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Preliminary Study on Antimicrobial Properties of Lactic Acid Bacteria Involved in the Fermentation of Corn Dough during *Doklu* Processing in Côte D'Ivoire

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ABSTRACT

This study aimed to assess the antimicrobial activities of lactic acid bacteria (LAB) isolated from corn dough for *doklu* production during spontaneous fermentation. Fermentation was conducted for 10 days by mixing corn dough with water at room temperature and biochemical analysis and LAB isolation was performed every 2 days. LAB isolated were then tested for potential antimicrobial activity. Biochemical analyses revealed the presence of 3 main organic acids in corn dough: tartaric acid, oxalic acid and lactic acid. Moreover, pH decreased while titratable acidity increased. Corn dough contains a diversity of LAB including homo- and heterofermentatives. From the 109 isolates, 83.33% showed the ability to inhibit the growth of a pathogen bacterium, *Staphylococcus aureus* ATCC 25915 with diameters of inhibition ranging from 10.4 ± 0.55 mm to 16 mm. Twenty six of these positive LAB produced inhibitory substances which could be bacteriocins with a large spectrum of action against several human pathogens.

Keywords: antibacterial activity, fermentation, LAB

INTRODUCTION

Maize is the basic staple grain for a large proportion of people in African countries as it provides significant amounts of the daily intake of calories, proteins and other nutrients (Obiri-Danso *et al*. 1997). The forms consumed in Côte d'Ivoire are quite variable, ranging from immature grain eaten after cooking in water (*kaba bleké*) or culling to braised to products processed from ripened shelled kernels by indigenous technologies such as fermentation for products such as *toh*, food for infants and young children, *doklu*, etc.

Doklu is a fermented maize dumpling cooked in dry maize leaves. It is a food consumed by people in southern and South-East of Côte d'Ivoire. The people often appreciate *doklu* for its sour taste due to fermentation. During the preparation of *doklu*, cleaned and washed whole maize grains are soaked in water for 2 or 3 days, milled, mixed into water and left to undergo spontaneous fermentation for 24 h. The dough is mixed a second time with water and then prebaked. The dough thus obtained is shaped into balls, wrapped in maize husks and boiled for about 2 h. During the fermentation process of corn dough, a succession of naturally occurring micro-organisms results in a population dominated by lactic acid bacteria, or LAB (Kostinek *et al*. 2005, 2007). These LAB are well-known for their capacity to produce a variety of inhibitors including metabolic end products such as organic acids, hydrogen peroxide (H_2O_2), reuterin, reutericyclin, antifungal peptides and bacteriocins. This capacity of LAB strains and/or their antimicrobial products to inhibit unwanted bacteria (pathogens) in food was introduced in the concept of biopreservation (Stiles 1996). In Côte d'Ivoire, foods for young children are often contaminated by many pathogenic microorganisms. Thus, children who eat these foods are exposed to gastroenteritis, diarrhea, etc. Dosso *et al.* (1998) and Michel *et al.* (2005) reported an outbreak of typhoid fever and gastroenteritis due to *Vibrio parahemolyticus* where more than 100 cases (patients) were hospitalized. Moreover, typhoid fever (a bacterial infection caused by *Salmonella enterica* serotype Typhi and Paratyphi A, B and C) remains very common in Côte d'Ivoire (Michel *et al.* 2005). In developing countries, one tenth of the children under five years of age die due to dehydration caused by incidences of diarrhea (Sahlin 1999).

The aim of this work was to highlight the antimicrobial activities of LAB involved in the fermentation of corn dough during *doklu* processing in order to use them in biopreservation of foods for young children.

MATERIALS AND METHODS

Maize

Dry white maize (*Zea mays* var. 'Saccharata') grown in Côte d'Ivoire was used in this study. This is a cultivar commonly used for confection of *doklu*. Maize has been bought at the market of Abobo (Abidjan, southern Côte d'Ivoire) and transported to the laboratory for experiments.

