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Lactic Acid Bacteria and Their Biopreservative Role in Attiéké (Ivorian traditional food)

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Abstract

A batch of thirty samples of ATTIÉKÉ from different regions of Côte d'Ivoire was subjected to microbiological analysis for highlighting the protective role of lactic flora located therein. A total of 15 Lactic acid bacteria isolates from Attieké samples were screened for antimicrobial activity against *Listeria monocytogenes*; an important pathogen linked to food. The results demonstrated that only three of them (*Enterococcus faecium, Pediococcus acidilacticci* and *Lactobacillus plantarum*) exhibited bacteriocinogenic activity towards the indicator strains (*Listeria monocytogenes*). Otherwise, the activity of these strains was lost after proteolytic enzymes treatment, showing the protein nature in the supernatant. When screened for bacteriocin-related genes using a bacteriocin PCR array based on know Lactic acid bacteria bacteriocin gene sequences in the NCBI GenBanK database, the result is positive to enterocin A, Pediocin PA-1, and plantaricin B.

Keywords: Attiéké, bacteriocinogenic strains, *Listeria monocytogenes*, PCR

1. Introduction

The attiéké is one of the major cassava transformation products developed by Ivorian women for years. It is a fermented food whose preparation requires the use of the ferment cassava (Assanvo , 2002). This traditional Ivorian food plays a vital and strategic role in food safety (Kakou 2000). It is also exported to Europe and other continents in his dehydrated form (Aboua, 1989). Three variants of attiéké occurred in Côte d'Ivoire namely "abodjaman coarse," "the abodjaman small grain" and "Attiéké garba." Unlike other Ivorian traditional foods such as rice, foutou or foufou, the attiéké is best kept at room temperature. This observation is probably due to the presence of protective strains naturally present in Attiéké whose action on spoilage flora leads to prevent their proliferation. Indeed, Attiéké's manufacturing process combines ferment called "magnan" in Ebrié language or "lidjrou" in Adjoukrou language. It is the main source of microorganisms involved in the early stages of manufacturing (Assanvo et al. 2000). It is therefore from this microbiological niche that provides the strains responsible for the inhibition of spoilage flora. However it is not excluded that this niche can harbor *Listeria*, a Gram positive, facultative, intracellular bacterium that contains ten species. One of these, *Listeria monocytogenes*, causes the disease listeriosis in humans and animals. It is able to multiply at temperatures as low as 2º to 35 °C, low pH and high salt concentration as well as salinity variations, which allows them to survive in diverse

ecological niches (ECDC, 2014). Worldwide, foodborne diseases have emerged as an important and growing public health and *L. monocytogenes* has been recognized as one of the most relevant foodborne bacteria pathogen.

Listeria monocytogenes can be found in many foods. Examples include smoked fish; meats; cheeses (especially soft cheeses) and raw vegetables. In fact, in 99% of cases listeriosis is due to foodborne transmission, but can also be transmitted from mother to fetus (Scallan et al., 2011). Clinical manifestations range from febrile gastroenteritis to septicemia, meningitis and encephalitis, as well as fetal infections that can result in abortion or postnatal health complications (Swaminathan and Gerner-Smidt, 2007).

The aim of this study was to identify bacteriocinogenic strains isolated from Attiéké's samples, evaluate the presence of bacteriocin genes by PCR and their ability to inhibit growth of harmful bacteria such as *Listeria monocytogenes*.

2. Material and methods

2.1. Isolation of lactic acid bacteria (LAB) from Attiéké

A batch of thirty bags 200 g of Attiéké were obtained from various Ivorian commercial producers stored in an icehouse (temperature 8^0C) and convoyed to laboratory of bio industry (University of Liège, Belgium) for analysis.

20-g samples were taken aseptically from each bags, diluted with 10 mL sterile saline solution (0.85% sodium chloride), and pressed manually in a Stomacher bag to extract as much liquid as possible, then, serial dilutions were made with sterile saline (0.85%, w/v NaCl), plated onto MRS agar (Difco) and incubated at 30°C for 24 h.

