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CHARACTERIZATION OF BACTERIOCIN-LIKE PRODUCING LACTIC ACID BACTERIA (LAB) ISOLATED FROM THE CRUDE AND TRADITIONALLY FERMENTED FISH MEAT (*GUEDJ*) IN SENEGAL

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ABSTRACT: *Nineteen (19) strains of Lactic Acid Bacteria (LAB) from meat of fresh and fermented fish, (Machoiron, Capitain and Sompatte) isolated on modified MRS agar (0.1% glucose) supplemented with polymyxin B (100 U/ml) and cycloheximide (50 g/ml) were preselected based on their antimicrobial potential demonstrated against Escherichia coli (O 157) and Listeria monocytogene (CWBI-B710), morphological and biochemical characteristics. The averages of LAB were between 3.8 10^4 to 7.4 10^5 CFU/ml for Machoiron, 4 10^3 to 6.2 10^5 CFU/ml for Captain and 3.1 10^4 to 8.2 10^5 CFU/ml for Sompatte. The phenotypes of the isolated bacteria helped identifying the following species: Lactobacillus (Lb) plantarum, Lb brevis, Lb curvatus, Lb fermetum, Lactococus(Lc) lactis spp lactis 1, Lc. lactislactis, Lc plantarum, Lc subsplactis, Enterococcus (Ec) sp, Ec. Faecium, Leuconostocs mesenteroides and Pediococcus pentasaceus. In addition, 21.05% of the strains have found to display an antagonistic activity against pathogenic bacteria strains.*

KEYWORDS: Lactic Acid Bacteria (LAB), Identification, Fermented Fish-*Guedj*, Pathogenic Bacteria, *Escherichia Coli* (O 157), *Listeria Monocytogene* (CWBI-B710).

INTRODUCTION

Guedj is a traditional Senegalese fermented and dried fish. The obtained product is brown and characterized by strong pungent odor. It is used as food or condiment in small quantities into stews and soups for local meal flavoring (Fall et al., 2014). Generally, *Guedj* is produced from spontaneous and uncontrolled fermentation of advanced microbial spoiled fish. The traditional fermentation of the fish is similar to that used for *Lanhouin* (Anihouvi et al., 2005) and *Adjuevan* (Koffi- Nervy et al., 2011). In Senegal, the tendency in traditional processing methods is to incorporate sodium chloride in large amounts (30-40%) and also an abusive use of chemical preservatives to reduce the growth of microorganisms and insects. The main concern about this indigenous fish product its safety. Nowadays, consumers demand products without preservatives and which still maintain long shelf life and safety.

To remedy to the technological constraint characterizing the traditional fermentation, search of optimization strategy has been undertaken. Some strategies of food fermentation optimization are based on starter cultures acidifying the matrix. Bacteriocinogenic LAB have been widely

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used as starter culture for food fermentation over recent decades (Olasupo et al., 1996; Agati et al., 1998; Sanni et al., 2002; Kostinek et al., 2007; Oguntoyinbo, 2007; Michel et al., 2010).

LAB are reported in many fermented fish products (Olympia, Ono, Shinmyo, &Takano, 1992; Ostergaard et al., 1998; Diop et al., 2007). The primary role of LAB is to ferment the available carbohydrates and thereby cause a pH decrease. They are known as producing different antibacterial metabolites, including organic acid, bacteriocins, diacetyl, hydrogen peroxide and reuterin. The combination of low pH, organic acids (mainly lactic acid) and salt is the main preservation factor in fermented fish products.

Up to now, little information on the LAB present on traditional handled fish or involved over fermentation for *Guedj*, is being reported. Thus, this study aimed to isolate and identify LAB from meat of fresh and fermented fish at different stages of the fermentation process and to evaluate their inhibitory action on the growth of pathogenic bacteria.

MATERIEL AND METHODS

Fish species and matrixes used for lab isolation

LABs were isolated from meat of three kind of fresh and fermented fish (fatty, moderately fat and lean fish), respectively Machoiron (*Arius latisculatus*), Capitain (*Pseudotolithus brachygnatus*) and Sompatte (*Pomadasys jubelini*). Samples were taken at different stages during the processing. The samples are recorded MP for the fresh fish (raw material); FR_{24H} for the fish after 24 hours of fermentation; FR_{48H} for the fish after 48 hours of fermentation; S_{24H} the dried fish taken after 24 hours of fermentation; S_{48H} the dried fish after 48 hours of fermentation; S_{72H} the dried fish after 72 hours fermentation.

