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Efficiency of Neutralized Antibacterial Culture Supernatant from Bacteriocinogenic Lactic Acid Bacteria Supplemented With Salt in Control of Microorganisms Present in Senegalese Artisanally Handled Fish by Immersion Preservative Technology During Guedj Seafood Processing at 10°C and 30°C

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Abstract

Senegal is a Western African country known for the importance of its marine resources. Artisanal fisheries contribute 85% of the total annual catch reaching 403,911 tons. Preserving approach during traditionally marine resource processing into indigenous seafood products is based on salt overuse. Guedj, a popular fermented indigenous seafood product, is the typical 30°C over salted transformed marine resource commodity across the country. One of the procedures for guedj production is immersing fish in water with sodium chloride at a concentration of over 30% (w/v) for preliminary fermentation during 24 hr to 48 hr to develop flavor followed by additional dry-salting and sun drying.

This study presents the level of microorganisms in fillets from lean, moderately fat and fatty artisanally handled fish, the efficacy of immersion in over salted [NaCl 60% (w/v)] water and 30°C incubation to control spoilage bacteria present in the fish matrixes, compared to those of lower salted [NaCl 14% (w/v)] antibacterial neutralized cell free culture supernatants (NCFCS) from two bacteriocinogenic lactic acid bacteria (LAB) and 10°C refrigeration. The two bacteriocinogenic bacteria (*Lactococcus lactis* subsp. lactis CWBI-B1410 and *Lactobacillus curvatus* CWBI-B28) were characterized in previous studies. Microbial population reduction in the treated fish was monitored using a 6 log10 CFU/g level at the end of storage.

The levels of the total viable microbial counts of raw flesh were similar for the three fish, and barely with the acceptable limit of 6 log10 CFU/g. The over salting preservation at 30°C did not enable reduction of microbial populations present in the fish. The total viable microbial, enteric and LAB counts of fillets immersed in the salted antimicrobial NCFCS from the bacteriocinogenic strains decreased and were maintained under the acceptable limit for 13 days to 18 days during incubation at 10°C.

These results indicate that the new preservative approach can reduce the need of abusive salt for guedj like products.

Keywords: Senegal; Fish; Fermentation; Preservatives; Salt; Bacteriocinogenic lactic acid bacteria

Introduction

Seafood products are known to be especially susceptible to microbiological and biochemical spoilage pathways. The early biochemical changes that occur in catches caused by autolytic enzymes make catabolites available for bacterial growth in the products [1-3].

Storage temperature is the most important environmental parameter influencing the growth rate and type of spoilage microorganisms in fish products. The raw fish matrixes must be rapidly chilled (-2°C to 0°C) as soon as possible after capture and eventually frozen (-20°C), for slowing down or preventing *in situ* bacterial changes that could lead to rejection for human consumption [4].

Good hygienic chilling practices are not widespread in most of the localized agrifood systems for fish commodities production in

developing countries, particularly in the hot climatic conditions characterizing costal Sub-Saharan African countries where marine resources contribute significant protein to the human diet. In these countries, traditional preserving of marine sources for food, such as fermentation, salting and smoking or their combination, are more widespread than modern technologies (refrigeration, freezing and canning).

Senegal is a western African country known for the importance of its marine resources with an annual fish production averaging 403,911 tons during the last decade. Industrial fisheries contribute only about 15% of the total seafood production compared with 85% from artisanal production. Catches are either handled at abusive temperature (over 30°C) or in small quantities of ice in the local fish marketing sites across the country. Marine food resources post-catching losses reach 25% of the total harvest [5,6].

The most common Senegalese indigenous fish products are "Ketiakh" (roasted, salted and sun dried fish), "guedj" (fermented, salted and sundried fish), "yet" (fermented, salted and sundried mollusks) and "tambadiang" (whole dried little fish species). About 600,000 women are organized into seafood processing microenterprises working with traditionally handled procedures for indigenous seafood products. Strategies for spoilage bacteria and microbial pathogen control during marine resources processing into indigenous seafood products are based on salt overuse. The procedure for guedj fish remain the typical example of salt overuse, as preserving approach, during marine resources transformation into indigenous seafood commodities across the country.

Guedj fish processing involves three steps; fermentation, salting and sun drying. Two procedures are described for the step popularly called fermentation. One is the practice in some Senegalese localized agrifood systems in the south of the country where the fish are subjected to fermentation at room temperature before being gutted, opened, abusively dry-salted and sun dried. However, the most frequently used technique for fish fermentation is immersion in water with salt added (sodium chloride). The amount of sodium chloride added in the immersion preservative solution in which the fish are allowed to ferment exceeds 30% (w/v). The fermentation duration of immersed fish is 24 hr to 48 hr for flavor development. Subsequently, the fermented fish is dry-salted and dried under the sun to obtain the final indigenous seafood product [6-8].