2.2. Screening for antiListerial activity by the double-agar layer test

Antilisterial activity of the strains was detected by double-agar layer test (Hanchi et al., 2014). To determine the inhibitory action of the isolates against *L. monocytogenes* M, all strains were grown as chopped of a pre-dried Trypticase Soy Agar (TSA, Himedia, Mumbai, India) and incubation for 18 h at 37 °C. Simultaneously, indicator organism was grown at 37 °C for 18 h and 100 µL of this culture with 10^6 CFU. mL-1 was inoculated into 10 mL Trypticase Soy Broth (TSB, Himedia, Mumbai, India) containing 0.7 % agar. This culture was equilibrated at 45 °C, mixed, and then poured as an overlay onto the plate with growth of microflora. Plates were incubated for 18 h at 37 °C before being examined by detection of inhibition zone.

2.3. Antimicrobial action of crude extraction from strains showing activity in double-agar layer test.

The crude extract bioassay of the strains, which showed activity in the double-agar layer test against *L. monocytogenes* M, was performed according Motta and Brandelli (2002). The strains were grown in 100 mL TSB at 37 °C for 18 h, and the cells were harvested by centrifugation at 3,680 x g for 15 min at 4 °C. The pH of the cell-free supernatants was adjusted to 6.5 with sterile 1 M NaOH to exclude acid production as the inhibitory mechanism, heated at 90 °C for 10 min to inactivate remaining cells, and then filtered through a 0.22 µm pore-size nylon syringe filter (Chromafil). The antimicrobial substance was kept at 4 °C for further experiment.

2.4. Effects of proteolytic enzymes on antimicrobial substance stability and antimicrobial activity quantification

To gain insight into the nature of the antimicrobial substance from the strains which showed activity in the double-agar layer test, the following treatments were used: the sensitivity to treatments with proteolytic enzymes was performed by addition of these enzymes to the

antimicrobial substance: trypsin (Sigma, St. Louis, MO, USA), papain (Merck, Darmstadt, Germany) and proteinase K (Merck) to a final concentration 2 mg. ml^{-1}. Incubations were performed at 35 °C for trypsin and papain and 37 °C for proteinase K for 1 hour. After the different treatments the antimicrobial activities were evaluated by the agar spot method and quantified according to an agar well diffusion assay described by Parente and Hill (1992) wherein Briefly, molten agar was seeded with an indicator strain (i.e., *L. monocytogenes*) and dispensed into Petri (20 ml per plate). Wells approximately 4.6 mm in diameter were bored in the agar. Two-fold serial dilutions of the supernatant were prepared and 60-ml aliquots of the various dilutions were loaded into separate wells. The plates were incubated overnight at 37°C, and bacteriocin activity was determined as the reciprocal of the highest dilution showing a definite zone of inhibition around the well. The bacteriocin activity in the undiluted extract was expressed in arbitrary units per milliliter (AU/ml), calculated by multiplying the reciprocal of the critical dilution by 1000/60.

2.5. PCR Detection of the potential bacteriocin Genes from the LAB showing activity in the double-agar layer test

The LAB strains of interest were analyzed by PCR to rapid screening of their producing bacteriocins genes that present on the bacterial chromosome or on plasmids. Total DNA was extracted from culture broth, 1.5 ml of culture broth pipetted into Eppendorf tubes then centrifuged at 4.300 x g for 5 min and the supernatant discarded; 200 µl of TE buffer was added, vortexes well, boiled for 10 min, and then put on ice immediately for 1 min; this was centrifuged again at 6,700 xg for 10 min and the supernatant was collected, which contains DNA for use as DNA template (Suwanjinda et al. 2007).

