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*Full Length Research Paper*

# *In vitro* detection and characterization of bacteriocin-like inhibitory activity of lactic acid bacteria (LAB) isolated from Senegalese local food products

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**The prevalence of lactic acid bacteria (LAB) in Senegalese local food products was determined to be $10^9$ CFU/g in millet flour and milk products, and $10^3$ CFU/g in seafood products. These food products are generally preserved by spontaneous fermentation (without addition of starters). Of 220 lactic acid bacteria strains randomly selected from such products, 12 isolates capable of producing bacteriocin-like substances (bac+) were detected. Based on the use of API 50 CH test kits and 16S rDNA sequencing, 11 isolates were characterized as Lactococcus lactis subsp. lactis strains and one as an Enterococcus faecium strain. Nisin- and enterocin B-encoding genes were respectively identified in the bac+ lactococcal strains and the E. faecium strain. Since the bac+ Lc. lactis strains were isolated from different products, it suggests a high potential of growth by these strains in variable ecological environments. Expression of the nisin gene was indicated for one of the lactococcal strains, designated Lc. lactis subsp. lactis CWBI-B1410, which showed the highest in vitro antibacterial activity. An antibacterial preparation prepared from the CWBI-B1410 strain showed many similarities with nisin with regards to its inhibitory effects, heat resistance, protease sensitivity profile, as well as retention time of the antibacterial substances on a $C_{18}$ column. These results suggest that a nisin-like substance is produced by the CWBI-B1410 strain. This strain has been selected for application as an additional barrier to supplementation with sodium chloride as a means to improve the bacterial quality of fish commodities in Senegal.**

**Key words:** *Lactococcus lactis*, antimicrobial, bacteriocins, Nisin-like substance.

## INTRODUCTION

Lactic acid bacteria (LAB) occur naturally in several raw materials (e.g. milk, meat, flour, etc.) used to produce foods (Rodriguez et al., 2000). They are the biological basis for the production of fermented foods in which they perform both acidification and production of flavour compounds. Moreover, LAB protects foods from spoil-age and pathogenic microorganisms due to the produc-tion of lactic and acetic acids, hydrogen peroxide, diacetyl, fatty acids, phenyllactic acid and/or bacterio-cins (Corsetti et al., 1998).

Bacteriocins are ribosomally synthesized; extracel-lularly released bioactive peptides or peptide com-plexes that have a bactericidal or bacteriostatic effect on other (usually closely related) bacterial species (Flynn et al., 2002). Many bacteriocins produced by LAB, such as nisin and lacticin 3147 produced by some *Lactococcus lactis* strains, have a broad spectrum of inhibition and are effective in preventing microbial food spoilage and to inhibit growth of pathogens in certain food systems (Abee et al., 1995; Einarsson and Lauzon, 1995; Ryan et al., 1996). Therefore, these proteinaceous inhibitors have become the focus of considerable interest in the field of food preservation. Bacteriocin-producing LAB originally isolated from a food are the best candidates for improving the micro-biological safety of these foods, because they are well adapted to the conditions in the foods and should therefore be more competitive than LAB isolated from other sources. However, the detection rate of bac-teriocin-producing LAB strains isolated from foods can be as low as 0.2% (Conventry et al., 1997; Garver et al., 1993).

Food fermentation by making use of LAB as natural Food fermentation by making use of LAB as natural

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Food fermentation by making use of LAB as natural Senegal. The resulting food commodities can be appro-priate ecological habitats for LAB that is capable of producing bacteriocins. The aim of the current study was to identify bacteriocin-producing LAB strains from Senegalese traditional foods, and to characterize their *in vitro* bactericidal activity with a view towards using them as biopreservatives for storage of fish products in Senegal.

## MATERIALS AND METHODS

### Bacterial strains and media

Modified MRS (MRSm) and M17 (M17m) media [MRS and M17 media containing 0.1% (w/v) glucose and supplemented with 50 µg/ml of cycloheximide (Sigma, St Louis, USA) and 100 UI/ml of polymixin B (Sigma)] were used for isolation of lactic acid bacteria from foods (Davidson and Cronin, 1973). The glucose concentration was reduced in the modified media to reduce the produc - tion of lactic and acetic acids by isolates *(*Conventry et al., 1997*)*. *S. aureus*, *L. monocytogenes*, *E. coli*, *S. infantis*, *S. typhimurium*, *P. pentosaseus* and *L. curvatus* were used as indicator strains for detection of antimicrobial and bacteriocin-like inhibitory activity. The bacterial strains were obtained from the culture collection of the Unit of Bio-Industries (Gembloux Agricultural University, Belgium).

### Source and nature of food samples

Thirty two (32) local food samples were used for the isolation of LAB. The foods were purchased from traditional markets in Dakar and Thies (Senegal), and included fermented and raw seafood, fermented milk and fermented millet flour products. These foods are generally stored at ambient temperatures (around 30°C) at the markets.

### Isolation and selection of LAB

LAB was isolated from foods by direct plating. A 10% (w/v) food sample in peptone (0.1%) water was homogenized, serially diluted (10-fold) and then 100 µl of each dilution was spread onto MRSm or M17m. Plates were incubated anaerobically (BD, BBL Campypak microaeroplilic system; Sparks, USA) for 48 h at 30°C. Agar plates with about 400 colonies were overlaid with 3 ml of soft (0.75%) agar medium that had been inoculated with 30 µl of the indicator bacterial cultures described above. Plates were incubated overnight at 37°C and colonies producing a zone of growth inhibition (clearing zone around the colony) in the indicator lawn were randomly selected and purified for further assays.