The efficiency of salt overuse in combination with 30°C incubation to control microorganisms present in artisanally handled fish during the fermentation for flavor development is a concern. Additionally, the excessive salt content of the indigenous guedj seafood products makes them unsuitable for consumption by people suffering from hypertension. Therefore, the search for an alternative preserving procedure to meet food hygiene and safety requirements and reduce the need for abusive salt levels for guedj fish products is an enormous challenge across the country.

Among alternative marine resources preservation technologies for lightly preserved seafood products, particular attention has been paid to biopreservation during the last twenty years. Biological preservation refers to the use of a natural or controlled microflora and or its antimicrobial metabolites to extend the shelf life and improve the safety of food. LAB, particularly those producing bacteriocins, are the category of microorganisms that offers the higher potential for bioprotective applications [9,10].

Bacteriocins are ribosomally synthesized peptides or proteins produced by certain strains inside the different bacterial species [11]. The defense mechanisms of bacteriocin producing LAB include organic acid and hydrogen peroxide production, completed by specific bactericidal activities. Considering that bacteriocin producing LAB are mostly isolated from food products, these antimicrobial substances have been consumed for a long time. Bacteriocins of LAB possess many advantages for application in food: they are thermo-resistant, are generally recognized as safe substances (GRAS), are not toxic to eukaryotic cells, are inactivated by proteases during digestion processes, and are active against food borne pathogens and food spoilage bacteria [12,13]. Several bacteriocins from LAB are very effective against certain specific foodborne pathogenic bacteria or have broad inhibitory spectra justifying various applications as antimicrobial agents in seafood products [9,10,14].

Lactococcus lactis subsp. lactis CWBI-B1410 and *Lactobacillus curvatus* CWBI-B28 are bacteriocinogenic LAB which were previously characterized for their specific antibacterial potential against a diversity of food spoilage and foodborne bacteria [15,16].

The application of LAB in food preservation offers several benefits, among them: its use reduces the need of chemical preservatives and can decrease the extension of thermal treatments [17,18]. The bacteriocinogenic LAB can be used as *ex situ* produced bacteriocin preparations or by inoculation with the bacteriocin producer strain. In situ bacteriocin production does not require a specific legislation approval, the strain must be able to grow and produce antibacterial metabolites including, amongst other compounds, lactic acid, hydrogen peroxide and the bacteriocin, in the concentration required to inhibit the target bacteria. In this sense, the nisinogenic *Lactococcus lactis* subsp. lactis CWBI-B-1410 has been successfully added to aid with the spontaneous fermentation that takes place during the Senegalese fish preparation for guedj. Two preserving procedure by starter culture use allow the reduction in abusive salting [19,20]. However, the level of starter cultures required for controlling the fermentation in the two procedures reached 10 log10 CFUC/g.

The application of *ex situ* antibacterial preparation from bacteriocinogenic LAB as part in preserving approach of immersion fermentation procedure enabling flavor development for guedj will have the advantage of limiting the number of total microorganisms present in the seafood system, when compared to the in situ biopreservation with CWBI-B1410 starter culture [19,20].

Ex situ antibacterial preparations from LAB are obtained by growing the producer strain followed by concentration and purifications which are needed to obtain a pure form of the bacteriocin. Since bacteriocin purification techniques allowing the recovery of high amounts of peptides in water or salt solution are usually not available, most of the studies aimed to assess effectiveness of bactericidal preparation as part of preserving approach in food system use bacteriocin containing supernatant or semi-purified fractions. There are several reports about antibacterial crude extract of culture supernatants from bacteriocinogenic LAB use in combination with refrigeration at 4°C to 5°C for light preservation of fish commodities during the last decades [21-23].

This study presents the results of an investigation aimed to assess the microorganisms present in Senegalese artisanally handled fish, the influence of storage temperature in the microbial changes, and the capacity of over salting immersion combined with 30°C incubation to control microorganisms in such fish, compared to the biopreservative

potential of antimicrobial neutralized cell-free culture supernatants from CWBI-B1410 and CWBI-B28 strains supplemented with sodium chloride in combination with 10°C refrigeration.

Materials and Methods

Origin, type of tested fish and pretreatment

Podamassys jubelini, Polydactilis quadrafilis and *Arius heudelotii*, respectively determined as lean, moderately fat and fatty fish [24] were purchased from a local market. The fish were scaled and eviscerated on site under the prevailing fish preparation conditions at traditional marketing sites [5]. Gutted fish were brought to the laboratory within 15 min in plastic bags purchased on site, and then rinsed in potable water. Filleting was done under sterile conditions. The obtained fillets were cut into small pieces using a pair of kitchen scissors. For each type of fish, a total of 410 g of unskinned small pieces was obtained. Such raw fish matrixes were used for monitoring initial microbial profile, changes occurring in the course of traditional over salting procedures by immersion combined with 30°C incubation, as well as biopreservation using antibacterial NCFCS from LAB supplemented with salt in combination with 10°C refrigeration.