PCR amplifications: Supernatant (9.5 µl) was used as a template and 0.5 µl (10µM) of each specific primers (table1) were added to 12.5 µl of PCR mixture (2X KAPA2G Fast Multiplex Mix). The PCR mixture contains KAPA2G Fast HotStart DNA Polymerase (1 U per 25 µl reaction), KAPA2G Buffer A (1.5X at 1X), dNTPs (0.2 mM each dNTP at 1X), $MgCl_2$ (3.0 mM at 1X) and stabilizers. The amplification profile was initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing 51°C for 45 s, extension 72°C for 45 sec and final extension 72°C for 5 min, The presence of PCR products was determined by gel electrophoresis in 1.0 % agarose gel containing ethidium bromide. Electrophoresis in 1XTris-borate-EDTA was performed at 100 volts, and photographed under an Ultraviolet transilluminator.

Table 1. Primers used for detection of bacteriocin genes.

Target gene	Technique	Sequence	Sense	Source
Sakacin G2 F Sakacin G2 R	PCR	5'-CGTTACAACAGAACTTCAAG-3' 5'-TGGAAGAATGAGTACTTGTT-3'	Forward Reverse	Todorov et al. 2011
Sakacin G1 F Sakacin G1 R		5'-TTAGAACTACACTGATCGTG-3' 5'-TGGAAGAATGAGTACTTGTT-3'	Forward Reverse	Todorov et al. 2011
Sakacin P F Sakacin P R		5'-ATGGAAAAGTTTATTGAATTA-3' 5'-TTATTTATTCCAGCCAGCGTT-3'	Forward Reverse	Remiger et al. 1996
Curvacin A F Curvacin A R		5'-GTAAAAGAATTAAGTATGACA-3' 5'-TTACATTCCAGCTAAACCACT-3'	Forward Reverse	Remiger et al. 1996
NisinF NisinR		5'-TTATTTGCTTACGTGAATAC-3' 5- AGATTTTAACTTGGATTTGC-3'	Forward Reverse	Gross et al.1971 Mulders et al. 1991
Enterocin A F Enterocin A R		5'-AAATATTATGGAGTGTAT-3' 5'-GCACTTCCCTGGAATTGCTC-3'	Forward Reverse	Aymerich et al. 1996
Enterocin B F Enterocin B R		5'-AAAATGTAAAAGAATTAAGTACG-3' 5'-AGAGTATACATTTGCTAACCC-3'	Forward Reverse	Du Toit et al. 2000
Enterocin L50A F Enterocin L50AR		5'-ATGGGAGCAATCGCAAAATTA-3' 5'-TTTGTTAATTGCCCATCCTTC-3'	Forward Reverse	Fouquié et al. 2003

Enterocin P F Enterocin P R		5'-ATGAGAAAAAAATTATTTAGTTT-3' 5'-TTAATGTCCCATACCTGCCAAACC-3'	Forward Reverse	Cintas et al. 1998
Pediocin F Pediocin R		5'-GGTAAGGCTACCACTTGCAT-3' 5'-CTACTAACGCTTGGCTGGCA-3'	Forward Reverse	Suwanjinda et al. 2007
Plantaricin F Plantaricin R		5'-GGCATAGTTAAAATTCCCCCC-3' R 5'-CAGGTTGCCGCAAAAAAAG-3'	Forward Reverse	Yi et al. 2010

3. Results

3.1. Antimicrobial activity producing strains

Among the 30 LAB strains grew on MRS agar (Fig 1A), six (designated St1, St2, St3, St4, St5 and St6) were showed active against *L. monocytogenes* M (Fig 1B) by double-agar layer test and therefore were chosen for quantification of antimicrobial activity.