Tested strains

Strains used for antimicrobial tests were Gram+ bacteria (*Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 25913) and Gram- bacteria (*Enterobacter cloacae* ATCC 13047, *Escherichia coli* ATCC 23355, *Klebsiella pneumoniae* ATCC 13883, *Proteus vulgaricus* ATCC 13315, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella Typhi* IPP 5534, *Salmonella Typhymurium* ATCC 14028, *Salmonella Enteritidis* ATCC 13076, *Shigella sonnei* ATCC 2571). These strains, which are often implicated in food spoilage and diseases, were obtained from the National Laboratory of Public Health. These indicator strains were maintained at 80°C in nutrient broth with 50% (v/v) glycerol and cultured twice aerobically on nutrient agar (Difco, Detroit, MI, USA) at 37°C, before use.

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Original Research Paper

Preparation and fermentation of corn dough

About 2 kg of corn were cleaned and soaked in 2 L of water for two days in closed pans. The water was then decanted and corn milled (Disc Attrition mill Model 10-2A, New Delhi, India). The flour obtained was sieved, mixed in water and allowed to left to spontaneously ferment at room temperature for 10 days. Every two days, samples of dough were collected for biochemical and microbial analyses.

Fermentation experiments were conducted in triplicate on two separate occasions. Results of all analyses represent the mean values of six replicate trial fermentations with duplicate determinations.

Biochemical analysis of the fermented corn dough

To determine pH, 10 g of fermented corn dough sample was blended with 30 mL of sterile distilled water and filtered through Whatman filter paper No. 1. The pH of the filtered solution was determined with a pH meter (P107. CONSORT, Bioblock Scientific, France). Total titratable acidity (TTA) was determined by using the method described by Kimaryo *et al.* (2000) with 10 mL of filtered solution.

For analysis of organic acids in the corn dough, 10 g of fermenting corn dough was ground in a mortar with 15 mL of 0.1 N H_2SO_4. The obtained mixture was centrifuged for 10 min at 12000 × *g* and then the supernatant was filtered through a 0.20 µm pore size Millipore membrane (Sartorium, Barcelona, Spain). Organic acids in the filtered solution were assayed by High-Pressure Liquid Chromatography (LC-6A, Shimadzu Corp., Japan) using an ion exclusion ORH-801 column (300 mm × 6.5 mm) (Interchrom, France). Running conditions were as follows: mobile phase, H_2SO_4, 40 mmol L^{-1}; flow rate, 0.8 ml min^{-1}; wavelength, 210 nm, room temperature (30 ± 2°C). The separated components were detected with a UV spectrophotometric detector (SPD-6A, Shimadzu Corp.).

Enumeration and isolation of microorganisms

Preparation of stock solutions, inoculation of agar plates, and cultivation and quantification of microorganisms were carried out according to Coulin *et al.* (2006). For all determinations, 10 g of the samples was homogenized in a stomacher bag with 90 mL of sterile peptone buffered watered (AES Laboratoire, Combourg, France). Ten-fold serial dilutions of stomacher fluid were prepared and spread-plated for counting microorganisms. Enumeration of coliforms was carried out using VRBL (Violet crystal and neutral Red Bile Lactose) plates containing agar (VRBL agar, Oxoid Ltd., Basingstore, UK) which were incubated for 24 h at 30°C for total coliforms and 44°C for fecal coliforms. Yeasts and molds were enumerated on plates of Sabouraud chloramphenicol agar (Fluka, Biochemica 89579, Sigma-Aldrich Chimie GmbH, India) incubated at 30°C for 4 days. Aerobic mesophiles were enumerated on plates of Count Agar (PCA Oxoid Ltd.) and incubated at 30°C for 2 days. Enumeration of LAB was carried out using plates of de Man, Rogosa and Sharpe (MRS) agar (Merck, Darmastadt, Germany) which were incubated in an anaerobic jar at 30°C for 3 days. Small colonies on MRS were then purified by successive streaking onto the same medium and tested for Gram and catalase determination, gas production from glucose and cultured on Bile Esculin Azide (BEA) (AES Laboratoire, Combourg, France) and Mayeux Sandine and Elliker (Biokar Diagnostics, Beauvais, France) agar for presumptive identification of genus. Only Gram+, catalase- rod, cocci and coccoids were considered as LAB in this study.