Determination of bacteria present in the artisanally handled fish products

Ten grams (10 g) of unskinned raw fish flesh pieces were suspended in 90 mL of sterile 0.8% saline in a sterile sealed plastic bag (BA6141/CLR, Seward, Worthington, UK) and homogenized in a stomacher (Blender 80, Seward) for 2 min. The resulting homogenate was tenfold serially diluted in sterile 0.8% saline water, and 100 µl of each dilution was spread in triplicate on PCA supplemented with 0.5% NaCl, MRS agar supplemented with 50 mg/L cycloheximide (Sigma, St. Louis, MO) and 100 UI/mL polymyxin b (Sigma)(4), Baird-Parker agar (Biokar Diagnostics, Beauvais, France), Rose-Gal BCIG agar (Biokar Diagnostics), and Hektoen enteric agar (Scharlau Chemie, Barcelona, Spain) for quantifying total viable microbial counts, LAB, *staphylococci*, *Escherichia coli*, and enteric bacteria, respectively. Colonies developing on the plates were counted after 48 hr of incubation at 30°C or 37°C (total viable microbial, LAB and, *staphylococci*) or 24 hr at 37°C (*E. coli* and enteric bacteria).

Assessment of temperature and salt influences on bacterial changes in fish

The growth of total viable microbial, enteric and lactic acid bacteria determined in the fish was first monitored during the course of their incubation at 10°C and 30°C. Subsequently, efficiency of salt overuse by immersion technology in combination with 30°C incubation and lightly or moderately salting combined with 10°C refrigeration to control spoilage bacteria has been determined. The salting was first performed by immersing the fish fillets in saline solutions prepared by addition of sodium chloride to potable water to reach 60% (w/v) combined with 30°C incubation. Subsequently, 8%, 12% and 14% (w/v) of saline water or NCFCS from LAB were used in combination with 10°C refrigeration. Fish pieces were placed in sterile 50 mL Falcon tubes, and were covered with the pasteurized (10 min heating at 80°C) preservative solutions at a ratio of 1:1 (w/v). Microbial population reduction in the treated fish was monitored using a 6 log10 CFU/g level at the end of storage.

LAB strains used for fish biopreservation assays

Lactococcus lactis subsp. lactis CWBI-B1410 and *Lactobacillus curvatus* CWBI-B28 strains were obtained from the Centre Wallon de Biologie Industrielle (CWBI) bacterial collection in the University of Liège Gembloux Agro Bio Tech (Belgium). The non bacteriocinogenic *Lactococcus lactis* subsp. lactis LMG 6890 and Lactobacillus curvatus LMG 21688 strains were used for production of neutralized culture supernatants without antibacterial potential, for use as a negative test of the biopreservative matrixes. The two bacterial strains were purchased from the collection of Gent University in Belgium.

Revitalization of cultures of LAB strains

A drop of defrosted stock cultures of the different bacteria strains was streaked on MRS. Plates were incubated at 30°C (LAB) for 48 hr and cell forming colonies were sub-cultured onto the same medium. Plates were incubated for 48 hr at 30°C to enable development of pure colonies of each different bacterium. After incubation, stock plates were maintained at 4°C and used to prepare cultures which were used for preparing neutralized cell free supernatants supplemented with salt to control microorganisms present in fish by immersion technique.

Culture conditions of LAB and treatment for NCFCS preparation

Colonies of the revitalized LAB were removed from the culture plates and sub-cultured in 10 mL of MRS which were incubated for 20 hr at 30°C. The 10 aliquots of the four lactic acid bacteria were separately inoculated in 1000 mL of MRS broth which were incubated at 30°C for 16 hr to allow *in situ* secretion of antibacterial peptides by bacteriocinogenic strains in the cultures [15,16]. The different LAB cultures were separately centrifuged at 2,280 × g for 20 min (2-4, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany). The supernatants were removed and neutralized at pH 6, by addition of NaOH (5N).

In vitro detection of antibacterial potential by LAB NCFCS

Detection of antibacterial potential in the neutralized cell-free supernatants from LAB cultures was performed by well diffusion technique, based on previous findings on bactericidal potentialities of *Lactobacillus curvatus* CWBI-B28 and *Lactococcus lactis* subsp. lactis CWBI-B1410. Strains of *Staphylococcus epidermis* and Listeria monocytogenes from CWBI bacterial collection were used as main indicators. Additionally, an antibacterial cross test of the different NCFCS from the LAB was performed against these cultures (CWBI-B1410, CWBI-B28, LMG 6890 or LMG 21 688). The complementary antibacterial cross test was performed to confirm effective resistance of each bacteriocinogenic strain to the antibacterial potential detected on its own NCFCS that is an example of indicator criterion for bacteriocin-like inhibitory action.