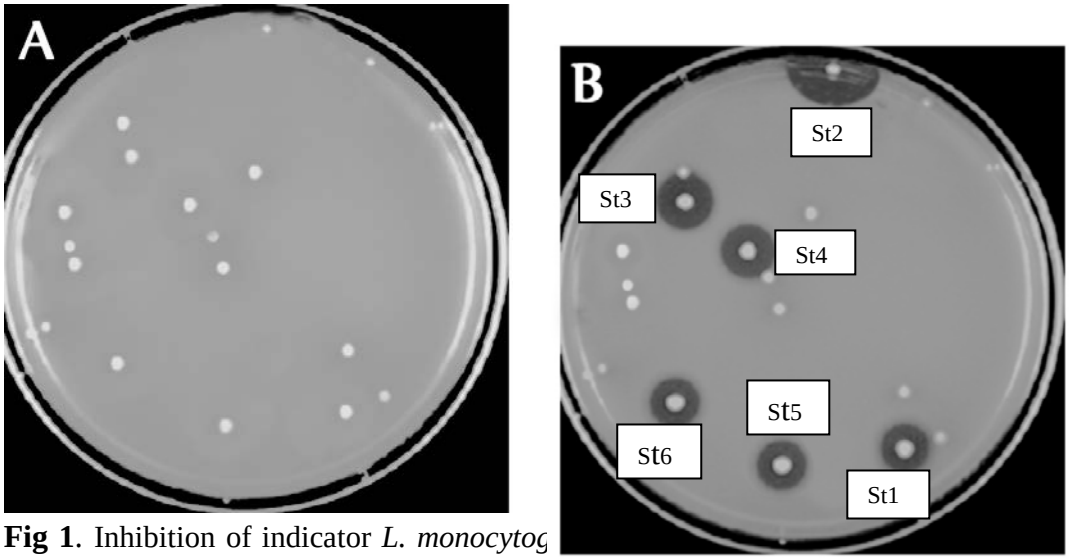

Fig 1. Inhibition of indicator *L. monocytog* om Attieke samples by double-agar layer test

3.2. Quantification of antimicrobial activity and antimicrobial substance stability

The production and evaluation of the antimicrobial compounds produced by the six strains were analyzed and observed at Figure 2, 3, 4 & 5, which indicates the growth curves of these strain (Fig. 2) and antilisterial activity in in vitro experiment (Fi. 3). All strains reached the mid exponential phase after 8 h of incubation (Fig. 2) and the highest (16 to 18 mm) inhibition zones (no shown) were observed at the stationary phase (12 to 40 h). Initial pH of all strains turns around 6.0, after 24 h passed around to 5.5, then up to 6.0 at 72 h growth. (no significant difference between the strains, $P > 0.05$) however, St2 alone showed a significant ($P<0.05$), approximately two unit decrease 4,75 in the pH over 8 h, but this was followed by an increase, reaching 5 by the end of the experiment at 72 h growth. (This decrease was significant, $P < 0.05$) (Fig.4). The highest antibacterial activities against *L. monocytogenes* M were observed with St2 (4266,66 AU. mL $^{-1}$) and St3 (3066,66 AU. mL $^{-1}$) (Fig. 5). Thus, cell-free supernatant pH was adjusted (pH 6.5) to verify the inhibition zone without interference of organic acids action. The activity of all antimicrobial substances was lost after

proteolytic enzymes treatment, showing the protein nature in the supernatant of the six strains (no show