Antibacterial activity of the LAB strains

The inhibitory activities of the isolates were studied using the agar-well diffusion assay. A well-isolated colony was selected from the MRS agar plate. The top of the colony was scooped with a sterilized loop and the growth was transferred into a tube containing 20 mL of sterile MRS broth. The broth culture was incubated at 35°C for 24 h. After incubation, the broth was then centrifuged at 3500 × *g* for 10 min in a Hermile ZK236 (Hermile GmbH and Co, Gosheim, Germany) centrifuge. The clear supernatant was removed and filter-sterilized (0.22 Am, Schleir and Schuell, GmbH, Dassel, Germany). Two portions (10 mL each) of the sterilized cell-free filtrates (CFF) were transferred aseptically into sterile sample bottles. One of these portions was neutralized (pH 6.5) using 0.1 M NaOH and treated with catalase (5 mg/mL) to eliminate organic acid and H_2O_2, respectively.

In this study, *Staphylococcus aureus* ATCC 25913 was used at first, and then the 11 pathogenic microorganisms, to assess the action spectra of the LAB strains. Tryptic casein soy agar was tempered to about 40-50°C and inoculated with 100 µL of a fresh culture (18 to 24 h at 37°C in brain heart broth) of each pathogenic strain. The plates were poured and allowed to solidify. Once solidified, wells 5 mm in diameter were then made using a sterile metallic borer and filled using 60 µL of (i) CFF of LAB or (ii) Neutralized CFF (pH 6.5).

The inoculated plates were kept in a refrigerator (4-5°C) for 2 h to allow the filtrate to diffuse into the agar, and then incubated for 24 h at 37°C. After incubation, these plates were examined. The antibacterial activity of LAB strains was shown by the appearance of inhibition zones around wells. The diameter of the inhibition zone was measured (average of two perpendicular diameters) with a transparent ruler. The results were expressed in mm, including the diameter of the wells. The results are presented as the means of duplicate tests.

Statistical analyses

All trials were repeated four times except for the agar well diffusion assay, which was performed twice. The different sample treatments were compared by performing one-way analysis of variance on the replicates at a 95% level of significance using the Statistica (99th Edn, Alabama, USA) statistical programme. Unless otherwise stated, significant results refer to $P < 0.05$. This software was also used to calculate mean values and standard deviations of the trials and to classify LAB isolates according their antibacterial activities (inhibition zones).

RESULTS AND DISCUSSION

Change in pH and total titratable acidity

pH is a critical factor in developing flavor and aroma of foods (Montet *et al.* 2006; Panda *et al*. 2007). In this study, the pH of the unfermented dough was 5.07 ± 0.17 (**Fig. 1**). During the fermentation, this value gradually decreased to reach 3.76 ± 0.05 after 6 days. The rate and extent of pH decline is indicative of the fermentation intensity. Unlike pH, titratable acidity increased during the first two days of fermentation from 0.45 ± 0.08% to 0.68 ± 0.02%, before it decreased until the end of the fermentation period (**Fig. 1**). The rise in TTA with the corresponding decline in pH during fermentation has been reported by several authors in studies on various African traditional foods such as *mawe*, sorghum beer and *ogi* (Hounhouigan *et al*. 1999; Sawadogo-Lingani *et al*. 2008; Oyarekua *et al*. 2008). Thus, the

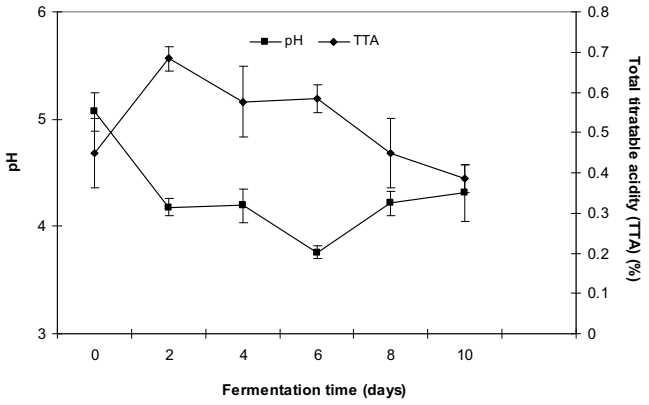

Fig. 1 Evolution of pH and total titratable acidity (TTA) of corn dough during fermentation. N = 6

Table 1 Concentrations of organic acids in corn dough during fermentation.