Monitoring fish fillet and salt influences on antibacterial potential of NCFCS

Bacteriocins are sensitive to certain proteases which can be present in food matrixes such as raw fish products. Additionally, the effectiveness of bactericidal preparations from LAB in food will depend on the interaction of the bacteriocin with the food components. Therefore, the influence of the raw fish fillets on the antibacterial potential of the neutralized cell free supernatants from

LAB following the preserving approach by immersion combined with 10°C refrigeration has been monitored in the presence or absence of salt. The monitoring was performed by measuring the initial and the residual *in vitro* antibacterial activity on the salted or unsalted NCFCS immersion of the fish during the course of refrigeration at 10°C. The initial and residual *in vitro* antibacterial activities in immersing preservative NCFCS were determined in AU/mL defined as the reciprocal of the highest dilution showing a definite inhibition zone on agar medium seeded with *Staphylococcus epidermis* or *Listeria monocytogenes* as indicators [25].

In this procedure, a sample of 1 mL of the preservative matrixes used for immersing the fish was centrifuged at 17,000 g for 15 min (Eppendorf AG, Hamburg, Germany). The supernatant was filter sterilized and serially twofold diluted in phosphate buffer (50 mM, pH 6.3). Samples of 50 µL aliquots for each dilution were loaded into separate wells cut in the agar medium seeded with 110 µL of Staphylococcus epidermidis or Listeria monocytogenes cultures. After initial incubation at 8°C for 20 min to enable diffusion, the plates were incubated overnight at 37°C. Inhibition was scored as positive in the presence of a detectable clearing zone around the well.

Technique of fish biopreservation combined with 10°C refrigeration

For each type of fish, the 410 g of unskinned small flesh pieces were divided into 41 portions of 10 g each which were placed in 10 sterile, disposable 50 mL Falcon culture tubes. The 40 Falcon culture tubes were divided into four (4) sets of 10, which were treated with pasteurized (80°C for 10 min), salted [NaCl added at 14% (w/v)] NCFCS from each of these four LAB strains: CWBI-B1410, CWBI-B28, LMG 21688 and LMG 6890. The salted NCFCS of LAB cultures was poured on the fish flesh pieces at a ratio of 1:1 (w/v) and totally immersed them. One tube of the different four sets of treated fish was randomly removed from the 10°C refrigeration incubator at 48h intervals and monitored for bacterial counts.

Monitoring of bacteria present in the fish growth during storage

Ten grams of fish flesh pieces immersed in 10 mL salted NCFCS of the different LAB strains were collected in a sterile 1L plastic stomacher bag (BA6141/CLR, Seward, and Worthington, UK). One hundred eighty (180) mL of sterile 0.8% (w/v) saline (NaCl) water were added in each preparation to make a tenfold dilution. Each suspension was serially diluted tenfold in sterile 0.8% (w/v) saline (NaCl) water and 100 µL aliquot dilutions were pour plated on PCA supplemented with 0.5% NaCl, MRS agar supplemented with 50 mg/liter cycloheximide (Sigma, St. Louis, MO) and 100 UI/mL polymyxin b (Sigma) (4), and Hektoen enteric agar (Scharlau Chemie, Barcelona, Spain) for quantifying total viable microbial, LAB and enteric bacteria counts, respectively. The data presented in this study are the mean and standard deviation from triplicate samples and three trials performed at different times.

Results and Discussion

Microbial spoilage and bacteria present in fillets from the fish

The microbial populations in the fillets from the three artisanally handled fish were similar, with *P. jubelini, P. quadrafilis* and *A. heudelotii* yielding total viable microbial counts of 5.75 ± 0.07, 5.35 ± 0.07 and 5.59 ± 0.12 log10 CFU/g, respectively (Figure 1).

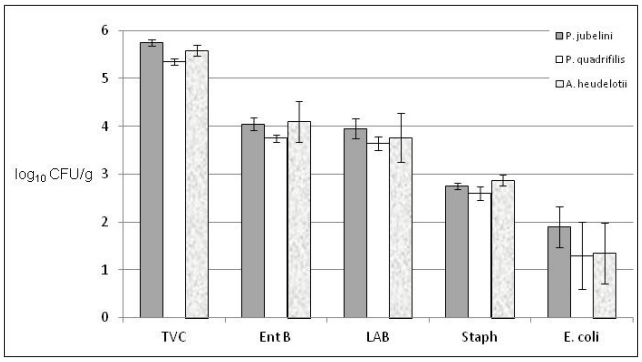

Figure 1: Microbial populations in raw fillets of artisanally handled lean *Podamasys jubelini*, moderately fat *Polydactylis quadrafilis*, and fatty *Arius heudelotii* purchased at a local Senegalese market. TVC: total viable microbial; Ent: enteric bacteria; LAB: lactic acid bacteria; Staph: *Staphylococcus aureus*; *E. coli: Escherichia coli*.