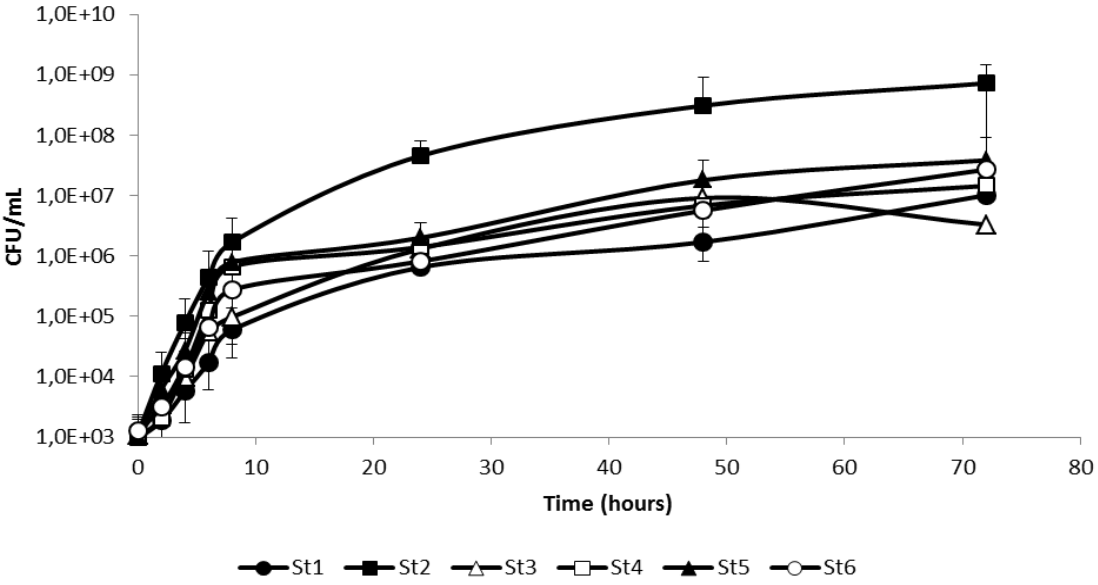

Fig 2. Growth curve of the six strains isolated from the attiéké showing an inhibition zone by double-agar layer test

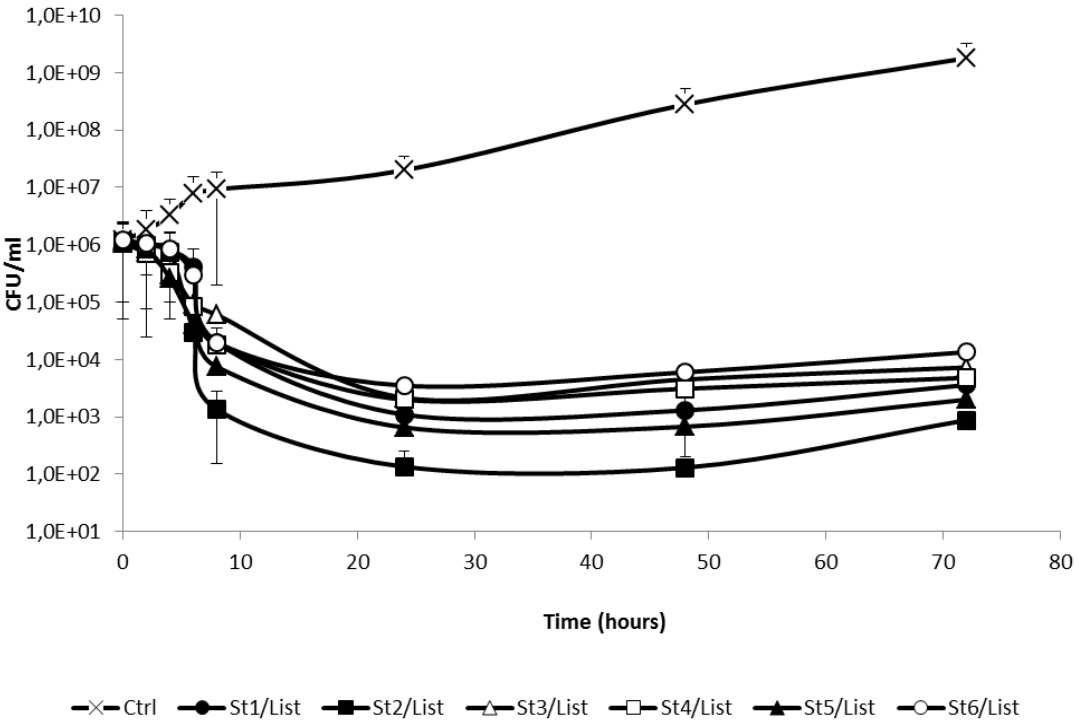

Fig 3. Evolution of the *Listeria monocytogenes* cfu count in the presence of each of the six strains isolated from the Attiéké. Growth of *Listeria monocytogenes* in a control without added antagonistic strain (x) was also monitored