### Detection of bacteriocin-like inhibitory substances produced by selected isolates

The detection of bacteriocin-like inhibitory substances in the neutralized cell-free culture supernatant (NCFS) of the selected isolates was performed by the agar well diffusion (WDA) technique, as described by Barefoot and Klaenhammer (1983). In order to eliminate the inhibitory effect of lactic acid and/or $H_2O_2$, the pH of the supernatants were adjusted to 6.5 with 5 N of NaOH and treated with catalase at a final concentration of 65 UI/ml, followed by filtration through a 0.22 µm pore size filter (Type Minisart NML; Sartorius GmbH, Göttingen, Germany). The antibacterial activity of the NCFS of the positive (bac+) strains was evaluated by critical dilution assays *(*Barefoot and Klaenhammer 1983*)*. The minimum inhibitory concentration (MIC) of each NCFS was defined as the reciprocal of the highest dilution showing a halo of inhibition, and it was expressed in arbitrary units per milliliter (AU/ml). To assess the sensitivity of the anti-microbial agent in the NCFS to proteolytic enzymes, 180 µl of diluted NCFS (160 AU/ml) were incubated for 90 min at 37°C with 20 µl of the following enzyme solutions: P 3911 protease (16.6 UI/ml), P XIV protease (7.9 UI/ml), P XVIII protease (0.66 UI/ml), proteinase K (59.2 UI/ml), chymotrypsin (70 UI/ml), pepsin (80 UI/ml) and trypsin (75 UI/ml). Controls were incubated with 20 µl of 50 mM phosphate buffer (pH 6.5). The residual activity was measured with the WDA assay. The stability of the inhibitor in the NCFS to heat was assessed at different pH values by diluting the NCFS to 1/4 in 50 mM phosphate buffers of which the pH was adjusted to 5, 7 and 9, respectively. Samples were placed in a water bath at 60, 70, 80, 90, 100, 110 and 121°C for 10 min. The antibacterial activity of the heated samples was assessed by critical dilution assays, as described above, and compared to those of non-heated control samples.

### Phenotypic characterization of the bacteriocin producers

Cellular morphology of the bacteriocin-producing strains were examined under a microscope, catalase activity was tested by the 3% $H_2O_2$ method and the Gram stain phenotype was determined by the KOH method *(*Buck 1982*)*. Biochemical tests and fermentation patterns of selected strains were determined using the API 50 CH test strip (Biomérieux, France). The results were obtained via the computerized database service provided by the manufacturer.

### Characterization of bacteriocin-producing strains by 16S rDNA sequencing

Total DNA was isolated from liquid cultures of the bacteria with the Wizard genomic DNA purification kit (Promega, Madison, USA), and used as a template for the amplification of the 16S rRNA genes by the polymerase chain reaction (PCR). This was performed with the primer pair SPO and SP6 (Table 1) targeted against regions of 16S rDNA. PCR products were resolved by electrophoresis in 1% (w/v) agarose gels, visualized by ethidium bromide staining and purified with the Microcon YM-100 kit (Bedford, MA, USA). The amplicons were sequenced using a set of eight universal primers (Table 1) and the BigDye Terminator v3.0 kit, as specified by the supplier. Analysis of the nucleotide sequences was performed using the Vector NTI (Version 8) software package (BD Biosciences, San Jose, USA).

### Genetic characterization of the nisin and enterocin B structural genes

To determine the presence of bacteriocin- and enterocin B-encoding genes in selected isolates, PCR and sequencing was performed, as described above, except that the specific primers presented in Table 1 were used. Sequences were analyzed using the Vector NTI (Version 8) software package, translated to proteins, and the deduced amino acid sequences were aligned to those of bacteriocin precursors collected from the EMBL or GenBank databases.

### Reverse transcription (RT)-PCR

Total RNA was isolated from the CWBI-B1410 isolate using the RNeasy Midi Kit (Qiagen, Hilden, Germany) and the first strand cDNA was synthesized using the First Strand cDNA Synthesis kit (Fermentas Life Sciences, Germany), as specified by the supplier. Subsequently, PCR was performed using the nisin gene-specific primer pair nisin F/nisin R (Table 1).

**Table 1**. Universal and specific primers used for PCR and sequencing of the 16S rDNA and bacteriocin genes from bac+ strains.

| Targeted genes | Technique | Primers | Sequence | Sense | Source |
|---|---|---|---|---|---|
| | PCR | 16SPO | 5'-AAGAGTTTGATCCTGGCTCAG-3' | Forward | Ventura et al. 2001 |
| | | 16SP6 | 5'-CTACGGCTACCTTGTTACGA-3' | Reverse | Ventura et al. 2001 |
| | | F1 | 5'-CTGGCTCAGGAYGAACG-3' | Forward | |
| 16S rDNA | | F2 | 5'-GAGGCAGCAGTRGGGAAT-3' | Forward | |
| | | F3 | 5'-ACACCARTGGCGAAGGC-3' | Forward | |
| | Sequencing* | F4 | 5'-GCACAAGCGGYGGAGCAT-3' | Forward | |
| | | R1 | 5'-CTGCTGGCACGTAGTTAG-3' | Reverse | |
| | | R2 | 5'-AATCCTGTTYGCTMCCCA-3' | Reverse | |
| | | R3 | 5'-CCAACATCTCACGACACG-3' | Reverse | |
| | | R4 | 5'-TGTGTAGCCCWGGTCRTAAG-3' | Reverse | |
| | | NisinF | 5'-TTATTTGCTTACGTGAATAC-3' | Forward | Gross et al.1971 |
| | | Nisin R | 5- AGATTTTAACTTGGATTTGC-3' | Reverse | Mulders et al. 1991 |
| | | Enterocin A F | 5'-AAATATTATGGAGTGTAT-3' | Forward | Fouquié et al. 2003 |
| Bacteriocin | PCR* | Enterocin A R | 5'-GCACTTCCCTGGAATTGCTC-3' | Reverse | Park et al. 2003 |
| genes | and | Enterocin B F | 5'-AAAATGTAAAAGAATTAAGTACG-3' | Forward | Fouquié et al. 2003 |
| | sequencing* | Enterocin B R | 5'-AGAGTATACATTTGCTAACCC-3' | Reverse | |
| | | Enterocin L50A F | 5'-ATGGGAGCAATCGCAAAATTA-3' | Forward | Fouquié et al. 2003 |
| | | Enterocin L50AR | 5'-TTTGTTAATTGCCCATCCTTC-3' | Reverse | |

*The primers were designed based on alignments of the 16S rDNA, nisin and enterocin genes collected from EMBL (Europeen Molecular Biology Laboratory) and Genbank databases, and were supplied by SIGMA-Proligo.

