tubes, which gives a blue-purplish coloration. Tubes were examined and biofilm formation was noted as absent (-), weak or moderate (+), and strong (++). The test was performed in triplicate for each isolate.

Ability of *Bacillus* and yeasts to produce biosurfactants

Ability of *Bacillus* isolates and yeasts to produce biosurfactants have been done as previously experimented (Elenga-Wilson et al., 2021). E24 represents the emulsification index which is the total height of the hydrocarbon (Ht) and the height of the emulsion (He) was measured using a graduated ruler. The values obtained allowed the calculation of the emulsification index E_{24} according to the formula:

$$E_{24} = \frac{HE}{HT} \times 100$$

where He= height of emulsion layer; Ht= total height of solution; E_{24}= emulsion index for 24 h.

Biosurfactant extraction by chloroform and ammonium sulfate

First of all, 5 mL of overnight culture were fuged. The supernatant coming from each isolate was added to an equal volume of chloroform (v/v). The mixture is strongly agitated by a vortex. After centrifugation at 6.000 rpm for 10 min, the non-aqueous phase is recovered. Evaporation of the solvent was completely done at room temperature. The residue is dissolved in a phosphate buffered saline (PBS) containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH 7.0. In terms of ammonium sulfate, an overnight culture has been fudged at 13.000 rpm for 15 min to separate the supernatant and pellet. The supernatant was then mixed with ammonium sulfate (80%) for 15 min. And finally, this has been incubated overnight. The mixture has been fuged at 6.000 rpm for 30 min. Pellets have been homogenized by using PBS. For both extractions (chloroform and ammonium sulfate), the emulsifying activity was tested in comparison with the supernatant at the start.

Antimicrobial activity of biosurfactant extracts

An overnight culture at 37°C of *Staphylococcus aureus*, *Escherichia coli,* and *Salmonella* species were done on specific media including Chapman medium, EMB medium, and S.S medium as well. A fraction of each pathogen strain was scraped and mixed with physiological water until reaching an OD corresponding to 0.5 McFarland. The next step consists of inoculating with a swab the inoculum of the pathogen previously obtained in physiological water on MH medium (or PCA) by making tight streaks in three directions. The Petri dishes are then dried for 20 to 30 min at 37°C. 20 µL of biosurfactant extracts from each *Bacillus* spp. are deposited on medium where the pathogenic strain to be tested has been previously inoculated. The dishes were incubated at 37°C for 24 h, and then the diameters of the inhibition halos were measured to judge the effectiveness of the biosurfactant.

Molecular identification of *Bacillus* isolates

To target strains such as *B. amyloliquefaciens*, *B. subtilis*, *B. pumilus*, *B. licheniformis*, and *B. safensis*, a PCR multiplex using the *fibE* gene encoding for fibrinolytic enzyme was used. The *fibE* primers were taken from the work first carried out and previously discribed (Kaya-Ongoto et al., 2020) Extraction and purification of genomic DNA of isolates were performed according to the NucleoSpin Microbial DNA (Macherey-NAGEL) kit. DNA purity was assessed by electrophoresis on a 1% agarose gel and by the ratio of optical densities of 260/280 nm. The genomic DNA obtained was used as a template for all PCR amplification experiments. In terms of yeast, a modified and adapted protocol was performed in our laboratory according to the protocol described in the previous study (Dymond, 2013).

Yeast identification by using ITS PCR amplification

A PCR reaction was carried out in a final volume of 50 µL containing 17 µL of distilled water, 4 µL of DNA, 2 µL of each primer (Table 1), and 25 µL of master mix. The 0.2 mL microtubes are then placed in a thermal cycler (Biorad, Singapore). PCR conditions included initial denaturation at 95°C for 5 min, followed by 25 cycles each comprising denaturation at 95°C for 30 s, hybridization at 60°C for 30 s, an extension at 72°C for 30 s and a final extension at 72°C for 7 min.

Colony multiplex PCR amplification of *epsH*, *tasA*, and other genes in *Bacillus* spp.

Genes involved in biofilm formation were also amplified in this work using the multiplex PCR colony approach. To achieve this, cultures of each bacterium were heated at 95°C for 15 min in a volume of 15 µL of sterile distilled water with a thermocycler to break cells for releasing genetic material that will be used as a matrix. Specific primers were generated from sequences uploaded to the NCBI portal (National Center for Biotechnology Information, https://blast.ncbi.nlm.nih.gov/Blast.cgi) genomic database of targeted strains. Microbesonline (http://www.microbesonline.org) has been used for checking lists. pDRAW32 software has been used for bioinformatics analysis. These different primers are listed in Table 2. A multiplex PCR reaction was carried out in a final volume of 50 µL comprising 25 µL of master mix, 2 µL of each

Table 2. Primers used for amplification of genes involved in *Bacillus* spp. biofilms.

Primers	Sequence (5'----3')	Size (pb)	Strain
BPymcAF	ACGCTTTATTCAAAAAAAGAGATTG	435	*Bacillus pumilus*
BPymcAR	TCATTTAATGGAGCAACTTGGT		
BLymcAF	GTGACGCTTTATACGAAAAAAGAGATT	432	*Bacillus licheniformis*
BLymcAR	TTATAGAGAACAGCTCGGTGAT		
BLyfiQF	TTTTGCTTCATGCGATATCAATGG	1089	
BLyfiQR	TTAACCTGCATGATGGCGC		
BStasA1F	ATGGGTATGAAAAAGAAATTGAGTT	786	*Bacillus subtilis*
BStasA2R	TTAATTTTTATCCTCGCTATGCG		
BSepsHF	AAACACCTGCGGTTAGTCTG	1035	
BSepsHR	TCACCCTCTGTTTCTCATTTTGTA		

Table 3. Distribution of isolates obtained by sample.

Sample	*Bacillus*	Yeast
Palm wine (VP)	46	9
Ginger wine (G)	32	14
Banana wine (VB)	17	9
Ntoba mbodi (NM)	39	-
Total	134	32

primer, 16 µL of ultrapure water, and 5 µL of matrix DNA. PCR conditions included initial denaturation at 95°C for 5 min, followed by 30 cycles comprising denaturation at 95°C for 30 s, a gradient hybridization from 55 to 60°C for 30 s, an extension at 72°C for 60 s and a final extension at 72°C for 7 min. 5 µL of each amplification product was mixed with 2 µL of loading buffer (BIOKE). Mixtures were subjected to electrophoresis on 1.5% agarose gel (w/v).