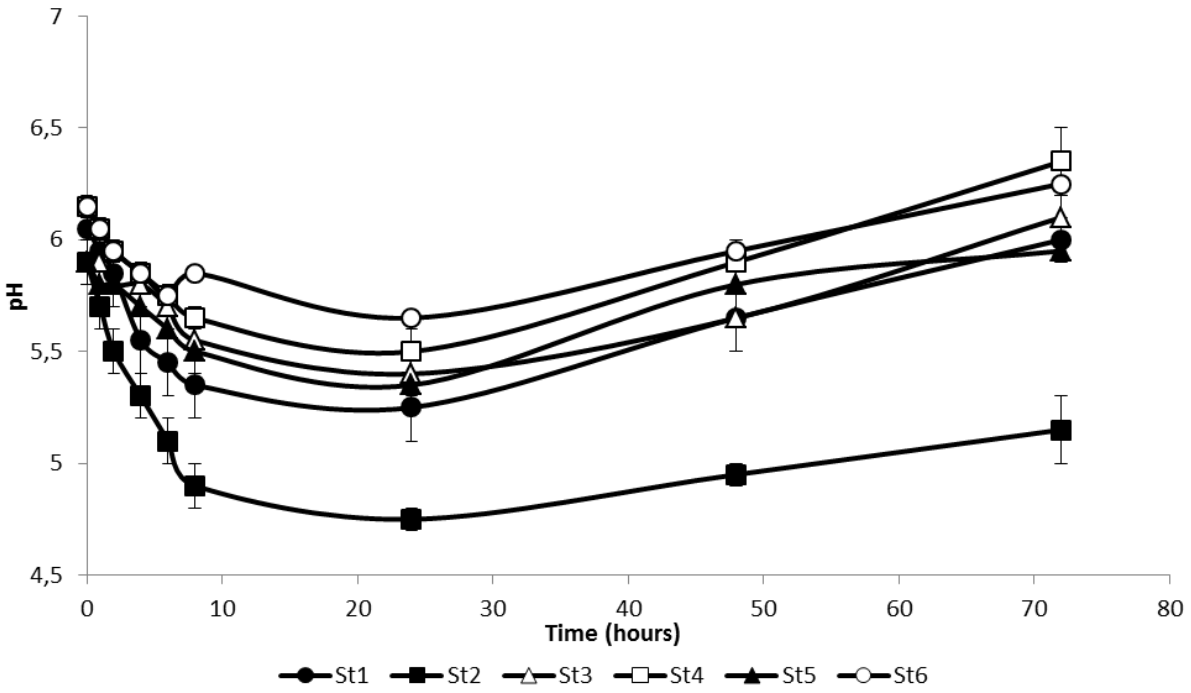

Fig 4. pH measurement in the supernatant of each of the six strains isolated from the Attieke by the double-agar layer test