These levels were barely within the limit of acceptability (6 log10 CFU/g) defined for seafood destined for human consumption [26] and similar to those reported by Diei-Ouadi [5] or Gram and Huss [27]. Populations of enteric bacteria in *P. jubelini, P. quadrafilis* and *A. heudelotii* were 4.08, 3.89 and 4.12 log CFU/g, respectively, and were higher than those of LAB. Populations of staphylococci were barely within the acceptable limit defined for *Staphylococcus aureus* (3 log CFU/g) in marine fish fillets destined for human consumption [28], whereas those of *E. coli* were above the limit defined for these bacteria (1.20 log CFU/g) (Figure 1). These results indicate that a severe preservation treatment or combination of many stress factors will be required for efficient control of bacteria present in marine resources from artisanal production in Senegal during storage or processing to ensure the safety and protection of the consumer against potential microbial hazard. Therefore, the influence of two incubation temperatures on microbial changes in Senegalese traditionally handled fish, was determined.

Influence of 10°C or 30°C incubation in bacterial changes in the fish

The growth of the total viable microbial, enteric bacteria and lactic acid bacteria present in fillets from artisanally handled sompat grunt during their incubation at 10°C and 30°C is presented in the Figure 2A. The total viable microbial of fillets increased from 5.57 to 10.27 and 6.68 log10 CFU/g within 48 hr of incubation at 30°C and 10°C, respectively. The enteric bacteria population increased from 4.02 to 9.65 and 5 log10 CFU/g when fish fillets were incubated at 30 and 10°C, respectively. These bacteria which can include pathogenic strains were higher than LAB present in the fish during the course of storage

at 30°C, while they were lower than the last group when the fish was incubated at 10°C (Figure 2A).

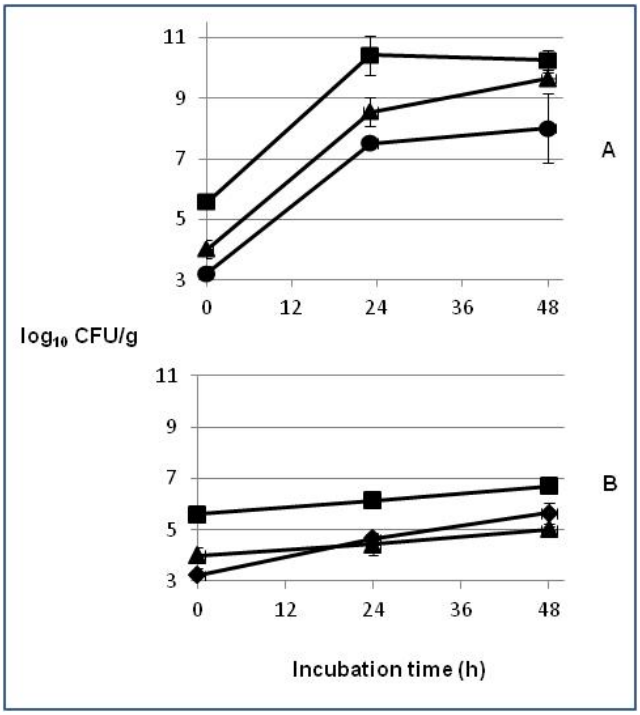

Figure 2: Evolution of total viable microbial (▪), enteric bacteria (▲) and lactic acid bacteria (●) present in fillets from artisanally handled lean *Podamassys jubelini* fish during the course of incubation at 30°C (A) and 10°C (B) for 48 hr.

When compared to 30°C, the fish fillets preserved by 10°C refrigeration had a reduction of 3.62 and 4.65 log10 CFU/g of the total viable microbial and enteric bacteria counts respectively. The significant growth of enteric bacteria in the fish during incubation at 30°C enhances the probability of *in situ* increase of pathogenic gram negative bacteria strains. These data indicate the importance of promoting fish processing at refrigeration temperatures across the country to enhance the safety and competitiveness of indigenous seafood commodities which are until now only sold at traditional markets because of hygienic concerns. Additionally, the effectiveness of salt overuse in combination with 30°C incubation to control bacteria that are present in traditionally handled fish based on the immersion wet salting approach during fermentation for guedj was undertaken.

Fish preservation potential by over salting combined with 30°C incubation

The total viable microbial, enteric bacteria and LAB counts increased from 5.6, 4.02 and 3.22 log10 CFU/g to reach 7.48, 6.75 and 6.02 log10 CFU/g (Figure 3). The number of enteric bacteria which can include pathogenic strains increased to 2.73 log10 CFU/g in the fish despite the over salting by immersion technology in pasteurized potable water with NaCl added at 60% (w/v) during incubation at 30°C. The increases of the microorganisms present in the over salted fish incubated at 30°C reached levels higher to those in the fish stored at 10°C as stress factor (Figure 2B). These results indicate the importance of handling or processing fish at low temperatures to meet hygienic conditions ensuring their safety, and justify continuing the investigation of combining 10°C refrigeration with light and moderate salting by immersion technology, to optimize preservative factors for fish from artisanal production in Senegal.