Interaction between *Bacillus* spp. and other microorganisms

Coculture between Bacillus spp. and yeasts

Each isolate's colony fraction was scraped off the agar and launched separately into 50 mL of nutrient broth, where it was incubated at 37°C at 150 rpm. To determine and/or to compare the type of interaction, 0.5 mL of each exponential growth culture was innoculated in single and mixed cultures. During growth, the enumeration in CFU.mL^{-1} of microorganisms has been done every 12 h. For yeast enumeration, Sabouraud agar medium supplemented with 0.1 mg/L of chloramphenicol has been used, and Mossel agar medium for *Bacillus* spp. Each experiment is done in triplicate.

Coculture between B. amyloliquefaciens-NM11 and L. louembei

The same protocole has been used. The Mossel medium was only used to enumerate the flora of *B. amyloliquefaciens* and *L. louembei* after incubation aerobically at 37°C. For *L. louembei*, CFU was then determined by subtracting the total CFU of coculture with *B. amyloliquefaciens* and CFU was found in coculture on Mossel medium supplemented with 6 mg/mL of gentamicin (an antibiotic used for the selection *B. amyloliquefaciens*). Each experiment was done in triplicate.

RESULTS

Isolation and characterization of *Bacillus* and yeasts strains

From the biological materials used, a total of 134 isolates were obtained from Mossel medium, of which 46 were obtained from palm wine samples, 17 from banana wine, 32 from ginger juice, and 39 from fermented cassava leaves. However, on Sabouraud + chloramphenicol medium, a total of 32 isolates were obtained, including: 9 from palm wine samples, 9 from banana wine, and 14 from ginger juice. As for the 32 yeast isolates, the characterization indicates that all of these yeasts divide by budding and are immobile. The majority (60%) of the cells were ovoid in shape (Table 3) with whitish coloration, regular outline and creamy consistency.

Detection of *Bacillus* spp. capacity to form biofilms

Phenotypic characterization of slime-producing ability

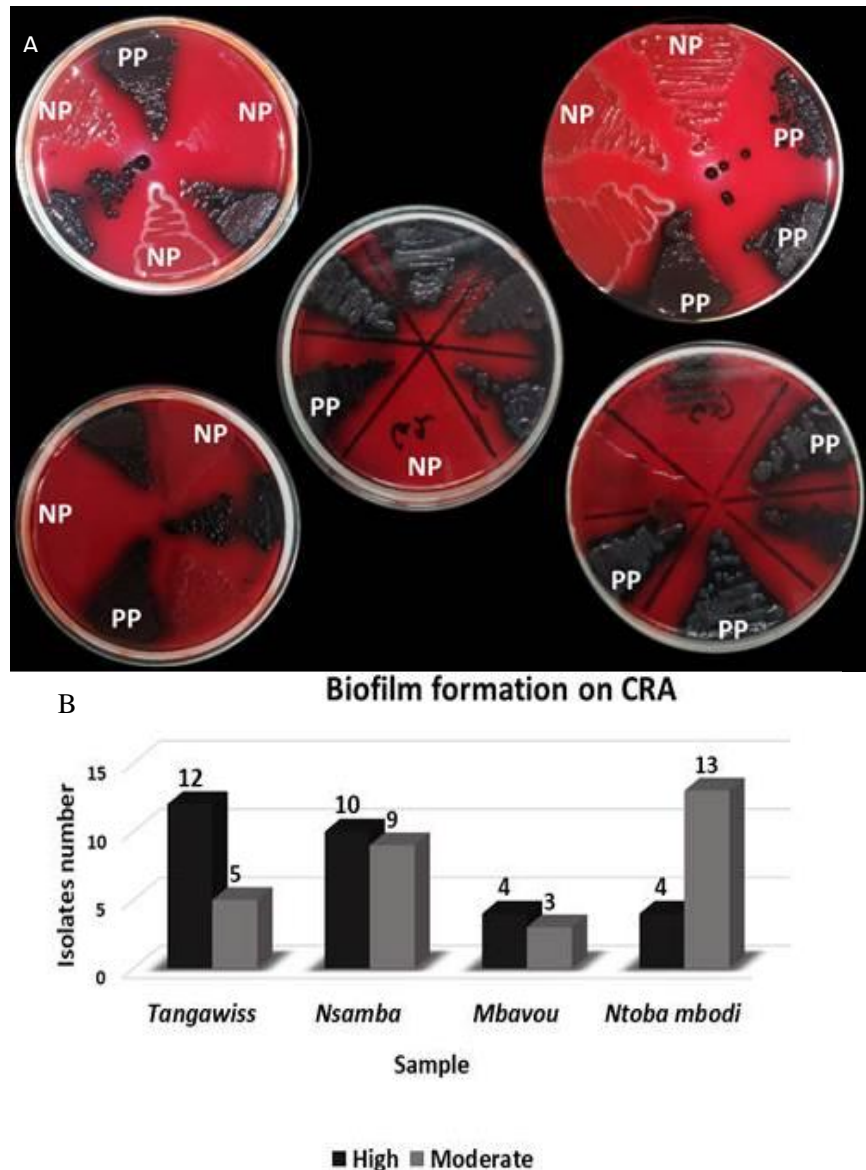The test of biofilm production by the Congo Red Agar

Figure 1. A: Capacity of strains for biofilm formation on Congo Red Agar (CRA) by *Bacillus* spp. NP: Negative phenotype, PP: positive phenotype. B: biofilm partitioning based on sample and phenotype.

method showed that, out of a total of 133 isolates tested, we have 60 isolates or 45% were positive. This positive phenotype is thus reflected by black colonies (strong or moderate coloration) with a crystalline lens of dry consistency on Congo Red Agar after incubation for 24 h at 37°C. This aspect is due to the reaction between Congo Red and exopolysaccharides produced by the isolates tested. 73 isolates or 55% were nonproducers of biofilms, presenting red colonies on the same agar medium. The phenotypic aspect of these results is as shown in Figure 1A. Additionally, Figure 1B shows the repartition of isolates capable of producing biofilm per sample.