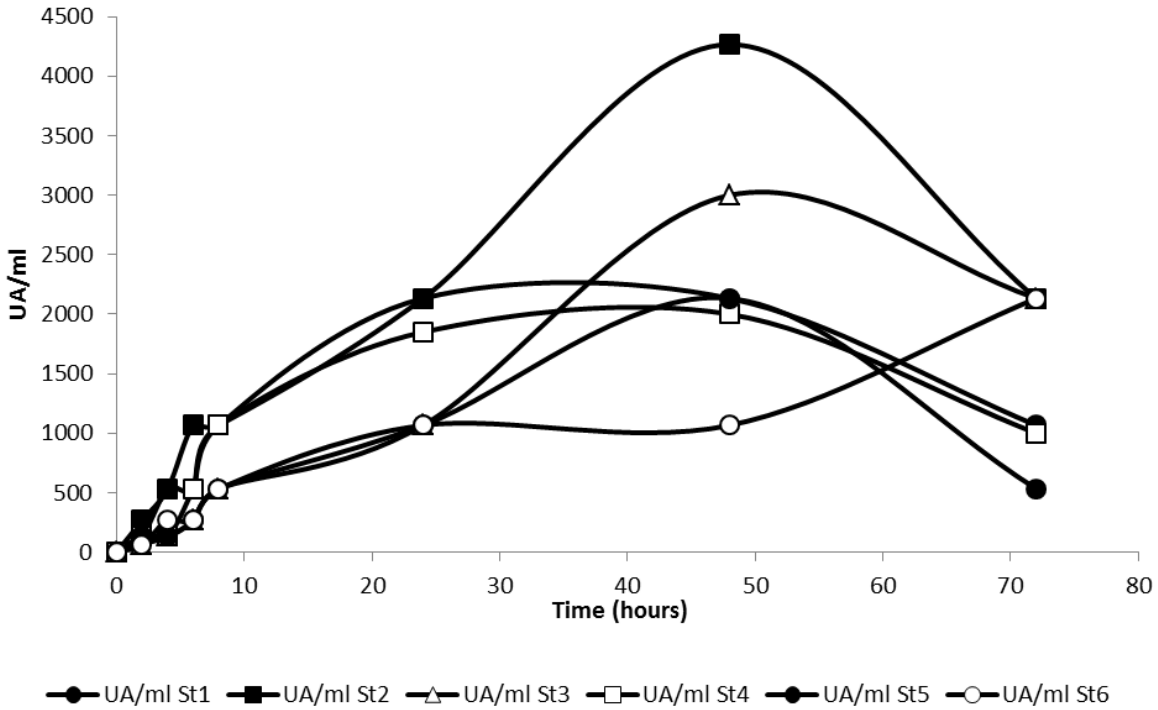

Fig 5. Bacteriocins activity measurement in the supernatant of each of the six strains isolated from the Attieke by the double-agar layer test

3.3. Identification of isolates St1, St2, St3, St4, St5 and St6 by PCR bacteriocin genes detection

Amplification of our six genomic DNA with genus-specific primers has been positive with St1 (226 bp) when Sakacin primer was used, positive with St2 (332 bp) when Pediocin primer was used, positive with St3 (412 bp) when enterocin primer was used, and positve with St4,St5 and St6 (428 bp) when plantaricin primer was used (Fig. 6). Isolates St1, St2, St3, and St4, 5&6 are thus considered to be respectively strains of *L. sakei*, *Pediococcus acidilactici*, *Enterococcus faecium* and *L. plantarum* for the last three.