**Comparison of the bacteriocin-like inhibitory substance of strain CWBI-B1410 with nisin**

A high potency commercial nisin powder (Fluka, Buchs, Deutschland) was used to prepare a nisin stock solution at a concentration of 2 mg/ml in deionized distilled water (dd$H_2O$), which was then filter-sterilized. The bac+ strain CWBI-B1410was grown at 30°C in 1.5 l of modified MRS broth [containing 0.1% (w/v) of each peptone, meat extract and yeast extract] to limit the casein content in the medium of protein and peptide compounds in the culture supernatant. A concentrated crude antimicrobial preparation was prepared from the culture supernatant by precipitation with solid $(NH_4)_2SO_4$ at a final concentration of 242 g/l at 4°C with stirring. The precipitate was collected by centrifugation at 17 000 x g for 20 min at 4°C, suspended in 50 ml of Milli-Q water and filter sterilized (0.45 µm pore size; Type Minisart NML; Sartorius GmbH, Göttingen, Germany). The antibacterial activity of the solution was assessed by the critical dilution assay. The concentrated crude antimicrobial preparation was desalted as follows: 25 ml of the preparation was passed through a 60 ml $C_{18}$ solid-phase extraction cartridge (Applied Separation, Allentown, USA) in a vacuum. The column was washed with 400 ml of Milli-Q water until discoloration. The antimicrobial substances were eluted from the cartridge by using 90 ml of 50 mM phosphate buffer (pH 6.5) containing 40% isopropanol. The liquid phase (water and isopropanol) were evaporated to dryness in a Speedy-vac (Savant, 110A). The residue was dissolved in 7.5 ml of Milli-Q water to yield a preparation of which the antibacterial activity was similar to that of a 10-fold dilution of the nisin solution stock. The inhibitory effects against various indicator strains, heat and protease sensitivities, as well as reverse-phase high performance liquid chromatography (RP-HPLC) separation of the two solutions were compared. The $C_{18}$ RP -HPLC was performed on a analytical column (Chromospher, 4.6 x 250 mm, 5 µm; Varian) by using a cycle similar to that described by Megrhrous et al. (1997). The eluates from the column were collected into different fractions according to UV absorbance (214 nm). Peak fractions were evaporated to dryness, as described above, and the residue was suspended in 500 µl of Milli-Q water, pasteurized (10 min, 80°C) and assayed for antibacterial activity with the WDA assay. The active fraction was analyzed by mass spectrum, which was carried out on a 4700 Proteomic Analyzer (Applied Biosystems), for determination of the molecular mass of the main constituents.

## RESULTS

### Detection of bacteriocin-producers and biochemical characterization of the bactericidal activity

The prevalence of LAB was determined to be $10^9$ CFU/g in traditionally fermented cereal and milk products, and $10^3$ CFU/g in seafood. Two-hundred-and-twenty (220) LAB isolates from these different food products were randomly selected, purified and assayed for production of bacteriocin-like inhibitory substances. The NCFS of twenty (20) of these isolates displayed antibacterial activity. Twelve (12) of the 20 isolates retained their activity following treatment of the NCFS with catalase (65 UI/ml). The antibacterial activity of the NCFS of the 12 isolates was either completely or partially inactivated by proteolytic enzymes (Figure 1), but was persistent after heat treatment, particularly at acidic and neutralized pHs (Figures 2A and 2B), indicating the presence of bacteriocin-like inhibitory substances (bac+) in these solutions.

The 12 bac+ isolates were catalase-negative and Gram-positive cocci (data not shown), while their carbohydrate fermentation profiles, as determined with API 50 CH test strips, showed a high similarity. Identifications made by the API database indicated that the isolates were all strains of *Lactococcus lactis* subsp. *lactis*, as shown in Table 3. Therefore, two stains, designated CWBI-B1410 and CWBI-B1426, were randomly selected from the seven and the three strains respectively isolated from the fermented cereal and the seafood samples. Two more strains,

**Table 2.** Repartition and API 50 CH characterization of bacteriocin-like substance producing lactic acid bacteria strains from Senegalese local foods.

| Nature of foods | Samples numbers | Prevalence of LAB (cfu/g) | LAB RSBA | NCSI LAB | Bac+ LAB | API 50 CHL Identification | FSY Bac+ LAB | Selected Bac+ LAB |
|---|---|---|---|---|---|---|---|---|
| Seafood | 16 | $10^5$ | 60 | 5 | 3 | *Lactococcus lactis* (93 %) | 1 | CWBI-B1426 |
| Millet flour | 8 | $10^9$ | 63 | 10 | 7 | *Lactococcus lactis* (95,7 %) | 1 | CWBI-B1410 |
| Dairy | 8 | $10^9$ | 97 | 5 | 2 | *Lactococcus lactis* (94 %)<br>*Lactococcus lactis* (91%) | 2 | CWBI-B1427<br>CWBI-B1411 |
| **Total** | 32 | | 220 | 20 | 12 | 4 | 4 | |

LAB-RSBA: lactic acid bacteria strains randomly selected from food samples for bacteriocin-like inhibitory assays
NCSI -LAB: lactic acid bacteria strains that displayed antibacterial activity in neutralized culture supernatant Bac+ LAB: lactic acid bacteria strains that showed bacteriocin-like inhibitory activity
FSY Bac+ LAB: number of food samples that yielded bac+ isolates.

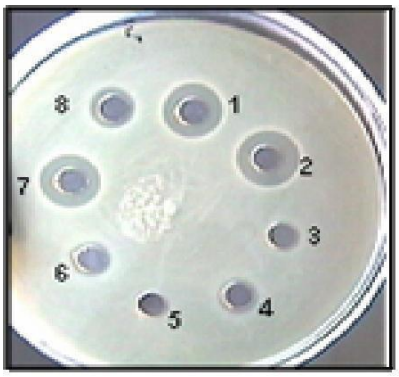


**Figure 1**. Effects of catalase and proteolytic enzymes on the antimicrobial activity of the neutralized culture supernatant of CWBI-B1410 against *P. pentosaseus.* Similar results were obtained for culture supernatants of the CWBI-B1426, CWBI-B1427 and CWBI-B1411 strains.**1:** not treated, **2:** catalase, **3:** P 3910, **4:** chymotrypsin, **5:** P XIV protease, **6**: P XVIII protease, **7**: pepsin, **8**: proteinase k