Methods for producing biofilms in a tube and staining with crystal violet/gentian violet

Twenty one positive isolates with a better profile of biofilm formation on Congo Red Agar (CRA), as well as 25 isolates negative for the CRA test, were selected for this test. Among the 21 isolates (previously positive for CRA) tested, 81% or 19 isolates remained positive (15++ and 2+), while 19% or 2 isolates were negative. On the other hand, of the 25 isolates negative for CRA, 36% or 9 isolates were positive (+) while 64% or 16 isolates remained negative. However, 9 isolates which were found to be positive when performing the test tube
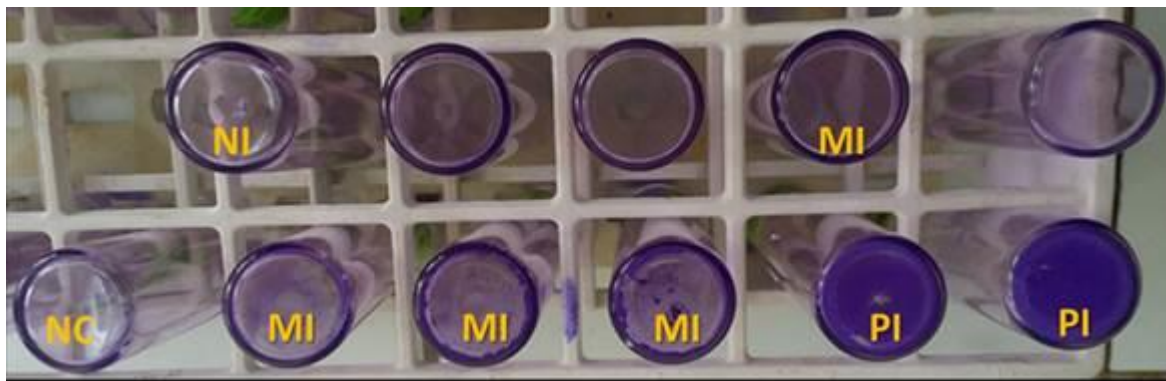
Figure 2. Screening of biofilm production by the tube method (TTM). Scores of 50 and 100%, were respectively, assigned to the MI: moderate isolate and PI: positive isolate. 0 was assigned to the isolate with negative results (Table 4). NI: Negative control, NC: negative control.

Table 4. Biofilm formation using TTM.

Bacillus isolates	Result	Assigned score (%)
G1, G5, VB11, VB18, NM14, NM53, VP16	Positive and strong	100
VP2, VP21, G14, G18, VB10, NM22	Positive and moderate	50
G6, G13, G20, G21, VP18, VP19	Negative	0

method with Trypticase Soy Broth supplemented with glucose were again negative for the test tube method using TSB supplemented with sucrose. Biofilm formation resulted in a film lining the bottom of the test tube and/or the wall (Figure 2 and Table 4).

Testing of *Bacillus'* ability and yeasts to produce biosurfactants

Isolates of *Bacillus* spp. have also shown a good ability to produce biosurfactants. At the end of the two methods used, we thus determined the emulsification index after 24 h (EI24), in particular, first from total culture, then from the cell-free supernatant after centrifugation. Consequently, out of the 60 isolates tested (able to form biofilm), 39 isolates or 65% were able to emulsify the gasoline with an EI24 between 5 and 100% after 24 h of incubation at room temperature. 11 of the 39 positive isolates obtained an EI24 ≥ 50% with the total culture, and 5 other isolates obtained an emulsification index EI24 ≥ 50% with the cell-free supernatant. This ability results in an emulsion of the fuel used (Figure 3A). The various indices obtained from the strains tested are represented in Figure 3B.

Antimicrobial activity of biosurfactant extracts

Biosurfactants have been reported to have antimicrobial

effects. In this study, *Bacillus* isolates showed good efficacy against *E. coli*, *Salmonella* spp., and *S. aureus* when taken as models of pathogens. Among the 16 isolates tested, 9 or 56% showed an inhibitory effect against the various pathogenic bacteria used in this study. Indeed, 6 extracts of biosurfactants were able to inhibit the growth of *S. aureus* and *Salmonella* spp., and 5 extracts were able to inhibit *E. coli* (Figure 4).

Molecular identification of *Bacillus* and yeast isolates

To confirm the identity of the 16 isolates presenting both better profiles in terms of their ability to produce biofilms and to emulsify hydrocarbons with an EI24 ≥ 50%, amplification of the *fib*E gene was carried out. A size of about 850 bp was obtained for the primers used, like *fib*EBs (targeting *B. subtilis*), *fib*EBp (targeting *B. pumilus*), *fib*EBsa (targeting *B. safensis*) and *fib*EB1 (targeting *B. licheniformis*). Isolates were identified as *B. pumilus* (12%), *B. subtilis* (12%), *B. safensis* (6%), *B. amyloliquefaciens* (6%), and *B. licheniformis* (6%) (Figure 5A). PCR performed allowed amplification of the universal genes encoding internal spacers transcribed in yeast isolates. In total, 8 strains were able to amplify the targeted gene. The amplicons revealed unique bands around the expected sizes. For all isolates selected, six including S1, S2, and S3 were able to amplify ITS 1/4 gene, with a band around 650 bp; on the other hand, P1 and P3 from palm wine were able to amplify the same
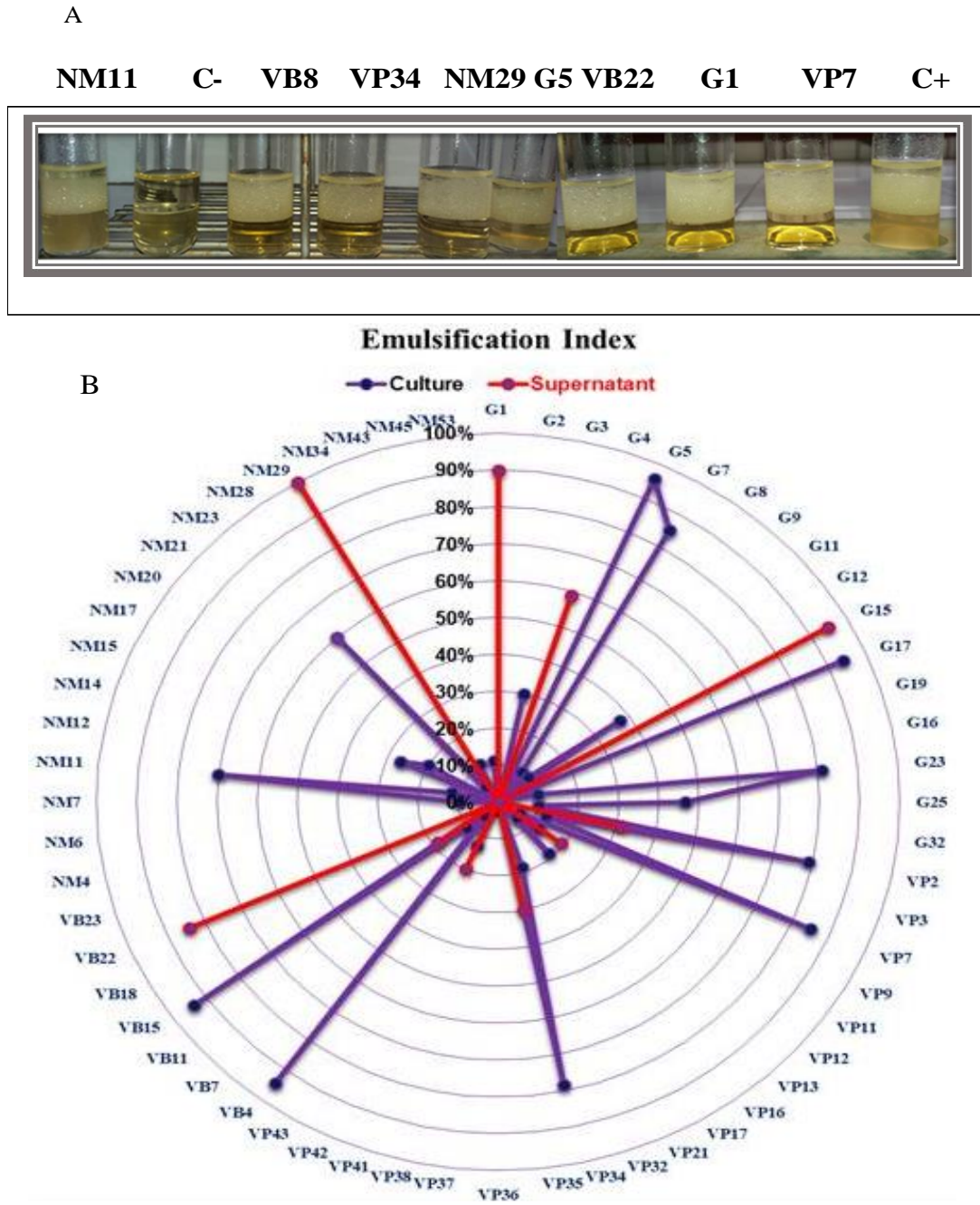
A