Organic acids (mg/100 g)	Fermentation time (days)					
	0	**2**	**4**	**6**	**8**	**10**
Oxalic acid	13.9 ± 4.1 a	18.8 ± 3.5 a	34.7 ± 6.6 b	39.2 ± 2.8 b	48.3 ± 3.5 c	36.9 ± 4.9 b
Tartaric acid	83.5 ± 5.6 a	0 b	0 b	0 b	18.3 ± 3.5 a	0 b
Lactic acid	23.6 ± 2.8 a	93.7 ± 4.2 b	274.5 ± 12.7 c	254.2 ± 19.1 d	213.1 ± 3.5 e	181.7 ± 12.7 f

Different letters within a line indicate significant differences according to Duncan's multiple test range ($P \leq 0.05$).

decrease in pH suggests the presence and activity of LAB during the spontaneous fermentation of corn dough. LAB produce organic acids, mainly lactic and acetic acids, which induce a lowering of pH (Coulin *et al.* 2006; Panda *et al.* 2007). Also, the decrease in titratable acidity associated with the increase of pH at the end of fermentation is due to the fact that some of the acid produced would be used in neutralizing the alkali before the excess is detected as acidity (Sefa-Dedeh *et al.* 2004).

Organic acids and effect on microbial growth

In this study, the organic acids detected were oxalic, tartaric and lactic acids (**Table 1**). However, in Ghanaian fermented maize dough the main organic acids were lactic, acetic, butyric and propionic acids (Olsen *et al.* 1995). The difference in the type of organic acids could be related to the composition of the corn used or the microorganisms involved in the fermentation of each maize-based food. At the beginning of fermentation, tartaric acid was the most abundant acid in *doklu* corn dough with a value of 83.5 ± 5.6 mg/100 g (**Table 1**). During the fermentation of dough, lactic acid was produced in the largest amount, reaching 274.5 ± 12.7 mg/100 g after 4 days. This is in accordance with the reports that LAB form lactic acid as the major acid end product. After reaching a maximum, the lactic acid content declined throughout the end of fermentation, indicating probable uptake and utilization of this acid by microorganisms. Unlike lactic acid, tartaric acid was reduced to undetectable levels within one day in the corn dough. This finding suggests the metabolization of tartaric acid throughout fermentation.

During fermentation, LAB, yeasts and moulds have similar growth but their initial counts (4.49 ± 0.70 and 7.81 ± 0.68 log cfu/g for yeasts and moulds, and LAB, respectively) were significantly different ($P < 0.05$) (**Fig. 2**). These counts increased, reaching highest values after 4 days for yeasts and moulds (5.55 ± 0.62 log cfu/g) and LAB (9.81 ± 0.59 log cfu/g) before decreasing slightly to 4.25 ± 0.53 and 7.70 ± 0.52 log cfu/g, respectively at the end of fermentation. These microorganisms (yeasts and LAB) are common in traditional fermented foods (Kunene *et al.* 2000; Nyanga *et al*. 2007). Co-metabolism of these microorganisms has been suggested (Gobetti *et al.* 1994). During this co-metabolism, LAB provide an acidic environment which encourages the growth of yeast, and yeasts provide vitamins and other growth factors for LAB. The important increase in lactic acid content would undoubtedly have inhibitory effects on microbial growth, particularly those which do not support high acid conditions since acid production correlated to a decrease in pH are known to extend the lag phase of sensitive organisms, including food-borne pathogens (Smulders *et al.* 1986). Indeed, with respective initial loads of 3.92 ± 0.97 and 6.02 ± 0.99 log cfu/g, faecal coliform and total coliform populations decreased rapidly and disappeared only after 2 and 6 days, respectively (**Fig. 2**) while the pH value and lactic acid content reached 3.76 ± 0.05 and 274.5 ± 12.7 mg/100 g, respectively. This corresponds to their death kinetics, also reported in similar naturally fermented plant materials (Nout *et al*. 1989; Masha *et al*. 1998). Nout (1991) and Masha *et al.* (1998) also reported the disappearance of coliforms as pH decreased to below 4.5, although Mensah *et al.* (1991) suggested that the anti-microbial effect of fermented maize dough porridge was not due to pH *per se* but probably to the presence of other antimicrobial compounds. Kingamkono *et al*. (1994, 1995) reported the anti-microbial effect of *togwa* (a Tanzanian fermented food) against several enteropathogens and its potential for decreasing the incidence of diarrhoea in children (Willumsen *et al*. 1997). Coliforms are usually active in the early stages of fermentation of cereal-based slurries and their activity is eliminated when an enriched culture is used (Mbugua 1987). These microorganisms have also been reported to be associated with the spoilage of fermented gruels (Mbugua 1984).