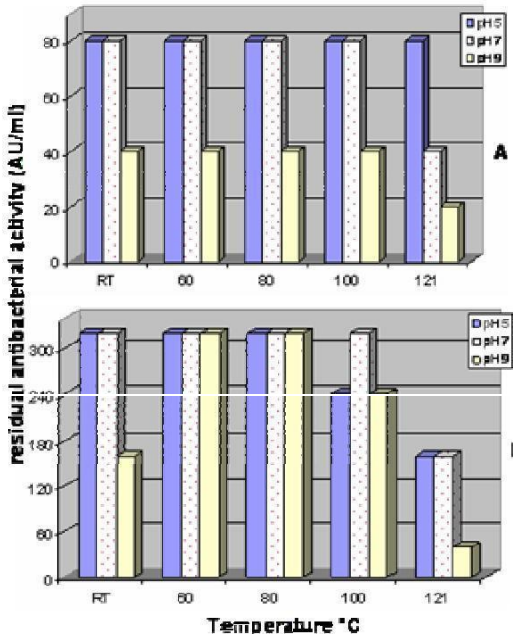


**Figure 2**. Effect of heating (60 to 121°C for 10 min) on the stability of the inhibitor in CWBI-B1410 (A) and CWBI-B1411 (B) neutralized culture supernatants at pH 5, 7 and 9. The residual activity was determined by critical dilution assays using *P. pentosaseus* and *Listeria monocytogenes* as indicator strains for CWBI-B1410 and CWBI-B1411, respectively. The heat sensitivities of CWBI -B1426 and CWBI-B1427 culture supernatants were similar to that of CWBI-B1410.

designated CWBI-B1427 and CWBI-B1411, isolated from two different samples of fermented milk products, were also selected for further investigation, as indicated in Table 2.

The sensitivity of 35 bacterial strains from different genera to the antibacterial activity of NCFS from cultures of the four strains is presented in Table 3. The NCFS of the CWBI-B1410, CWBI-B1427 and CWBI-B1426 strains had similar spectra of inhibition, which were broader than that of the CWBI-B1411 strain. All of the NCFS showed bactericidal activity against *Bacillus coagulans*, which is involved in food spoilage (Garcia et al., 2003), and against the food-borne pathogen *L. monocytogenes*. The NCFS of CWBI-B1410, CWBI-B1426 and CWBI-B1427 displayed bactericidal activity against *Bacillus cereus*.

Antibacterial activity of the NCFS and crude concentrated bacteriocin preparations of the four strains were determined by using a set of 10 indicator bacteria, including the selected four strains (Table 4). In contrast to *L. curvatus* and *B. cereus*, certain indicator strains such as *B. coagulans* showed high sensitivity ($10^4$ to $10^5$ AU/ml) to the antibacterial solutions. The level of sensitivity of *P. pentosaseus* and *Bacillus fusiformis* to

**Table 3.** Inhibitory spectrum of neutralized supernatants from the CWBI-B1410 (A10), CWBI-B1411 (A11), CWBI-B1426 (A26) and CWBI-B1427 (A27) cultures.

| No. | Indicator bacteria* | Neutralized culture supernatants | | | |
|---|---|---|---|---|---|
| | | A10 | A11 | A26 | A27 |
| 1. | A10 | - | + | - | - |
| 2. | A11 | + | - | ± | ± |
| 3. | A26 | + | + | - | - |
| 4. | A27 | - | + | - | - |
| 5. | *Bacillus cereus* BS12 | + | + | + | + |
| 6. | *Bacillus coagulans* LMG 6326 | + | + | + | + |
| 7. | *Bacillus subtilis* | + | - | ± | + |
| 8. | *Bacillus subtilis* 5499 GR1 | - | - | - | - |
| 9. | *Candida albicans* | - | - | - | - |
| 10. | *Candida glabrata* | - | - | - | - |
| 11. | *Enterococcus faecium* | ± | + | ± | + |
| 12. | *Erwinia chrysanthemi* | - | - | - | - |
| 13. | *Erwinia uedovora* | - | - | - | - |
| 14. | *Lactobacillus brevis* 7761 | + | - | + | + |
| 15. | *Lactobacillus curvatus* CWBI B28 | + | + | + | + |
| 16. | *Lactobacillus curvatus* LMG21688 | + | + | + | + |
| 17. | *Lactobacillus doderlein* THT | + | - | + | + |
| 18. | *Lactobacillus fermentum* MU1 | + | - | + | + |
| 19. | *Lactobacillus helveticus* LMG 6413T | + | - | + | + |
| 20. | *Lactobacillus paracasei* ssp. *paracasei* LMG 9192 | - | - | - | - |
| 21. | *Lactobacillus plantarum* πr | + | - | + | + |
| 22. | *Lactobacillus plantarum gobetti* | + | + | + | + |
| 23. | *Lactobacillus rhamnosus* | + | - | + | + |
| 24. | *Lactobacillus rhamnosus mbanick Senegal* | + | - | + | + |
| 25. | *Lactococcus lactis ssp. lactis* LMG6890 | + | + | + | + |
| 26. | *Leuconostoc ezal 2* | - | - | - | - |
| 27. | *Listeria monocytogenes* | + | + | + | + |
| 28. | *Pediococcus acidilactici* | - | + | - | - |
| 29. | *Pediococcus pentosaseus* | + | - | + | + |
| 30. | *Salmonella infantis* | - | - | - | - |
| 31. | *Salmonella typhimurium* | - | - | - | - |
| 32. | *Staphylococcus aureus* | - | - | - | - |
| 33. | *Staphylococcus aureus* ATCC 29213 | - | - | - | - |
| 34. | *Staphylococcus carnosus* | + | - | + | + |
| 35. | *Streptococcus thermophilus* | + | + | + | + |

* All indicator bacteria were tested for inhibition of growth, determined by an area of inhibition surrounding each well, which was cut in the agar medium and inoculated with 110 µl of the bacterial culture of the indicator bacteria. Sixty µl of filter-sterilized neutralized (pH 6.5) cell-free supernatant was inoculated into each well. A zone of growth inhibition ≥ 8mm was expressed as positive reaction (+), lack of inhibition as negative inhibition (-); and a weak area of inhibition as < 8 mm (±).

antimicrobial solutions of the CWBI-B1410, CWBI-B1426 and CWBI-B1427 strains was between those of these two groups. *L. monocytogenes* was more sensitive to the antimicrobial solution of CWBI-B1411 than to those of the three other strains.