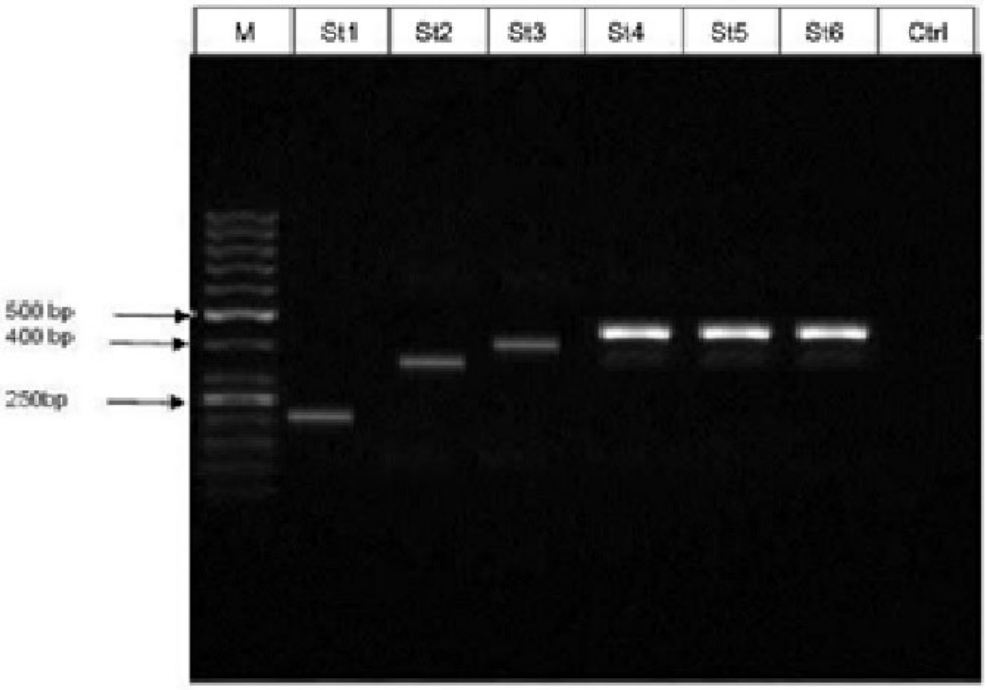

Fig. 6. Agarose gels showing DNA fragments obtained after PCR with species-specific primers. Lane M: O'GeneRulertm 50 bp DNA Ladder (Fermentas), lane St1 to St6 (positive control), lane Ctrl: no DNA loaded (negative control),

4. Discussion

Based on the performed PCR reactions targeting bacteriocins genes in the total DNA of each strain, which showed activity in the double-agar layer test against *L. monocytogenes* M, positive results were obtained, pointing that these strains carry these genes in the genomic DNA. However it should be noted that further more thorough study as for example sequencing is necessary to identify unambiguously the corresponding strains. Remiger et al. (1996) have shown that unspecific amplification was recorded when performed PCR targeting enterocin B gene in L. sakei ST22Ch and L. sakei ST153Ch. This underlines the importance of sequencing of the PCR products for confirming the identity of the generated PCR products.

All off the six isolates *L. sakei*, *Pediococcus acidilactici*, *Enterococcus faecium* and the three *L. plantarum* examined in this study had inhibitory activity against *L. monocytogenes* which was not attributed to bacteriophage, acid inhibition, or hydrogen peroxide and was eliminated by treatment with protease, complying with the classic definition of bacteriocins (Jagannathan et al. 2014). Lactobacillus, Pediococcus and Enterococci have been well documented for the production of bacteriocins which may give them an environmental advantage against susceptible bacteria (Nes et al. 2006). Certains of these bacteriocin-producing strains have been characterized for use as probiotics in commercial products (Cebrián et al. 2012). Now it is well known that bacteriocins produced by these strains belong to class IIa (Nes & Holo, 2000). The anti-listerial activity of this class IIa bacteriocins is allocated to the conserved sequence YGNGV on the bacterial genome (drider, fimland, héchard, McMullen, & prevost, 2006; Nes & Holo, 2000). Typical LAB bacteriocins have narrow antibacterial spectrum and inactivate closely related bacteria only. The presence of these strains in our Attiéké could therefore explain the extension of shelf life compared to the other traditional foods mentioned above.
As shown in Fig 2 & 5, Bacteriocins are produced at higher levels during the stationary phase of fermentation. This suggests that the six bacteriocins may be produced at high levels during all phases of (fermented) Attieke processing. The antibacterial spectrum of activity of these strains (St1 to St6), indicates their potential for use in a mixed starter culture for the fermentation of Attieké products. Further research on their technological properties, safety and the production of specific flavour compounds is in progress.

Conclusion

In brief, it appears that the Attiéké samples collected in the area of Abidjan and Dabou include certain kinds of lactic acid bacteria (LAB).
However, PCR identification alone though it is a fundamental preliminary step remains sketchy and imprecise, herefore is not sufficient. Sequencing of the PCR fragments is required for a better strains authentication.
On the other hand, the study of bactericidal activity (bacteriocinogenic and acidifying) of these six strains has shown that this action may extend the life of Attiéké by its influence on the development of the total flora responsible for the deterioration of Attiéké.
In perspective, another studies could be considered to evalute the capacity of these strains in the conservation of other food matrix.

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