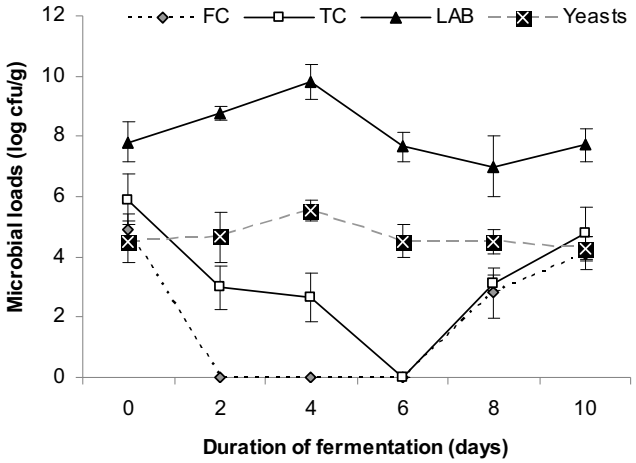

Fig. 2 Evolution of microbial loads during fermentation of corn dough. FC, faecal coliforms; TC, total coliforms; LAB, lactic acid bacteria; N = 6

Growth and fermentative types of LAB

A total of 109 LAB was isolated from fermenting corn dough and were characterized by phenotypic tests. According to Aguirre and Collins (1993), the term LAB is used to described a broad group of Gram-positive, catalase-negative, non-sporing rods and cocci, usually non-motile, that utilize carbohydrates fermentatively and form lactic acid as the major end product. These LAB could be divided into four groups comprising homofermentative rods, heterofermentative rods, homofermentative cocci and heterofermentative cocci in decreasing order of predominance (**Fig. 3**). Homofermentative LAB produce lactic acid as the major or sole end product from glucose fermentation while heterofermentatives produce an equimolar amount of lactate, CO_2 and ethanol from glucose (Tamime and O'Connor 1995). Most of the homofermentative rods (the dominant group representing 60% of the isolates) were presumptively identified as *Lactobacillus sp*. The next predominant group of LAB (20% of total isolates) consisted of heterofermentative rods belonging to either the genera *Lactobacillus* or *Weissella*, while the homofermentative cocci (16.99% of isolates) belonged either to the genera *Enterococcus*, *Pediococcus* or *Streptococcus*. These different genera identified in our study were similar to those reported by several authors (Mugula *et al.* 2003; Kostinek *et al*. 2005, 2007) in several traditional fermented foods such as *togwa* (a Tanzanian fermented food) and *gari*.