### Genetic characterization of the strains

Identification by 16S rDNA sequencing confirmed the biochemical identification (*Lactococcus lactis* subsp. *lactis* strains) of the CWBI-B1410, CWBI-B1426 and CWBI-B1427 strains. However, the 16S rDNA sequence of the CWBI-B1411 strain matched best with those of *Enterococcus faecium* strains (Figure 3). The closest match for the CWBI-B1411 strain, that is, *E. aecium*, was different from the identification determined by the API method (Table 2). However, we prioritized the genetic identification because of the high percentage of similarity (99%) obtained with this technique, compared to that obtained using the API 50 CH test strip. The lower percentage of identity obtained with the latter can be due to the inadequacy of API 50 CH for accurate and unequivocal identification of *Enterococcus* strains. The 16rDNA sequences of the four

**Table 4.** Antibacterial activity of neutralized culture supernatants and crude concentrated bacteriocin-like inhibitory preparations of strains CWBI-B1410 (A10), CWBI-B1411 (A11), CWBI-B1426 (A26), and CWBI-B1427 (A27).

| Indicators bacteria | Bacteriocin-like inhibitory Activity (x $10^2$ AU/ml) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A26 | | A10 | | A27 | | A11 | |
| | CFS | CCB | CFS | CCB | CFS | CCB | CFS | CCB |
| *Bacillus cereus* | 0.20 | 0.40 | 0.4 | 0.8 | 0.20 | 0.40 | - | - |
| *Bacillus coagulans LMG 6326* | 51.2 | 4010 | 102 | 4010 | 25.6 | 102 | 51.2 | 4010 |
| *Bacillus fusiformis* | 3.20 | 12.80 | 6.4 | 25.6 | 6.4 | 12.80 | - | - |
| CWBI-B1410 | - | - | - | - | - | - | 0.8 | 3.2 |
| CWBI-B1411 | 0.2 | 0.8 | 1.6 | 6.4 | 0.2 | 0.8 | - | - |
| CWBI-B1426 | - | - | 0.8 | 3.2 | - | - | 0.8 | 3.2 |
| CWBI-B1427 | - | - | - | - | - | - | 0.8 | 3.2 |
| *Lactobacillus curvatus* | 0.80 | 1.6 | 3.2 | 6.4 | 0.2 | 1.6 | 0.2 | 1.6 |
| *Lactococcus lactis* subsp. *lactis* LMG 6890 | 0.2 | 0.8 | 1.6 | 6.4 | 0.2 | 0.8 | 0.8 | 3.2 |
| *Listeria monocytogenes* | 0.80 | 1.60 | 1.60 | 3.20 | 0.80 | 1.60 | 6.40 | 51.20 |
| *Pediococcus pentosaseus* | 3.20 | 12.8 | 6.4 | 25.6 | 0.8 | 6.4 | - | - |

CCB: crude concentrated bacteriocin-like substance solution, CFS: cell-free supernatant, -: no inhibition
CCB were prepared from the culture supernatant by precipitation with solid $(NH_4)_2SO_4$ at a final concentration of 246 g/l. The precipitate collected by centrifugation was suspended in 200 ml of Milli-Q water.

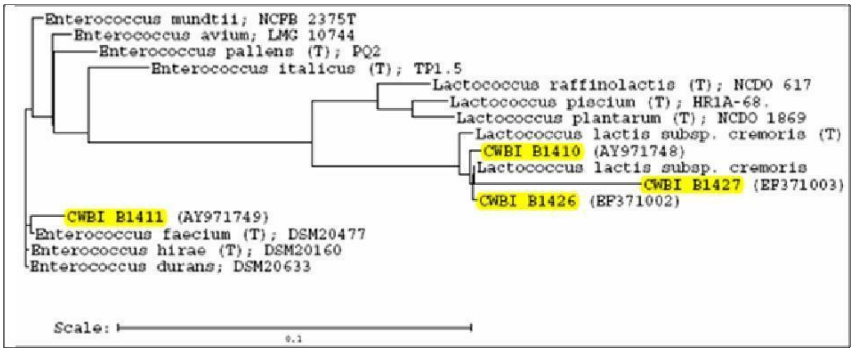


**Figure 3**. Dendogramme based on similarities of 16S rDNA sequences of the bac+ isolates with those of type strains from GenBank database. GenBank accession numbers of the new bacteriocin-producing isolates are indicated in the brackets.

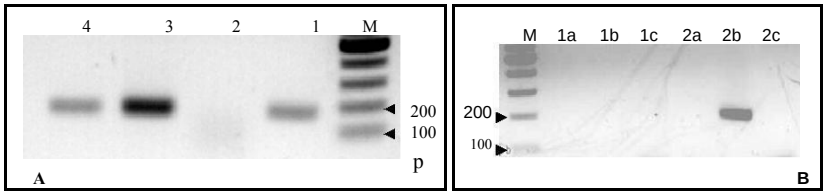


**Figure 4**. Agarose gel electrophoresis of PCR products generated from total DNA of bacteriocin-producing strains. In A, primer pair nisinF/nisinR was used: Lanes 1: CWBI-B1410; 2: CWBI-B1411; 3: CWBI-B1426; 4: CWBI-B1427. In B, the following primer pairs were used: a, enterocin AF/enterocin AR; b, enterocin BF/enterocin BR; and c, enterocin L50AF/enterocin L50AR. Lanes 1: CWBI-B1410; 2: CWBI-B1411. The DNA molecular size marker (Smart Ladder 100 bp) is indicated in lanes M.

bacteriocin- producing strains have been registered in the GenBank database under the accession numbers AY971748 (CWBI-B1410), AY971749 (CWBI-B1411), EF371002 (CWBI-B1426), and EF371003 (CWBI-B1427).