Isolation and selection of lab

A pre-culture of LAB from each fish was carried out in flasks (250 ml) by adding 10 g of meat fish in 90 ml of MRS broth supplemented with 0.1% glucose, 100 U/ml polymyxin and 50 µg/ml cycloheximide with modifications as reported by Diop et al. (2008). The cycloheximide and polymyxin B were used respectively to inhibit the proliferation of yeast and Gram-negative bacteria present in the samples, and to make the medium selective. The culture was incubated for 8h at 30°C. Subsequently, dilutions series were prepared from the homogenate in sterile peptone water (0.1% w/v) from 10^{-1} to 10^{-5}. Then, 100 µL of each dilution were plated on MRS and MRS supplemented with 0.1% glucose and incubated anaerobically at 37°C for 48 h (De Man et al., 1960; Wanchai et al., 2007). Bacteria colonies with clear zones on the plates were individually picked and then re-streaked on MRS agar plates to obtain pure cultures. Each of the isolates strains were identified based on their macroscopic, microscopic and biochemical characteristics by performing the catalase test, the oxidase test and the Gram's stain. Carbohydrate fermentation of the strains isolates was characterized by using the API 50 CHL kit (API system BioMerieux, France) according to supplier's recommendations. Biochemical profiles obtained were read by using an API LAB software identification (BioMérieux, France).

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Detection of inhibitory activity of selected lab isolates

Bacteriocin activity of LAB was determined by overlay method and agar well diffusion assay against *Listeria monocytogenes* (CWBI-B710) and *Escherichia coli* (O 157). The overlay method was performed as described by Magnusson and Schnurer

(2001) and the agar well diffusion assay was determined as reported by Barefoot et al., 1983. MRS agar and MRS supplemented with 0.1% glucose was used for cultivation LAB at 37°C for 24 hrs. Pathogenic strains were grown on the 868 medium and incubated at 37°C for 16 to 18 hours.

Overlay method: Plates providing a total of 300 colonies were covered with 3 ml of a surface layer of soft agar (863 medium with 0.75% agar) previously inoculated with 30 µL of an overnight culture of the indicator strains *Escherichia coli* or *Listeria monocytogenes*. After 24 hours of incubation at 30°C, plates were examined for the detection of suspected bacteriocin activity. The inhibitory activity was displayed by the presence of a clear halo around the LAB colonies. The colonies producing zones of growth inhibition in the indicator lawn were removed from the MRS (0.1% glucose) and MRS agar with the tapered end of a sterile Pasteur pipette and inoculated into 10 ml of MRS broth then incubated for 24 h at 30°C.

The well method was used to confirm the inhibitory activity observed by overlay method.

Well method: Strains of LAB isolates were grown for 24 hours at 30°C into 10 ml of MRS broth (0.1% glucose). 5 ml of each culture were centrifuged at 4000 rpm for 10 min and were then adjusted to pH 6.5 with NaOH (5 N) and sub-sequently filtered through 0.2 µm membrane filters. They are then sterilized at 80°C for 10 min. Twenty ml of 868 agar medium (50°C) were poured on sterile plates and inoculated with 100 µL of each pathogenic bacteria. After solidification, wells of size (6mm) were perforated with a sterile pipette Pasteur. Aliquots (60 µL) of the cell free culture supernatant (CFS) were dispensed in each well. The plates were incubated at 30°C and examined after 48 h for clear zones showing the pathogenic bacteria inhibition.

Inhibition test was performed in triplicate.

Effect of pH, hydrogen peroxide and proteolytic enzymes on bacteriocin

In order to eliminate the organic acid effect, different strains to be tested were grown on MRS (0.1% glucose) for 24 h. The CFS were centrifuged at 4000 rpm for 5 minutes and supernatants were collected. The pH value of the CFS was adjusted to 6.0 with NaOH (5N), then filtered and sterilized. Iinhibitory activity was performed by agar well diffusion assay as described above.

To confirm the inhibitor effect of the bacteriocin, the CFS were incubated at 25°C for 1 h with 30 µL of catalase at a final concentration of 1 mg/mL and the same supernatants without catalase were used as control to eliminate the possible inhibitory action of hydrogen peroxide. The well diffusion assay was replicated. After eliminating the effects of organic acid and hydrogen peroxide, the CFS still retaining antimicrobial activity were selected to determine the type of peptide from the detected antagonistic compound. Four enzymes (peroxidase 158 IU / mg, pepsin 367 IU / mg, protease 4 IU / mg and trypsin NB FIP- 4581 IU / mg) brought to a final concentration of 10mg/ml were used. Twenty microliters (20 µL) of each enzyme solution were added to 180 ml of the CFS from each pre-selected LAB. CFS were incubated at 37°C

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for 2h and then cooled to 4°C for 20 minutes to stop the reaction, whereas controls were added 20 µL of sterilized distilled water. Iinhibitory activity was then carried out using the agar well diffusion assay.