NM11   C-   VB8   VP34   NM29 G5 VB22   G1   VP7   C+

Emulsification Index

B

Figure 3. A: Emulsion of gasoline by some isolates of *Bacillus* spp. B: Emulsion index –(EI24) of *Bacillus* spp. Isolates C-: negative control; C+: positive control, *Bacillus* isolates NM4, 6, 7, 11, 12, 14 15, 17, 20, 21, 23, 28, 29, 34, 45, 53. G1, 2, 3, 4, 5, 7, 8, 9, 11, 12, 15, 16, 17, 19, 23, 25, 32. VB4, 7, 11, 15, 18, 22, 23, and VP2, 3, 7, 9, 11, 12, 13, 16, 17, 21, 32, 34, 35, 36, 37, 38, 41, 42, 43.

gene with a band around 850 bp. Figure 5B illustrates the electropherogram resulting from these amplifications.

Multiplex colony PCR of genes involved in biofilms

To demonstrate the presence of the genes encoding

biofilm markers, multiplex PCR targeting *eps*H, *tas*A, *ymc*A, and *yfi*Q genes has been performed. *Bacillus* isolates VB15 and G23 were identified as *B. pumilus; Bacillus* isolates G7 and G26 were identified as *B. subtilis;* and *Bacillus* isolate G33 was identified as *B. licheniformis eps*H and *tas*A genes were positive in *B. subtilis*. *ymc*A and *yfi*Q genes were positive in *B.*
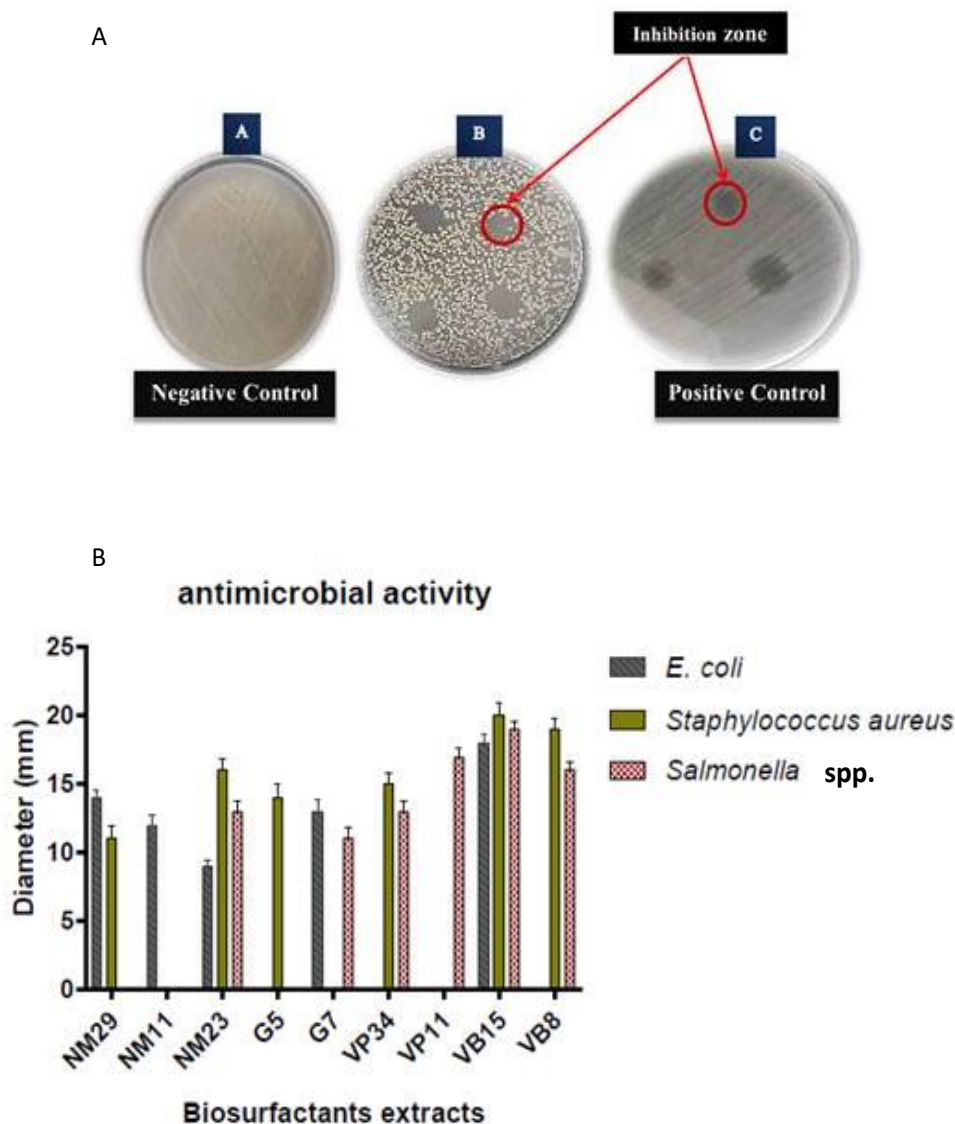
Figure 4. Antimicrobial activity of *Bacillus* biosurfactants on pathogenic models. A: activity illustration, B: Diameters of inhibition. MM29, MN11; MN23: Isolates from Ntoba Mbodi sample. G5, G7: Isolates from ginger wine. VP34, VP1: Isolates from palm wine. VB15 and VB8: Isolates from Banana wine.