The results obtained following screening of the bac+ isolates DNA by PCR for bacteriocin-encoding genes are presented in Figure 4. A structural gene encoding the nisin A prepeptide (Gross and Morell, 1971) was detected in CWBI-B1410 DNA, a structural gene en-

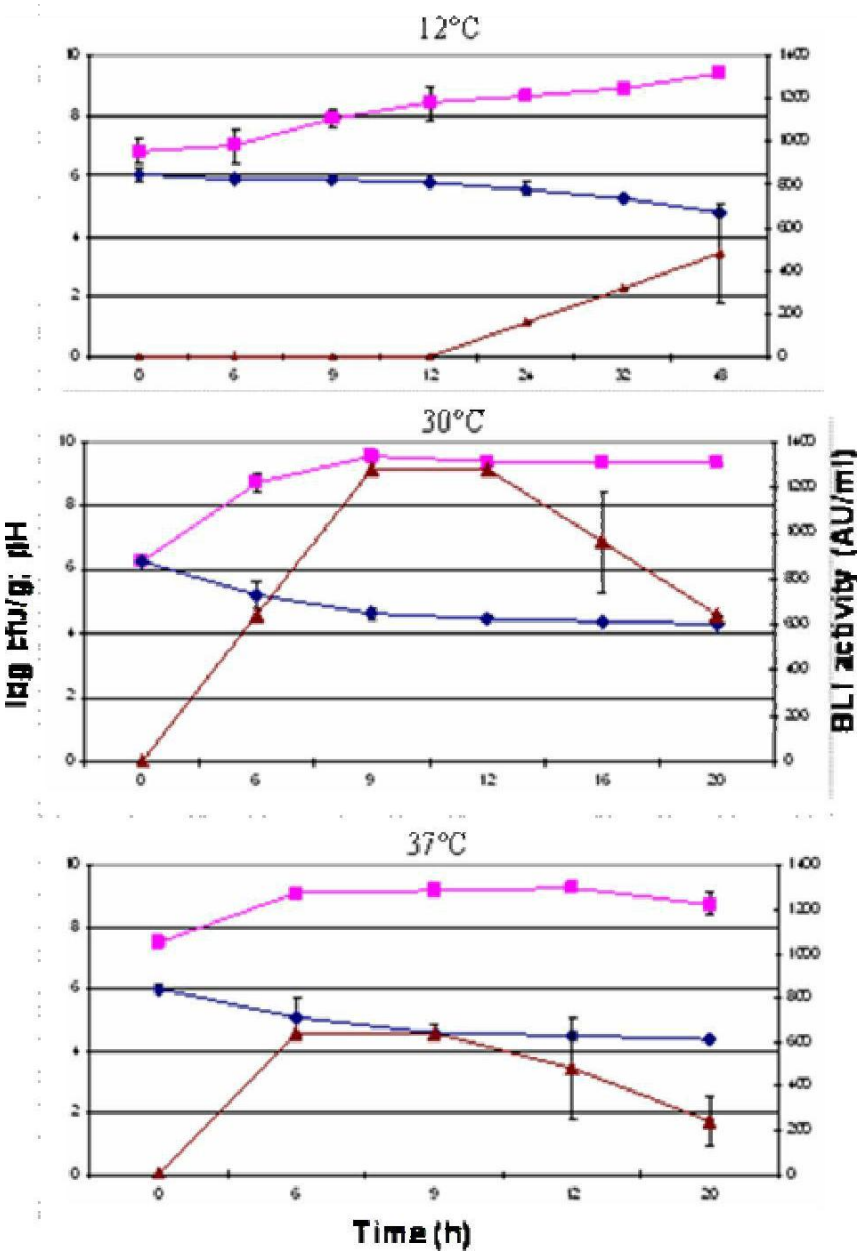


**Figure 5**. Relationship between *Lactococcus lactis* CWBI-B1410 growth and its bacteriocin-like inhibitory substance production in MRS broth at 12, 30 and 37°C. Symbols: Bacterial counts on MRS ( ); Culture pH ( ); Bacteriocin-like inhibitory activity in the culture supernatant ( ). The bacteriocin-like inhibitory activity was assessed by critical dilution assays using *P. pentosaseus* as indicator strain.

coding the nisin Z prepeptide (Mulders et al., 1991) was detected in both CWBI-B1426 and CWBI-B1427 DNA, and a structural gene encoding the enterocin B prepeptide (Causas et al., 1997) was detected in CWBI-B1411 DNA. The nucleotide sequences of these genes and their resulting prepeptide amino acid sequences have been deposited in the GenBank database under the accession numbers (EF371000 and ABN45880), (EU128491 and ABV64388), (EU 128485 and ABV64387), and (EF371004 and ABN45881) for the CWBI-B1410, CWBI-B1426, CWBI-B1427 and CWBI-B1411 strains, respectively. Because of its high antibacterial activity, the CWBI-B1410 strain was se-lected for more detailed characterization regarding its bacteriocin-like inhibitory substance.

**Similarities of the bacteriocin-like inhibitory substance of *Lactococcus lactis* CWBI-B1410 with nisin**

The relationship between *Lactococcus lactis* subsp. *lactis* CWBI-B1410 growth and production of its bacte-

**Table 5**. Comparison of nisin and the CWBI-B1410 bacteriocin-like inhibitory substance (BLIS) with regards to inhibitory effects, sensitivity to proteolytic enzymes and retention time on a $C_{18}$ column.

| | | nisin | | | | | | CWBI-B1410 BLIS | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Indicator bacteria | **NTS** | **1** | **2** | **3** | **4** | **5** | **NTS** | **1** | **2** | **3** | **4** | **5** |
| | *B. coagulans* LMG 6326 | + | - | - | - | + | - | + | - | - | - | + | - |
| | *Enterococcus faecium* | + | - | - | - | + | - | + | - | - | - | + | - |
| | *Lactobacillus curvatus* | + | - | - | - | + | - | + | - | - | - | + | - |
| | *Lc. lactis* CWBI 1410 | **+** | **-** | **-** | **-** | **+** | **-** | - | **-** | **-** | **-** | **-** | **-** |
| **WDA** | *Lc. lactis* LMG6890 | + | - | - | - | + | - | + | - | - | - | + | - |
| | *Listeria monocytogenes* | + | - | - | - | + | - | + | - | - | - | + | - |
| | *Pediococcus pentosaseus* | + | - | - | - | + | - | + | - | - | - | + | - |
| | *S. epidermis* CWBI-B1433 | - | - | - | - | - | - | + | - | - | - | + | - |
| | *Streptococcus thermophilus* | + | - | - | - | + | - | + | - | - | - | + | - |
| | *Weissella confusa* CWBI-B1438 | + | - | - | - | + | - | + | - | - | - | + | - |
| **$C_{18}$ RP-HPLC time retention** | | 31.5 min. | | | | | | 31.5 min. | | | | | |

CWBI-B1410 bactericion solution was prepared from the culture supernatant and had an activity similar to that of the commercial nisin solution, estimated at 1280 AU/ml by the well diffusion assay (WDA) using *P. pentosaseus* as indicator. All indicator strains (except CWBI-B1410 itself) were previously found to be sensitive to CWBI-B1410 neutralized culture supernatant activity as shown in Table 3.
NTS: not treated samples; enzymatic treatments: (1: protease P XIV; 2: Protease P XVIII; 3: chymotrypsin; 4: trypsin; 5: proteinase K).