RESULTS

Microorganism counts

The LAB density increased from 3.8 10^4 to 7.4 10^5, 4 10^3 to 6.2 10^5, and 3.1 10^4 to 8.2 10^5 CFU / ml respectively for Mahoiron, Captain, and Sompatte. These results allowed to indicate that LAB density increased during *Guedj* processing for all samples with a decrease at the end of the process.

Morphological, physiological and biochemical properties of lab

A total of 28 strains, (11 from fresh and 17 from fermented fish) were characterized as LAB. These isolates were gram-positive, rods (bacilli) (32%) - cocci (66%), catalase and oxidase negative. Furthermore, nine (09) strains were from Machoiron, eleven (11) from Captain (C), and eight (08) from Sompatte (S). Biologically, five (05) different genera including Lactobacilli (9 isolates, 33%), Lactococci (7 isolates, 26%), Enterococci (5 isolates, 17%), Leuconeustocs (4 isolates, 14%) and Pediococci (3 isolates, 10%) were identified and represented in (**Fig 1**). Thereby, among the 28 strains analyzed, 19 strains, were identified. They were composed by 6 strains of *Lactobacillus spp* (2 *Lb plantarum*, 1 *Lb brevis*, 1 *Lb curvatus*, 1 *Lb sp* and 1 *Lb fermentum*); 5 strains of *Lactococus spp* (2 *Lc. lactis ssp lactis 1*, 1 *Lc lactis lactis*, 1 *Lc plantarum* and 1 *Lc subsp lactis*); 3 strains of *Enterococcus spp* (2 *Ec sp* and 1 *Ec faecium*), 3 strains of *Leuconeustocs mesenteroides* and 2 strains of *Pediococcus pentasaceus* (**Tab 1**).

Antibacterial activities

A total of 19 LAB investigated, 16 strains showed a good antimicrobial activity against *L. monocytogenes* and *Escherichia coli* (**Tab 2**; **Fig 2**). After the elimination of the organic acid, only 9 strains exhibited distinct inhibitory activity against the two indicators. Further, after applying catalase treatment on 5 strains tested against *E. coli*, an antagonistic activity loss was observed on two of them. Whereas, an antimicrobial activity of CFS was noticed on 4 strains tested against *L. monocytogenes*. After treatment with peroxidase, pepsin, protease and trypsin, on the 4 tested strains, 3 of them (2 *Lb platarum* and 1 *Lb brevis* respectively from captain and sompatte) still exhibited residual antimicrobial activity against (*E. coli* and *L. monocytogenes*) and the remained strain (*Lc lactis spp Latis 1* from Machoiron*)* showed positive reaction against *L. monocytogenes*.

DISCUSSION

The number of LAB in *Guedj*, the most popular indigenous fermented fish in Senegal was in the range of 10^3 to 10^5 CFU/g. These values were in agreement with those found by Koffi-Nervy et al., (2011) for *Adjeuvan*, a traditional Ivorian fermented fish.

Compared to the morphological characteristic of gram's staining, the percentage of spherical shape cells (66%) (Cocci) is higher than rods shaped cells (32%) (Bacilli) in the studied

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bacteria. Similar results were previously reported by Dortu (2002). Cocci were salt tolerant microorganisms that can grow in salty products such as sausages. It was not surprising to find them in *Guedj* salty. In addition, Mauguin and Novel (1994) found that *Lactococcus* was the major flora isolated in fish. The gender and species of isolated and identified LAB from *Guedj* were represented by 33% of *Lactobacili*, 26% *Lactococci*, 17% of *Enterococci*, 14% of *Leuconostoc* and 10% of *Pediococci*. Many authors reported a wide varieties of LAB obtained from fish fermentation products in different African regions (Paludan-Muler et al.,2002; Kopermsub and Yunchalard, 2010). Same results were reported by Nerquaye et al. (1978); Yankah (1998), Sanni et al. (2002), and Essuman (1992), with the isolation of *Bacillus*, *Lactobacillus*, *Pseudomonas*, *Pediococcus*, *Staphylococcus*, *Klebsiella*, *Debryomyces*, *Hansenula* and *Aspergillus* in *Momoni*, a Ghanaian fermented fish condiment. Similarly, to *Momoni*, various microorganism species s including *Bacillus spp*., *Staphylococcus* spp., *Micrococcus* spp., *Streptococcus* spp. and *Corynebacterium* spp. were isolated and identified in the fermentation of *Lanhouin*. However, *Bacillus* spp. and *Staphylococcus* spp. were the most predominant identified microorganisms. Meanwhile, Diop (2008) reported *Proteus* spp. *Shewanella putrefaciens* and *Bacillus* spp as the most predominant microbial populations associated with *Guedj* fermentation.