licheniformis. *ymc*A gene was positive in *B. pumilus*. Thus, the targeted genes were amplified and obtained at good sizes, as shown in Figure 6 and confirmed with controls.

Interactions between yeasts and *Bacillus* spp.

Isolates with a percentage of EI24 ranging from 50 to 100% were selected for the characterization of the type of interaction between yeasts and *Bacillus* spp. The difference between the growth in single culture (pure culture) and coculture was evaluated after enumeration

by comparing the values of the growth rates during time.

Coculture between Bacillus spp. strain VP11 and S. cerevisiae strain P3

Both strains were isolated from palm wine. The values of the growth rate in CFU/mL were multiplied by the "logarithm". Thus, the analysis of the growth curves in single and in coculture (Figure 7A) shows that in coculture as in single culture, the two isolates follow the same growth logic, with a slight regression in the growth rate (Log CFU/mL) during time in coculture compared to
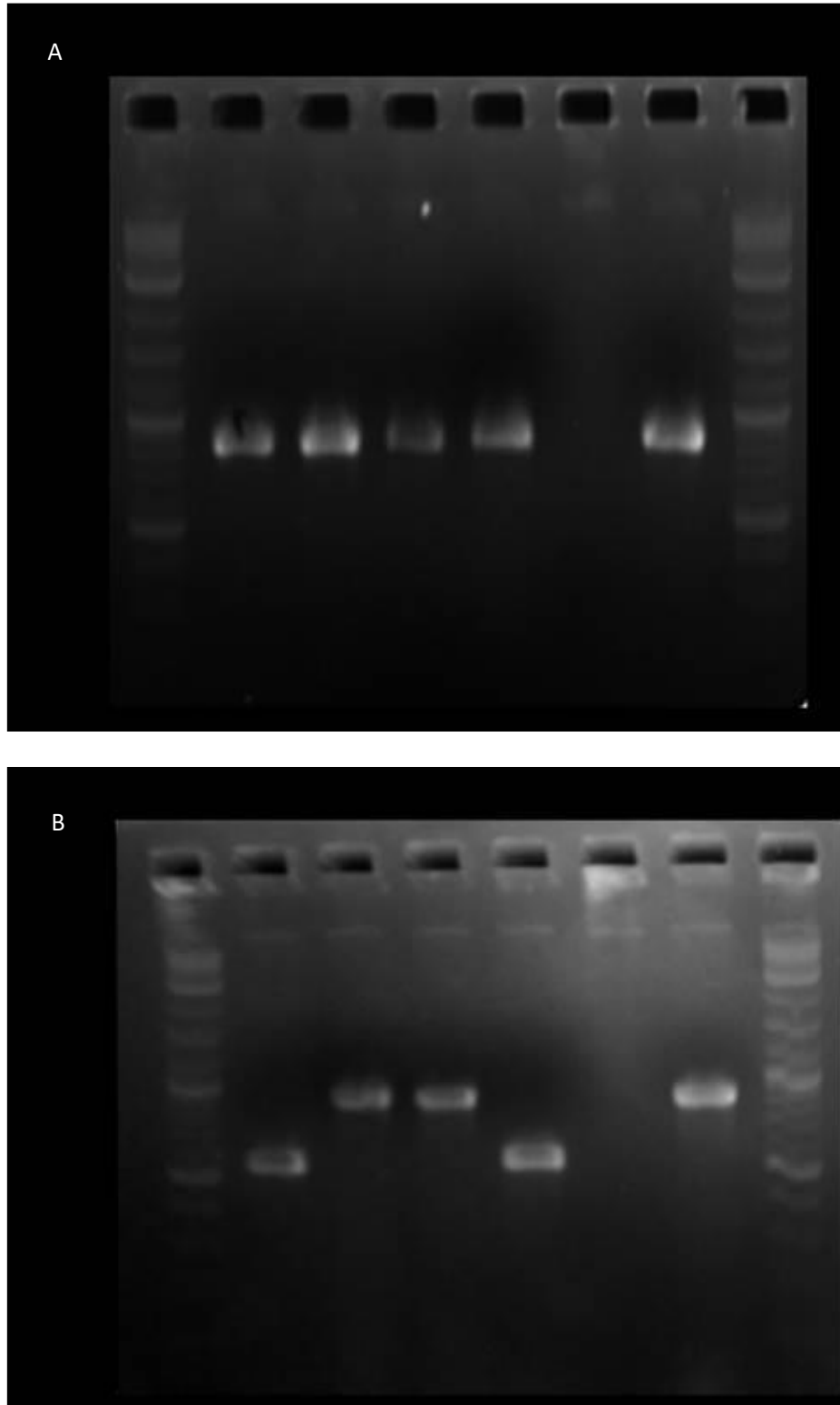
Figure 5. (A): 1% agarose gel electrophoretic profile of the fibE gene PCR amplicons from *Bacillus* spp. isolates (MP: Molecular Weight Marker, lane 1: *Bacillus* spp. isolate G7, lane 2: *Bacillus* spp. isolate VB15, lane 3: *Bacillus* spp. isolate G17, lane 4: *Bacillus* spp. isolate NM23, lane 5: negative control, 6: positive control. B: Electrophoretic profile on a 1.5% agarose gel of PCR products of the ITS region of rDNA from yeast isolates. MP: molecular weight marker; lanes 1 and 2: *Bacillus* spp. isolate V1, lane 3: *S. cerevisiae* strain P3, lane 4: *S. cerevisiae* strain S2, lane 5: negative control, and lane 6: positive control.
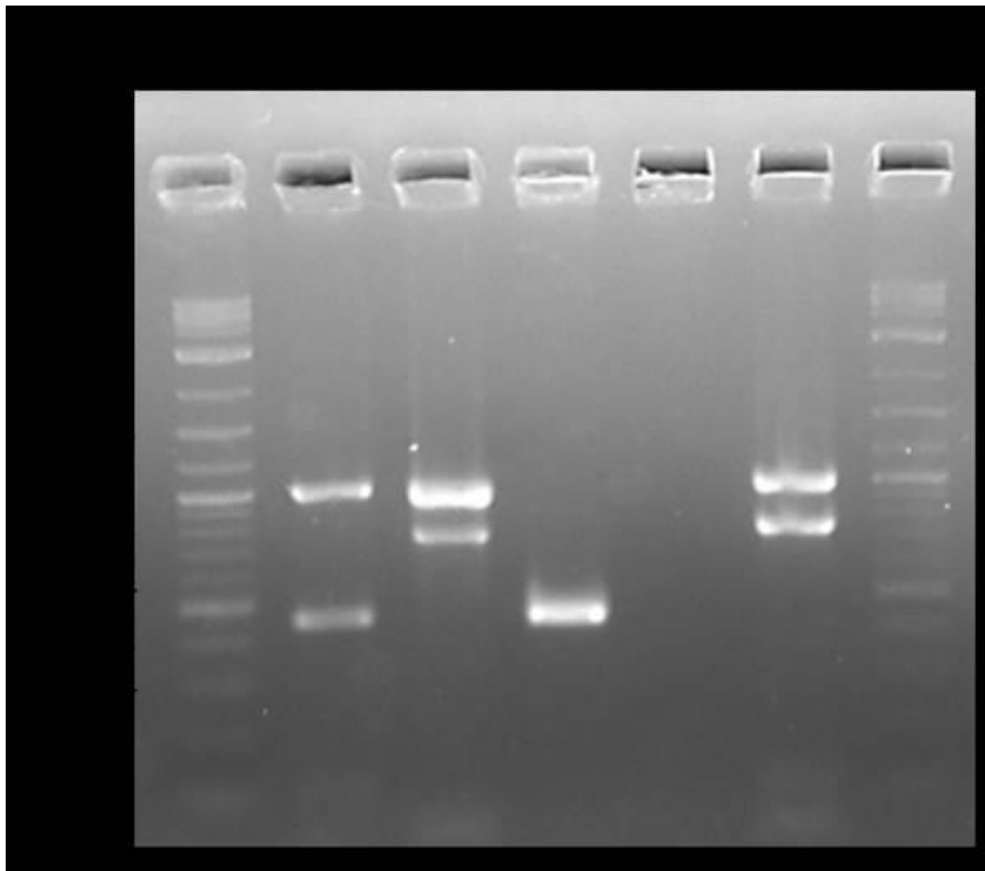
Figure 6. 2% agarose gel electrophoretic profile of PCR products of *eps*H, *tas*A, *ymc*A and *yfi*Q genes from *Bacillus* isolates. (MP: 2-Log molecular weight marker, lane 1: *Bacillus* isolates G33 (*yfi*Q≈1089 bp and *ymc*A≈432 bp genes), lane 2: *Bacillus* isolates G7 (*eps*H ≈1035 bp and *tas*A≈786 bp genes), lane 3: *Bacillus* isolates VB15 (gene *ymc*A≈435 pb), lane 4: negative control, lane 5: *Bacillus subtilis* was used as a positive control.