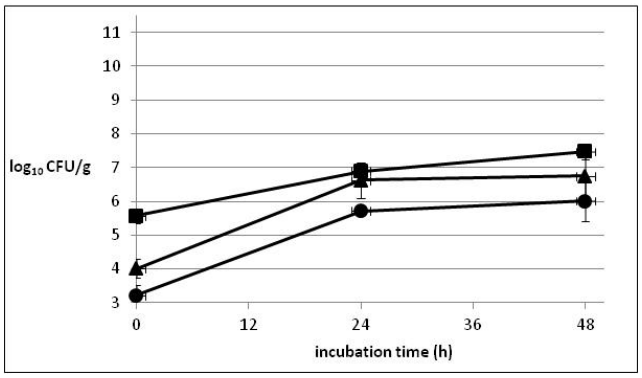

Figure 3: Evolution of the total viable microbial (▪), enteric bacteria (▲) and lactic acid bacteria (●) counts of fillets from artisanally handled *Podamassys jubelini* fish immersed in pasteurized over salted water used as hurdle in combination with 30°C incubation to control spoilage bacteria based on immersion fermentation for guedj seafood products in Senegal. The amount of NaCl added in the water for fish preservation was 60% (w/v).

Fish preservation potential by salting combined with 10°C refrigeration

The growth of the total viable microbial in fillets from artisanally handled sompat grunt treated by immersion in pasteurized 8%, 12% and 14% (w/v) salted (sodium chloride) water and incubated at 10°C are presented in Figure 4.

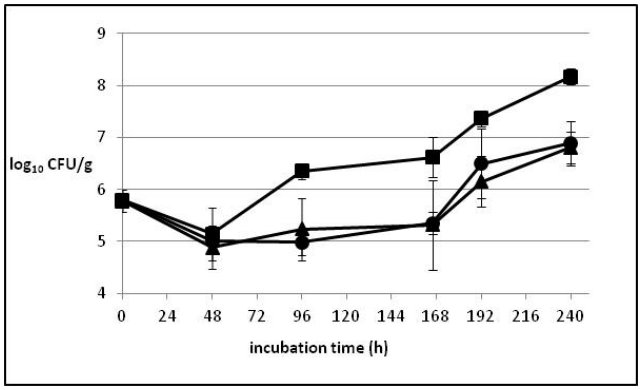

Figure 4: Evolution of total viable microbial of fillets from artisanally handled *Podamassys jubelini* fish immersed in pasteurized 8 (▪), 12 (●) and 14% (▲) (w/v) salted (sodium chloride) water and incubated at 10°C.

The total viable microbial of fillets preserved in the three salted solution declined 1 log10 CFU/g within 2 days of incubation at 10°C. The 6 log10 CFU/g was reached after 3.5, 6.5 and 7 days of storage for

the fish immersed in 8%, 12% and 14% saline solutions, respectively. These results indicate an improvement in spoilage bacteria control in the fish compared to the use of 10°C refrigeration as a unique stress factor of microorganisms (Figure 2B). Subsequently, the evaluation of the preservative capacity of 10°C in combination with neutralized cell-free culture supernatants from CWBI-B1410, CWBI-B28, LMG 6890 and LMG 21688 (LAB) strains supplemented with sodium chloride has been undertaken to optimize the preservative factors. These assays were performed following a preliminary assessment of antibacterial potential in NCFCS from the four LAB.

In vitro antibacterial potential of NCFCS from LAB

The inhibitory potential of the NCFCS from CWBI-B1410, LMG 6890, CWBI-B28 and LMG 21688 LAB strains against 6 bacterial strains from different genera including themselves is presented in Table 1.

Bacteria tested as indicators	Inhibitory potential* in the NCFCS of lactic acid bacteria			
	Lactobacillus curvatus strains		*Lactococcus lactis* subsp. lactis strains	
	CWBI-B28	LMG 21688	CWBI-B1410	LMG 6890
Lactobacillus curvatus CWBI-B28	-	-	+	-
Lactobacillus curvatus LMG 21688	+	-	+	-
Lactococcus lactis subsp. lactis CWBI-B1410	-	-	-	-
Lactococcus lactis subsp. lactis LMG 6890	-	-	+	-
Listeria monocytogenes	+	-	+	-
Staphylococcus epidermis	-	-	+	-

Table 1: Detection of inhibitory potential in the pasteurized (80°C, 10 min) NCFCS from CWBI-B28, LMG 21688, CWBI-B1410 and LMG 6890 bacterial strains. 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 previously inoculated with 110 µL of the indicator bacterial cultures. Sixty µL of pasteurized neutralized (pH 6.5) NCFCS were poured into each well. Detection of a clear zone of growth inhibition around well was expressed as positive reaction (+), lack of inhibition as negative inhibition (-).