In contrast, the recent work carried out by Koffi-Nevry *et al.* (2011) on *Adjuevan* showed that the fermentation was dominated by LAB, and the genera and species isolated and identified were *Leuconostoc lactis*, *Lactobacillus fermentum*, *Pediococcus* sp. and *Streptococcus* sp. These results agreed with the findings by various authors on fermented fish products obtained with a mixture of fish and carbohydrate source such as rice Adams et al., (1987).

Like other foods, seafood can be re-contaminated during processing by bacteria, such as *Staphylococcus aureus*, *Salmonella spp*., *Shigella spp*., *Clostridium perfringens*, *Bacillus cereus*, *Yersinia enterocolitica*, *Listeria monocytogenes* and *Escherichia coli*. Indeed, according to Mauguin (1991), isolated lactic strains of marine fish were low acidifying components. Similar results were found in our study. Thus, among the 19 isolates of LAB tested, only 3 bacterial strains (*Lb plantarum 1*, *Lb brevis* and *Lc lactis ssp lactis 1*) were detected with antagonistic effects against *E. coli* and *L. monocytogenes*. Bacteriocins could be one of the inhibitory substances produced by these three LAB isolates through their inhibitory effects of organic acids and hydrogen peroxide, eliminated by pH adjustment to 7 and addition of catalase. Isolation of LAB originally from aquatic with antibacterial activity was already reported in previous work. Indeed, Essid et al., (2008) found that *Lb plantarum* inhibited *Salmonella arizonae, Sptaphylococcus aureus, Psedomonas aeruginosa* and *E. coli*. Moreover, Buntin *et al*., (2008) reported that three strains of LAB, *P. pentosaceus* APa4, *P. pentosaceus* AIa1, and *Enterococcus faecium* ARa1 were able to inhibit growth of *S. aureus*, *Salmonella* sp., *E. coli*, and *L. monocytogenes* using agar method.

CONCLUSION

The results of this present study clearly state that three bacteriocin-like producing LAB strains, *Lb plantarum 1*, *Lb brevis* and *Lc lactis subsp. Lactis 1* with technological properties could potentially be used as starter's cultures to produce traditional fermented fish known as *Guedj*. These LAB strains could provide significant health benefits and enhance safety of the products. However, genetic complementary characterization contributes to more identification indeed of the bacteriocin-like producing LAB isolated from the indigenous fish products.

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APPENDIX

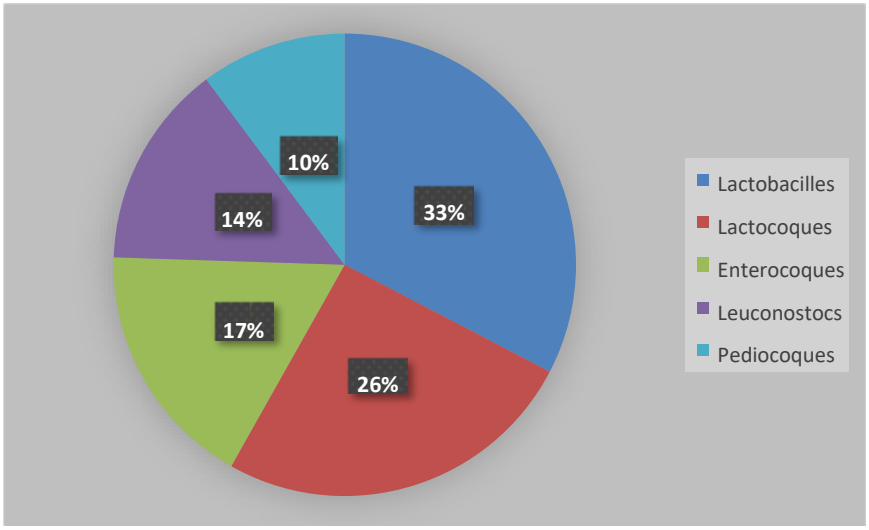

Figure 1: Distribution of Lactic acid bacteria (LAB) isolated during the *Guedj* processing
Table 1: Iidentification and characterization of LAB *Guedj* processing by API 50 CHL