that observed in the single culture. This observation shows that there is an interaction between the two microbial populations (Figure 7A).

Coculture between B. subtilis strain G7 and yeast strain S2

The strains tested were isolated from *Tangawiss* (ginger juice). The second mixed culture was carried out between *B. subtilis* strain G7 and yeast isolate S2 in LB broth. The count was made with a volume of the inoculum of 100 µL. After enumeration, the analysis of the growth profiles for the pure and mixed cultures (Figure 7B) was carried out according to the same procedures as the first. Furthermore, it showed that the flora of *B. subtilis* strain G7 in coculture increased considerably compared to that in the single cultures. In addition, the yeast isolate S2 regresses slightly more in mixed culture than in single culture. It emerges from this observation that the cohabitation of the two isolates promotes the growth of

G7 and that there is an interaction between the two microbial populations (Figure 7B).

Coculture between B. pumilus strain VB15 and yeast strain V3

A third mixed culture was carried out between strains V3 and VB15 from "*Mbamvu*", the growth of the two types of microorganisms was followed according to the same methods as the others. The comparison of the growth profiles showed on one hand that when V3 grows in the presence of VB15, the growth rate (Log CFU/mL) increases considerably compared to V3 in single culture and a slight decrease in the V3 flora is observed in coculture with 96 h. On the other hand, the growth of VB15 remains almost unchanged over time. This observation suggests that the growth of V3 in mixed culture is stimulated by VB15 and that there is a positive interaction between the two microbial populations (Figure 7C).
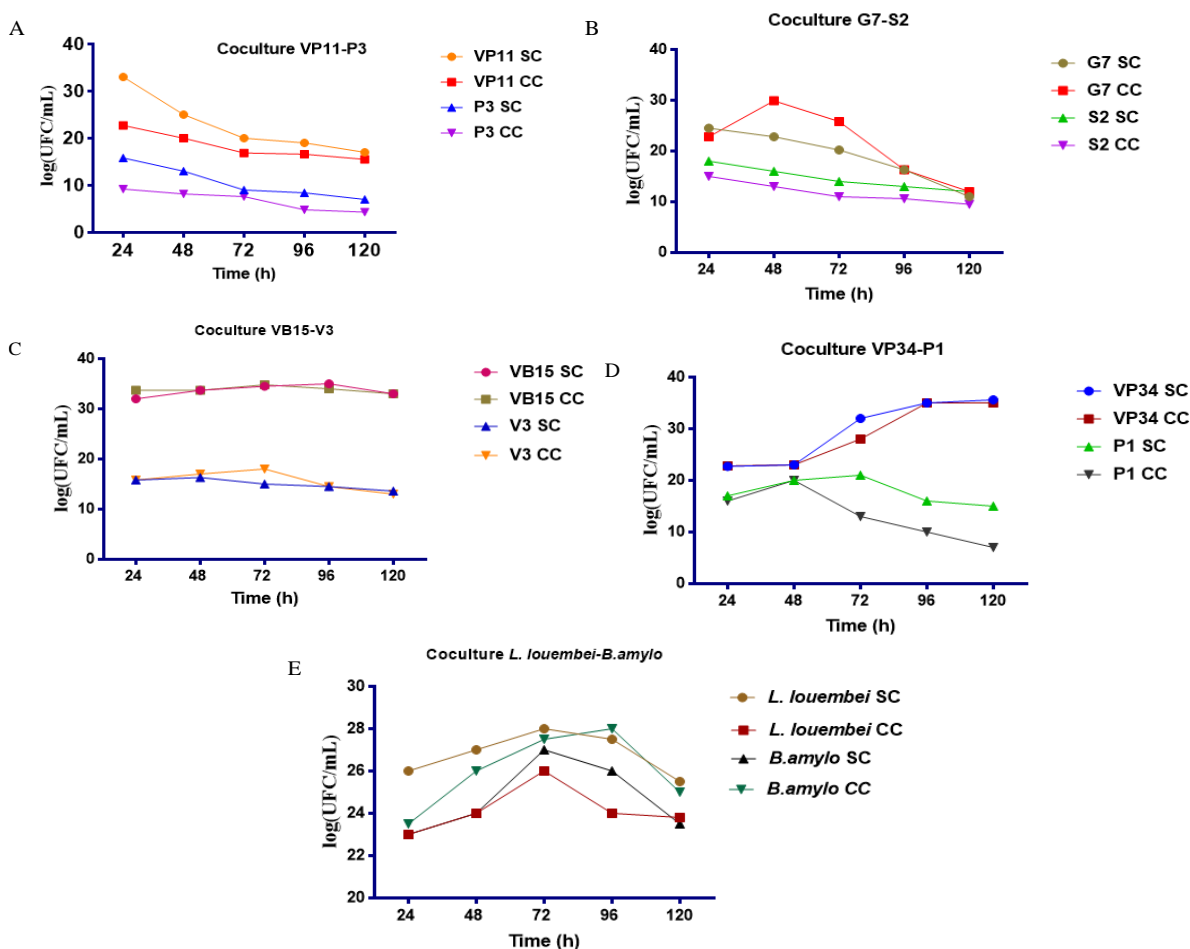
Figure 7. Single culture and coculture growth profiles. A: between *Bacillus* spp. isolate VP11 and *S. cerevisiae* isolate P3. B: between *Bacillus subtilis* strain G7 and yeast isolate S2. C: between *Bacillus pumilus* isolate VB15 and yeast isolate V3. D: between isolates *Bacillus* spp. Isolate VP34 and *S. cerevisiae* strain P1. E: *Bacillus amyloliquefaciens* strain NM11 and *L. louembei*. CC: coculture, SC: single culture.

Coculture between Bacillus spp. strain VP34 and S. cerevisiae strain P1

Two strains used were isolated from the same sample, "palm wine". In this case, the comparison of the growth profiles shows that from 48 h when P1 is in coculture with VP34, its flora regresses compared to the single culture of P1 and that the growth of VP34 in pure and mixed culture remains almost unchanged with the growing flora. This observation shows that there is an interaction between the two isolates and that the presence of VP34 in the same medium disadvantages the growth of P1 (Figure 7D).

Coculture between *B. amyloliquefaciens* and *L. louembei*

L. louembei has been previously identified and isolated

from "*Ntoba Mbodi*" in our laboratory. This strain was tested in mixed culture with *B. amyloliquefaciens* (NM11) from the same sample, to study the interaction between both microorganisms. This fact bears resemblance to that observed in the case of the mixed culture of *B. pumilus* strain VB15 and yeast strain V3 *B. pumilus* strain VB15 and the yeast strain V3. This is because *L. louembei* stimulates the growth of *B. amyloliquefaciens*. This is a positive interaction during which the cohabitation of microorganisms is favourable to one of them (*B. amyloliquefaciens*) by impacting the growth of *L. louembei* (Figure 7E).