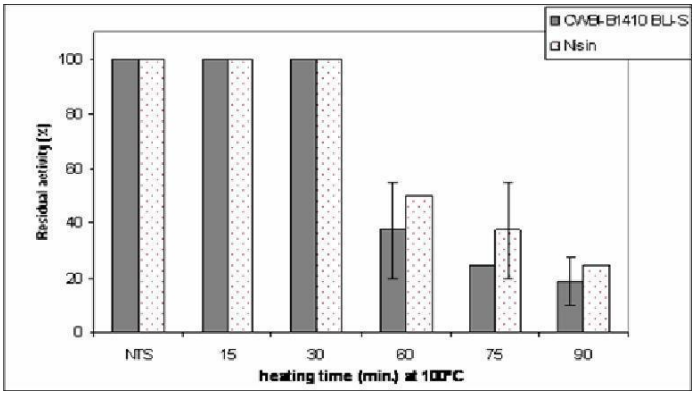


**Figure 6**. Comparison of heat stability of nisin and the CWBI-B1410 bacteriocin-like inhibitory substance. The residual antibacterial activity determined by critical dilution assays using *P. pentosaseus* as indicator strain was expressed as a percentage of the activity of the non-heated sample (NTS), which was considered as 100%.

riocin-like inhibitory substance in MRS broth at 12, 30 and 37°C is shown in Figure 5. As can be seen from this figure, the production of the bacteriocin-like inhibitory substance started at the exponential phase and reached its maximum during the stationary phase. The bacteriocin-like inhibitory activity remained constant for up to 9 h, and decreased as the CWBI- B1410 strain entered late stationary phase (12 - 13 h). The production of the antibacterial agent was more pronounced at 30°C.

To determine whether the nisin gene was transcribed into mRNA, total RNA was extracted from the CWBI-B1410 isolate at the end of the exponential growth phase (culture of 10 h at 30°C in MRS broth). A DNA fragment similar in size to the nisin structural gene (GenBank number EF371000) was amplified from the cDNA by PCR using primers nisin F/nisin R.No PCR products were observed when either the CWBI-B1410 total RNA or the cDNA synthesized of RNA isolated from the CWBI-B1411 strain were used as templates. In contrast 16S rDNA PCR products were detected for both strains (CWBI-B1410 and CWBI-B1411) by using the universal SPO/SP6 primers pair and their respecttive cDNA as templates (data not presented). These results indicated that the nisin structural gene identified in the CWBI-B1410 strain was transcribed into mRNA as required for biosynthesis of a nisin-like inhibitory substance during the growth of this strain.

Similarities between the inhibitory effects against different indicator bacteria, protease sensitivity, heat stability, as well as retention time on a $C_{18}$ column of the CWBI-B1410 bacteriocin-like bioactive agent and nisin

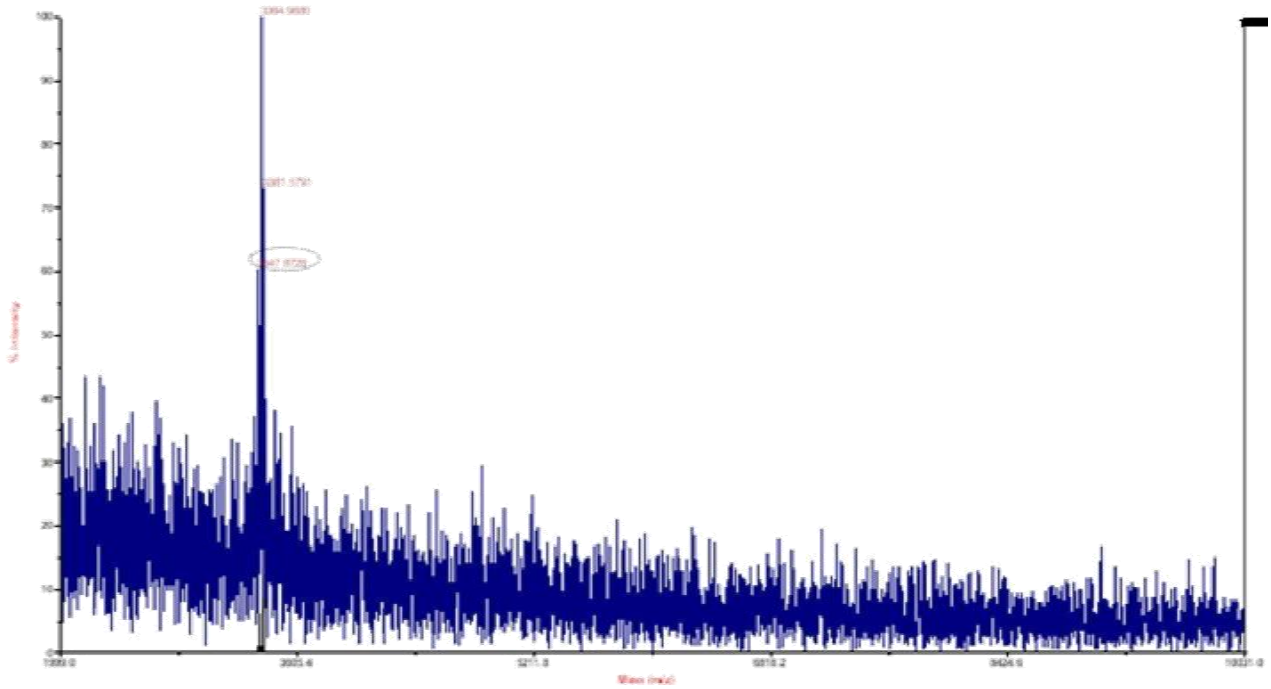

**Figure 7**. Mass spectrum of the positive fraction isolated from the crude bacteriocin-like inhibitory solution of the CWBI-B1410 strain by RP-HPLC separation on a $C_{18}$ column. A compound with a molecular mass identical to nisin ($C_{143}H_{228}N_{42}O_{37}S_7$) (Handary Bio-engineering BV, Oosterhout, Netherlands) is encircled.