N° de la souche	Groupe	Identification API 50CHL	Pourcentage d'identification (%I.D)
$MP_{M/1}$	Lactocoques	*Lactococcus lactis spp lactis 1*	54.4%
$MP_{C/2}$	Lactocoques	Non identifié	-
$MP_{C/3}$	Lactocoques	*Lactococcus lactis lactis*	80,9 %
$MP_{S/2}$	Lactocoques	Non identifié	-
FRM24H/ 5	Lactocoques	*Lactococcus plantarum*	93%
FRC 24H/2	Lactocoques	*Lactococcus lactis subsp lactis*	88%
SM48 H/3	Lactocoques	*Lactococcus latis spp lactis 1*	95,9%
$MP_{M/2}$	Enterocoques	Non identifié	-
$MP_{C/4}$	Enterocoques	*Enterococcus sp*	90%
$MP_{S/1}$	Enterocoques	Non identifié	-
FRM24H/1	Enterocoques	*Enterococcus feacium*	98%
FRC48H/4	Enterocoques	*Enterococcus sp*	87%
$MP_{C/1}$	Leuconeustocs	Non identifié	-
$MP_{S/3}$	Leuconeustocs	*Leuconeustocs mesenteroides*	11,4%
SS48H/4	Leuconeustocs	*Leuconeustocs mesenteroides*	98%
SM48H/2	Leuconeustocs	*Leuconeustocs mesenteroides*	93%
$MP_{M/3}$	Pediocoques	Non identifié	-
FRS 24H/1	Pediocoques	*Pediococus pentasaceus*	88,6%

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FRM24H/3	Pediocoques	*Pediococus pentasaceus*	13,5%
MPC/5	Lactobacilles	Non identifié	-
FRC24H/4	Lactobacilles	*Lactobacillus plantarum1*	99,7%
FRC48H/2	Lactobacilles	*Lactobacillus fermentum*	92%
SC24 H/3	Lactobacilles	*Lactobacillus plantarum 1*	89,4
SC72 H/2	Lactobacilles	Non identifié	-
FRS24H/2	Lactobacilles	*Lactobacillus sp*	84,6%
SS24 H/ 1	Lactobacilles	*Lactobacillus curvatus*	78%
Ss48 H/1	Lactobacilles	*Lactobacillus brevis*	99,8%
SM72 H/2	Lactobacilles	Non identifié	-

Table 2: LAB isolates during *Guedj* processing showing inhibitory activity on *Escherichia coli and Listeria monocytogene* by overlay method

N° de la souche	**Bactéries lactiques/Souches indicatrices**	***Escherichia coli***	***Listéria monocytogene***
MPM/1	*Lactococcus lactis spp lactis 1*	+	+
MPC/3	*Lactococcus lactis lactis*	+	+
FRM24H/ 5	*Lactococcus plantarum*	+	+
FRC24H/2	*Lactococcus lactis subsp lactis*	+	+
SM48 H/3	*Lactococcus latis spp lactis 1*	+	+
MPC/4	*Enterococcus sp*	+	+
FRM24H/1	*Enterococcus feacium*	+	+
FRC48H/4	*Enterococcus sp*	+	+
$MP_{S/3}$	*Leuconeustocs mesenteroides*	-	±
SS48H/4	*Leuconeustocs mesenteroides*	-	±
SM48H/2	*Leuconeustocs mesenteroides*	-	±
FRS 24H/1	*Pediococus pentasaceus*	+	+
FRM24H/3	*Pediococus pentasaceus*	+	+
FRC24H/4	*Lactobacillus plantarum1*	+	+
FRC48H/2	*Lactobacillus fermentum*	+	+
SC24 H/3	*Lactobacillus plantarum 1*	+	+
FRS24H/2	*Lactobacillus sp*	+	+
Ss24 H/1	*Lactobacillus curvatus*	+	+
Ss48 H/1	*Lactobacillus brevis*	+	+

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Figure 2: Inhibition of *Listeria monocytogenes* and *Escherichia coli* around the streak lines of LAB strain ($MP_{C/3}$; $FR_{M24H/3}$; $MP_{M/1;}$ $S_{s48\ H/1)}$ from the Captain, Machoiron and Sompatte by overlay method.

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