Only CWBI-B1410 and CWBI-B28 CFS showed antibacterial activity in the NCFCS. The CWBI-B1410 CFS confirmed a broader spectrum of activity [15] compared to that of CWBI-B28 (Table 2). This later demonstrated highest inhibitory potential against Listeria monocytogenes. The two strains resisted to the antibacterial potential detected on their NCFCS. This is one of the indicator criteria of bactericidal activities by *ex situ* preparation from CWBI-B28 and CWBI-B1410 for which a production of new bacteriocin and nisin-like substance were respectively demonstrated in previous studies [15,16].

In vivo antimicrobial potential of LAB NCFCS on fish refrigerated at 10°C

Bacteriocin-containing cell-free culture supernatants from LAB can lose their antibacterial potential or effectiveness in food matrixes, due to enzymatic degradation and interactions with food components such as proteins and lipids [18,29]. Therefore, the influence of the raw fillets from traditionally handled sumpat grunt fish in the antibacterial potential determined for NCFCS from CWBI-B1410 and CWBI-B28 strains has been monitored in the presence or absence of salt.

The *in vitro* inhibitory potentials of the NCFCS from the two bacterial fish immersion solutions in combination with 10°C refrigeration, as additional stress factor, are presented in the Table 3.

Indicator bacteria	Antibacterial activity* on NCFCS of the bacteriocinogenic lactic acid bacteria (× 10^2 AU/mL)	
	Lactobacillus curvatus CWBI-B28	*Lactococcus lactis* subsp. lactis CWBI-B1410
Listeria monocytogenes	4 096	1.60
Staphylococcus epidermis	0	12.8

Table 2: *In vitro* antibacterial activity* of pasteurized (80°C, 10 min) NCFCS from lactic acid bacteria which showed inhibitory potential. *Inhibitory activity of the pasteurized NCFCS from the two lactic acid bacteria were expressed in arbitrary units (AU) per milliliter, defined as the reciprocal of the highest dilution of the NCFCS showing a definite inhibition zone on agar medium seeded with the indicator bacterium [25].

Incubation time (h) of immersed fish fillets stored at 10°C	*In vitro* antimicrobial activity* (× 10^2 AU/mL) of pasteurized NCFCS from the bacteriocinogenic LAB immersing the fish fillets	
	CWBI-B28 NCFCS	CWBI-B1410 NCFCS
0	204.80	6.40
48	102.40	3.20
96	19.20	0.80
144	1.60	0.60
192	0	0
240	0	0
288	0	0

Table 3: Evolution of the *in vitro* antibacterial activity of CWBI-B28 and CWBI-B1410 bacterial fish immersion NCFCS during storage at 10°C. Tests were performed with *Podamassy jubelini* fillets; inhibitory activities of the two pasteurized bacterial fish immersion NCFCS was expressed in arbitrary units (AU) per milliliter, defined as the reciprocal of the highest twofold dilution of the immersion matrixes showing a definite inhibition zone on agar medium seeded with Listeria monocytogenes or Staphylococcus epidermis, respectively.

The antibacterial potential of the NCFCS fish immersion solutions progressively decreased during the course of treated fish storage. The cell free culture supernatants from the two bacterial fish immersion

solutions completely lost their antimicrobial potential against the targeted bacteria after 8 days of storage at 10°C (Table 3). When the treated fish were incubated at 30°C, the *in vitro* antibacterial potential in the preservative solutions were entirely lost within 48hr of fish storage (data not shown). Several authors described similar antibacterial activity loss or inefficiency of bactericidal preparation from bacteriocinogenic LAB. They were linked with the possible degradation of bacteriocin by endogenous proteases or by molecular interaction with the food matrix [30-32].

Incubation time of immersed fish fillets stored at 10°C (hr)	Antibacterial activity* (× 10^2 AU/mL) pasteurized salted (NaCl) CWBI-B28 NCFCS immersing the raw fish fillets		
	[8% (NaCl, w/v)]	[12% (NaCl, w/v)]	[14% (NaCl, w/v)]
0	204.80	204.80	204.80
48	204.80	204.80	204.80
96	102.40	153.60	153.60
144	51.20	102.40	102.40
192	38.40	51.20	102.40
240	12.80	38.40	51.20
288	6.40	13.60	51.20

Table 4: Evolution of the *in vitro* antibacterial activity of pasteurized 8%, 12% and 14% (w/v) salted (sodium chloride) CWBI-B28 bacterial fish immersion NCFCS during storage at 10°C. Tests were performed with *Podamassy jubelini* fillets; inhibitory activity of the pasteurized 8%, 12% and 14% (w/v) salted CWBI-B28 bacterial fish immersion NCFCS was expressed in arbitrary units (AU) per milliliter, defined as the reciprocal of the highest twofold dilution of the preservative matrixes showing a definite inhibition zone on agar medium seeded with *Listeria monocytogenes*.