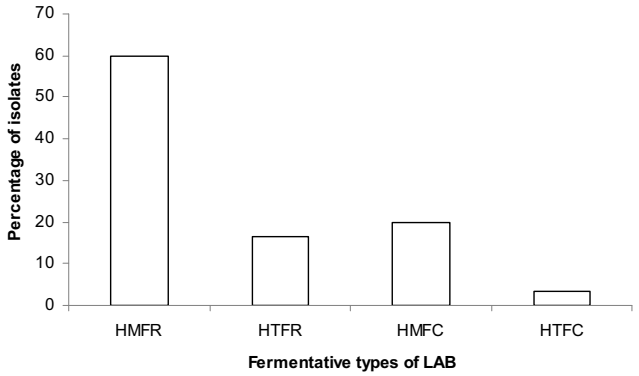

Fig. 3 Fermentative types of LAB isolated from fermented corn dough. HMFR, homofermentative rods; HTFR, heterofermentative rods; HMFC, homofermentative cocci; HTFC, heterofermentative cocci; N = 109 isolates

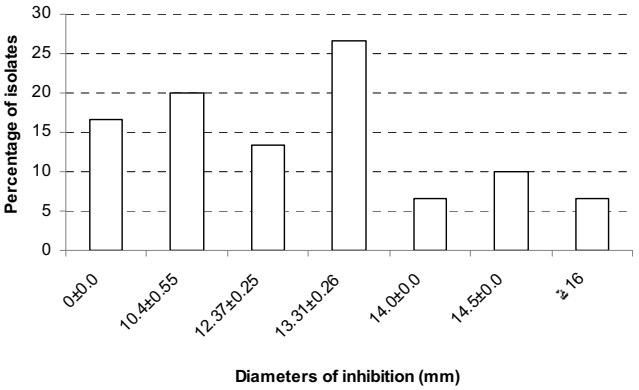

Fig. 5 Distribution of LAB isolates with antimicrobial activity against *S. aureus* ATCC 25913 according to the diameters of inhibition. N = 109 isolates

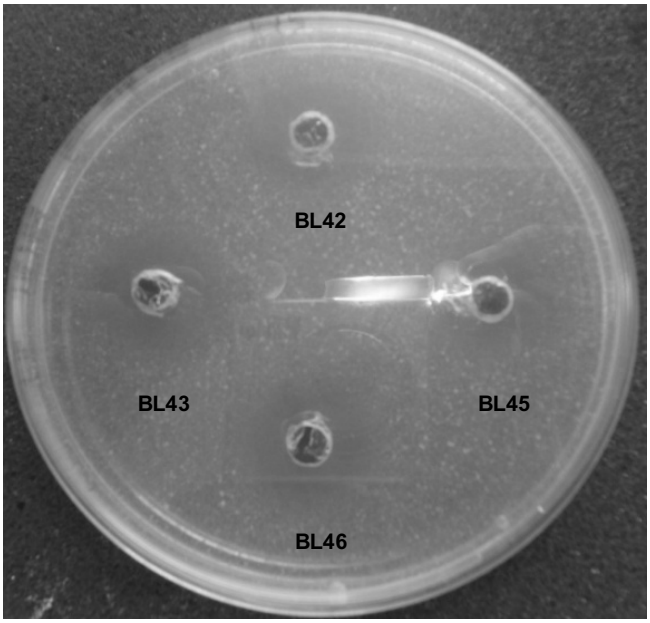

Fig. 4 Photograph of a Petri dish showing the inhibitory effect of LAB against *S. aureus* ATCC 25913.

Antibacterial activity of LAB

The inhibition of *S. aureus* ATCC 25913 was scored as positive if the width of the clear zone around the wells was 1 mm or larger (**Fig. 4**). From a total of 109 LAB strains, 83.33% of MRS (Man Rogosa and Sharpe) supernatants of LAB strains yielded inhibition zones when tested against *S. aureus*. The diameters of the inhibition zones ranged from 10.4 ± 0.55 to 16 mm and more. The majority of isolates (≈ 30) presented an inhibition zone of 13.31 ± 0.26 mm (**Fig. 5**). Savadogo *et al*. (2004) reported that extracts of LAB strains isolated from milk fermented in Burkina Faso gave zones of inhibition ranging from 8 to 12 mm against pathogenic strains (*S. aureus* and *E. coli*). Mufandaedza *et al*. (2006) and Valerio *et al*. (2009) also made similar observations during studies on the antimicrobial properties of LAB cultures isolated from traditional fermented milk of Zimbabwe and Italian semolina ecosystem against pathogenic bacteria and fungal strains.