DISCUSSION

This work aimed to identify bacteria at the molecular level and to study the interactions between microorganisms isolated from 4 fermented foods found in the Republic of

Congo. 133 bacterial isolates belonging to the *Bacillus* genus and 32 isolates of yeast were obtained from fermented cassava leaves, ginger juice, palm wine, or banana wine. Several studies have shown that bacteria of the *Bacillus* genus and yeasts constitute ecological niches in fermented foods and actively participate in the fermentation quality (Ouoba et al., 2008). Fermented foods are complex ecosystems harbouring microorganisms that are able to interact with each other by producing biomolecules of interest and by forming biofilms (Marchut-Mikolajczyk et al., 2021; Qin et al., 2022; Jumpathong et al., 2022).

Biofilms are bacteria lifestyles in natural environments, and they severely impact much of the bacterial composition. In *Bacillus* spp., the extracellular matrix mainly comprises many components: exopolysaccharides (EPS), proteins (TasA, TapA, and BslA) and some nucleic acids (Pandit et al., 2020). This work showed that 45% of *Bacillus* isolates were positive on CRA. It has been shown that 60% of food-borne bacteria, including the genus *Bacillus* are capable of producing biofilm-like structures using the CRA method. In addition, some phenotypic differences have been seen, namely, the strong or moderate black coloration (Arnaouteli et al., 2021; Qin et al., 2022; Blair et al., 2008). This could be explained by the level of gene expression involved in the exopolysaccharide biosynthesis and the substrate specificity as well. This could justify the two stages of biofilm formation: adhesion to the surface followed by the production of exopolysaccharides (Pandit et al., 2020). Exopolysaccharides are multifunctional compounds that have interesting applications in both the pharmaceutical and food industries (Elzeini et al., 2021).

Sensitivity of CRA could be explained by the fact that the formation of biofilm or the production of exopolysaccharides can be conditioned by the type of substrate metabolized, especially since in this test sucrose was substituted by glucose. These results indicate that the capacity for biofilm formation depends on substrates linked to environmental factors (Xu et al., 2010).

Bacillus has long been known for its ability to produce biosurfactants such as lipopeptide, surfactin, iturin, fengycin, and lichenysin (Eras-Munoz et al., 2022). However, it has been reported that surfactin is also a quorum sensing molecule which has a positive effect on biofilm formation, especially in *B. subtilis* (Pandit et al., 2020). In this work, we demonstrate the correlation between biofilm formation and biosurfactant production. 65% of *Bacillus* isolates were able to emulsify the gasoline with an EI24 between 5 and 100% after 24 h of incubation at room temperature. Those bacteria were also capable of producing BLS. This emulsification has also been observed in bacteria such as *Pseudomonas aeruginosa, B. subtilis,* and *B. licheniformis,* which have been widely used in the bioremediation of polluted soils (Stancu, 2020; Wu et al., 2022). As for the yeasts, 69% of

the isolates were able to emulsify gasoline, with an emulsifying activity of the strains ranging from 46 to 100%. Many studies have demonstrated the same phenotype (Zara et al., 2020; Jezierska et al., 2018; Konishi et al., 2011).