were investigated. As shown in Table 5, all the indicator bacteria were sensitive to the bacteriocin- like inhibitory substance of strain CWBI-B1410 and they were also all sensitive to the inhibitory activity of nisin, except *Staphylococcus epidermis*. *Lc. lactis* CWBI -B1410 was inhibited by nisin, but resistant to its own bacteriocin-like substance, probably because of the immunity principle of bacteriocin producers (De Jong et al., 2006). The two bioactive agents showed identical protease sensitivity profiles, and identical retention times on the

C18 column (31.5 min), as well as similar heat stability (Figure 6 and Table 5). Only trypsin was unable to inactivate the two bioactive agents, which were not affected by heat treatment for 30 min at 100°C. Analysis of the mass spectrum of the active fraction isolated from the crude antibacterial solution ofthe CWBI-B1410 strain by RP-HPLC separation revealed the presence of three major compounds with molecular masses of 3364.96, 3381.58 and 3347.87 Da, respecttively (Figure 7).

## DISCUSSION

The aim of the current investigation was to isolate and identify bacteriocin-producing LAB from foods that could be used locally for improving food preservation (biopreservation) and biosafety. Seven isolates from fermented millet flour samples (the main cereal product in Sub-Saharan Africa), three isolates from raw sumpat grunt fish fillet, and two isolates from two different fermented milk samples, representing 5.45% of the tested strains, produced antibacterial compounds. The antibacterial compounds were resistant to catalase and heat, but were inactivated by proteolytic enzymes, indi-cating them to be bacteriocin-like substances according to Tagg et al. (1976) and Jack et al. (1995). Our detec-tion rate was higher than that reported by Ennahar et al. (1996) and Lasagno et al. (2002) in similar studies. Four of the 32 tested food samples yielded LAB isolates producing bacteriocin-like inhibitory substances (12.5% incidence rate). Graver and Muriana (1993) obtained a similar incidence rate in the isolation of bac+ LAB strains from foods by direct plating.

Since the seven bac+ isolates from fermented millet flour samples and the three isolates from raw sumpat grunt fillet showed many similarities (morphology, spectrum of inhibition against a set of ten indicator bacteria, identical profiles of carbohydrate fermentation), they could be considered as being the same strain. Based on their food sources, four bac+ strains from four different food samples were selected and examined in greater detail. Isolates CWBI-B1410, CWBI-B1426 and CWBI-B1427 that were isolated respectively from fer-mented millet flour, sumpat grunt fish, and fermented milk were identified as strains of *Lc. lactis* subsp. *lactis*, while isolate CWBI-B1411, isolated from fermented milk, was identified as a strain of *E. faecium* by 16S rDNA sequence analysis. As nisin-encoding genes could be detected in the three lactococcal strains and an enterocin B-encoding gene in the *E. faecium* strain, their bacteriocin-like substances could preliminary be regarded as compounds belonging to respectively Group 1 and Group 2, as proposed by Klaenhammer (1993).

*E. faecium* CWBI-B1411 showed a narrow spectrum of inhibition in comparison to that of the lactococcal strains. Moreover, enterococci are a frequent cause of a variety of infections in humans (Jett et al. 1994). Therefore, the CWBI-B1411 strain was discarded for use as a food biopreservative. The spectra of inhibition of the three bac+ lactococcal strains (CWBI-B1410, CWBI-B26 and CWBI-B27) included different food spoi-lage and pathogenic bacteria such as *B. coagulans, L. monocytogenes* and

***B. cereus***. The antibacterial activity of crude bactericidal preparations of the new isolates against *B. coagulans* was similar to that reported for *Lactobacillus salivarius* subsp. *Salivarius* (Flynn et al., 2002). The high prevalence of bacteriocin-producing *L. lactis* subsp. *lactis* strains and their detection in different food products indicates a high potential by these strains to grow and dominate the microbial population in the ecological environments of the foods. This predomi-nance could be explained in part by the conditions of local food product processing and storage. Raw food materials are processed (natural fermentation) and stored at ambient temperatures around 30-35°C that are similar to the optimal growth and bacteriocin pro-duction temperatures of these strains, as demonstrated for the *Lc. lactis* CWBI-B1410 strain. Nisin gene expression was confirmed for the CWBI-B1410 strain. A crude bactericidal solution prepared of the CWBI-B1410 strain also showed many similarities with nisin (identical inhibitory activity, profile of sensitivity to proteolytic enzymes, heat sensitivity, as well

as time retention on a $C_{18}$ column). Moreover, three main compounds with molecular mass below 3400 Da were detected in the active fraction isolated by RF-HPLC separation of the CWBI-B1410 crude bacteriocin-like inhibitory solution. One of these compounds showed a molecular mass of 3347.87 Da, which is similar to that

predicted from its formula ($C_{143}H_{228}N_{42}O_{37}S_7$) (Handary Bio-engineering BV, Oosterhout, Netherlands), and to the molecular mass of nisin (3346.39 Da) described by Megrhrous et al. (1997). These data suggest that the CWBI-B1410 strain produces a nisin-like substance.

**Nisin-producing *Lc. lactis*** strains are generally considered as GRAS (generally recognized as safe) and used as starters or protective cultures in a diversity of foods products like milk and cheese (Hirsch et al., 1951), lightly preserved fish products (Wessels and Huss, 1996) and vegetable products (Cai et al., 1997). The ability of the CWBI-B1410 strain to inhibit a wide range of food spoilage and pathogenic bacteria is of special interest for food safety, especially in the Senegalese environment with perennial problems of poor food hygiene. The use of such a strain as a biopreservative could be a suitable means of enhancing the quality and safety of the local food products.

## Conclusion

Senegalese local food products provide an appropriate ecological habitat for harboring bacteriocin-producing lactic acid bacteria. A high prevalence of bacteriocin-like substance producing *Lc lactis* strains was observed, indicating a high potential of growth of these strains in the ecological environments of the local foods. The presence of a nisin-encoding gene and its expression were confirmed in the *Lc. lactis* strain, designated CWBI-B1410. The data suggested that the *Lc. lactis* CWBI-B1410 strain produces a nisin-like inhibitory substance. *Lc. lactis* CWBI -B1410 can be selected for application to improve the traditional process of fish preservation in Senegal.

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