After elimination of organic acid and H_2O_2, only 23.85% (26 LAB) of LAB isolates showed positive antibacterial activity against *S. aureus* ATCC 25913 with diameters of inhibition ranging between 7 and 10 mm. In fact, bacterial infections caused by the genus *Staphylococcus* represent a grave threat to both humans and animals and they are of major concern to health authorities. Over the last few decades, methicillin-resistant *Staphylococcus aureus* (MRSA) has been recognized as the principal nosocomial pathogen worldwide (Graffunder and Venezia 2002; Anderson *et al*. 2008). Thus, there is an urgent need for a new program to control the rapidly progressing dissemination of MRSA in the human and animal populations (Karska-Wysocki *et al*. 2010). For this reason, the application of new food additives to food products, which can stimulate growth of lactic acid bacteria and increase their antagonistic activity expressed by LAB (probiotic) toward most pathogenic organisms, opens new perspectives for research.

The inhibitory substances produced by these isolates were also active against a broad range of 8 other pathogens (**Table 2**). Similar observations were made by Abdelbasset and Djamila (2008) who studied the antimicrobial activity of autochthonous LAB isolated from Algerian traditional fermented milk *raïb*. Thus, for the remaining isolates, representing 76.15% of all isolates, the inhibitory effect was fully eliminated by pH neutralization and elimination of H_2O_2. That implies that the antimicrobial activity previously observed for these isolates was essentially due to the effect of organic acids and H_2O_2. Olsen *et al.* (1995) also suggested the effect of organic acids and H_2O_2 on the antimicrobial activity of LAB strains while Karska-Wysocki *et al*. (2010) demonstrated that a direct interaction of LAB and methicillin-resistant *Staphylococcus aureus* (MRSA) in a mixture led to the elimination of 99% of the MRSA cells. However, Mensah *et al.* (1991) and Pringsuala *et al*. (2012) suggested that the antimicrobial effect of LAB isolated from fermented maize dough is not only due to the effect of pH or H_2O_2, but is also related to the presence of other antibacterial compounds such as bacteriocins. However, since the protein nature of the inhibitory compound was not tested with proteinase in this study, we could only presume a bacteriocin activity to be involved. Moreover, these antimicrobial substances detected were not active against *P. vulgaricus* and *E. coli*.

CONCLUSION

This study showed that corn dough contains a diversity of LAB. The supernatants of LAB cultures (after elimination of organic acid and H_2O_2) showed antibacterial activity against *Staphylococcus aureus*. These supernatants were tested against 11 pathogenic microorganisms. 23.33% of the tested strains produced antibacterial compounds that could be bacteriocins. The characteristics of the bacteriocins and other compounds with antimicrobial activity should, however, be explored in more detail before the use of these bacteria as starter cultures in food biopreservation.

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Table 2 Spectral action against the pathogens by lactic acid bacteria isolated from fermented corn dough.

Pathogens strains	Lactic acid bacteria tested						
	LAB 9	**LAB 46**	**LAB 54**	**LAB 83**	**LAB 84**	**LAB 86**	**LAB 109**
Staphylococcus aureus	+	++	+	+	++	+	++
Klebsiella pneumoniae	+	++	+	++	-	-	-
Pseudomonas aeruginosa	++	++	-	-	++	++	-
Proteus vulgaricus	-	-	-	-	-	-	-
Shigella sonneï	-	+	-	-	-	-	-
Salmonella Typhi	++	+	++	-	-	-	-
Escherichia coli	-	-	-	-	-	-	-
Bacillus subtilis	-	+	+	+	-	-	-
Salmonella enteritidis	+	-	-	-	++	-	-
Salmonella Typhymurium	+	-	-	-	-	+	+
Enterobacter cloacae	-	+	-	-	-	-	-

– : no inhibition ; + : 6-8 mm of diameter of inhibition, ++ : > 8 mm of diameter of inhibition

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