Isolates with an EI24 ≥ 50% were selected for biosurfactant extraction. This made it possible to confirm that these biosurfactants are extractable, as shown in the work carried out with *B. subtilis* (Cheng et al., 2013; Mnif et al., 2013). Subsequently, we showed that the extracted biosurfactants of 56% *Bacillus* isolates have antimicrobial activity, as they are able to inhibit the growth of pathogenic bacteria such as *E. coli*, *S. aureus*, and *Salmonella* spp. This is an interesting observation because *Bacillus* spp. and yeasts would play an important role in the preservation of local fermented products through their biosurfactant-like molecules, due to their antimicrobial properties. It was previously shown that the lipopeptides of *B. subtilis* exhibited antibacterial and anti-biofilm activity against opportunistic bacteria or pathogenic agents such as: *Alcaligenes faecalis*, *Achromobacter xylosoxidans*, *Pseudomonas alcaligenes,* and *Pseudomonas putida*; with partial inhibition also observed against *Klebsiella aerogenes*, *E. coli* and *Pseudomonas aeruginosa* (de Souza et al., 2020). As previously reported, *Bacillus methylotrophicus* through its BLM has antibacterial activity by protecting against *Salmonella enterica* and *Xanthomonas campestris* contamination (Rani et al., 2020).

It has recently been shown that the *fib*E gene can be used in the molecular identification of *Bacillus* spp. belonging to phylogenetic group I (Kaya-Ongoto et al., 2019). This method is reliable, fast and has great discriminatory power. On the basis of a combination of phenotypic and molecular characterisation using fibE PCR multiplex, species of *Bacillus* isolated from fermented food were identified as *B. pumilus* (12%), and *B. subtilis* (12%), *B. safensis* (6%), *B. amyloliquefaciens* (6%), and *B. licheniformis* (6%). Similar results were obtained showing the presence of *Bacillus* genera (Parkouda et al., 2009).

A new method of multiplex PCR using biofilm genes, including *eps*H, *tas*A, *ymc*A, and *yfi*Q has been found in this work. *B. subtilis, B. licheniformis,* and *B. pumilus* have been identified and confirmed. The method is correlated with the technology using *fib*E gene amplification.

To identify yeast isolates at the molecular level, the direct PCR approach for the identification of yeast species has been chosen among the techniques described in the literature for its discriminatory power and ease of implementation. In the context of this work, it was shown that *S. cerevisiae* strain P1 and strain P3 have been identified. ITS1/ITS4 of 5.8S rRNA gene with a fragment size of around 850 bp, and isolates V1, V2, and V3 from banana wine, as well as isolates S10 and S13 from ginger juice have been linked to other genera or

species of yeast. The identification of *S. cerevisiae* species showed a fragment size about 850 bp and those of other species around 650 bp (David et al., 2014). The results of the present study showed identification of *S. cerevisiae* in palm wine. Similar result has been previously obtained. The predominance of this species in palm wine is due to the high sugar and alcohol content in palm wine, unlike ginger juice and "*Mbamvu*". Unlike other species, *S. cerevisiae* can tolerate moderately high levels of sugar and alcohol (Guo et al., 2020).

Fermented foods are the products of complex interactions between molds, yeasts, and bacteria. It is therefore important to understand interactions between different groups. The present study focused on the interactions between *Bacillus* and yeasts, as well as and *Bacillus* spp. and *L. louembei*. In some cases, it has been observed that when two strains grow together, they regress compared to a single culture (SC). This observation suggests that there is a negative interaction when the two strains coexist, reflecting competition because the two strains compete. Such was the case between *Bacillus* spp. strain VP11 and *S. cerevisiae* strain P3. This kind of interaction occurs when two strains in a medium all consume one or more common nutrients for their growth during fermentation. Competitive interaction for carbon, nitrogen, and iron between yeasts and bacteria isolated from the soil has been demonstrated (Zhou et al., 2022; Arnold, 2022). Likewise, Guo et al. (2020) noted a competing interaction for nutrients between lactic acid bacteria and yeasts, which produce metabolic substances that inhibit their growth together. These harmful substances can be lytic enzymes, antimicrobial peptides. Meng et al. (2015) observed a negative effect of *S. cerevisiae* on the growth of *B. licheniformis* in the fermentation process of Chinese liquor Maotai-flavor. Likewise, some studies have shown that an amensalism interaction between *S. cerevisiae* and *Torulaspora delbruecki*; *S. cerevisiae* normally grows in single and coculture while *T. delbruecki* shows a drop in its growth (Fernandez et al., 2013; Frey-Klett et al., 2011).

In other cases, we have observed a positive effect of the cohabitation of microorganisms, in particular commensalism, as the presence of one stimulates the growth of the other. Our results are similar to those of Fossi et al. (2014), who showed a commensalism interaction between *B. amyloliquefaciens* and *S. cerevisiae* and to those of Sieuwerts et al. (2008) who showed that *Lactobacillus delbrueckii bulgaris* supplied amino acids to *Streptococcus thermophilus* which in turn produced formic acid and CO_2 favorable to growth *L. delbrueckii* subsp. *bulgaris*. Commensalism like interaction has been found in our study between *B. amyloliquefaciens-L. louembei*, and *B. pumilus* strain VB15-yeast strain V3 cocultures. This work comes at the right time to give added value to fermented foods in all their diversity.

The importance of studying *Bacillus*-Yeast interactions is a contribution to the understanding of bacteria-bacteria, bacteria-yeast communication; knowing the identity card of the molecules secreted by these microorganisms, means reconsidering the interactions of microorganisms. Mastery of interactomes would enable long-term control of food quality. When communication between microorganisms is disrupted this could lead to the loss of organoleptic characteristics. Good knowledge of molecules such as biosurfactants which are responsible for *Bacillus*-Yeast communication could lead to the development of starters in the sustainable preservation of food because these are the same molecules which play assigned roles in the survival mechanisms of quorum bacteria, sensing and quorum quenching.

Conclusion

The present work demonstrates the microbial grouping in different fermented foods and sheds light on the interactions between *Bacillus* species with *S. cerevisiae* and *L. louembei.* These results will help industries at a national level with the quality control of fermented foods by rationally singling out starters and optimizing their microbiota that are still mischaracterized.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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