Incubation time (hr) of immersed fish fillets stored at 10°C	Antimicrobial activity* 0028 × 10^2 AU/mL) on pasteurized salted (NaCl) NCFCS from CWBI-B1410 strain immersing the raw fish fillets		
	[8% (NaCl, w/v)]	[12% (NaCl, w/v)]	[14% (NaCl, w/v)]
0	6.40	6.40	6.40
48	6.40	6.40	6.40
96	3.20	3.20	6.40
144	1.60	3.20	3.20
192	1.20	1.60	3.20
240	0.60	0.80	1.60
288	0.20	0.80	1.60

Table 5: Evolution of the *in vitro* antibacterial activity of pasteurized 8%, 12% and 14% (w/v) salted (sodium chloride) CWBI-1410 bacterial fish immersion NCFCS during storage at 10°C. Tests were performed with *Podamassy jubelini* fillets; inhibitory activity of the pasteurized 8%, 12% and 14% (w/v) salted CWBI-B28 bacterial fish immersion was expressed in arbitrary units (AU) per milliliter, defined as the reciprocal of the highest twofold dilution of the preservative matrixes showing a definite inhibition zone on agar medium seeded with *Staphylococcus epidermis*.

The evolutions of the antibacterial potential for NCFCS from the two bacteriocinogenic LAB with added sodium chloride fish immersion solutions are presented in the Tables 4 and 5. When compared with Table 3, the decrease of the antibacterial potential was less accurate when the NCFCS have been previously salted. When the NCFCS from the two bacteriocinogenic bacteria have been preliminarily supplemented with sodium chloride added at 14% (w/v) before treating fish, the two preservative solutions kept an antibacterial potential corresponding to 25% of the initial level after 12 days (288 hr) of treated fillets incubation at 10°C (Tables 4 and 5). The results indicate that the supplementation of the NCFCS from the two bacteriocinogenic bacteria can be a strategy of limiting the negative actions by factors present in the fish matrix enabling reducing the in situ antibacterial potential of the biopreservative solutions.

Therefore, the efficiency of salted [NaCl added at 14% (w/v)] antibacterial NCFCS as alternative hurdles by immersion technology in combination with 10°C refrigeration to improve control of spoilage bacteria present in fillets from artisanally handled fish, has been tested and compared to those of similar saline solution performed in using water and non-antibacterial NCFCS from non bacteriocinogenic LAB used as negative controls.

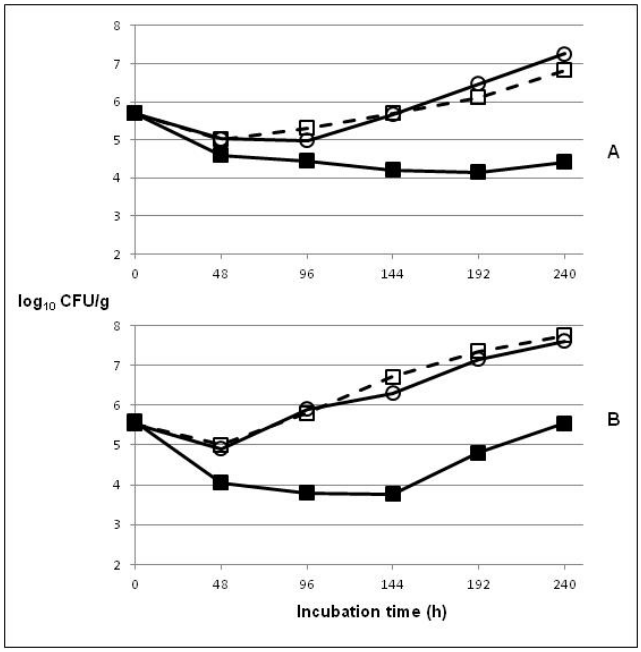

Figure 5: Evolution of total viable microbial of fillets from artisanally handled *Podamassys jubelini* (A) and *Arius heudelotii* (B) fish immersed in pasteurized 14% (w/v) salted water (○), NCFCS from *Lactococcus lactis* sp. lactis LMG 6890 (□) and NCFCS from *Lactococcus lactis* sp. lactis CWBI-B1410 (▪) in combination with 10°C refrigeration for improving spoilage bacteria control. The NCFCS from CWBI-B1410 demonstrated an *in vitro* antibacterial potential contrarily to that from LMG 6890.

Fish preservation potential by salted NCFCS combined with 10°C refrigeration

The evolution of the total viable microbial of sumpat grunt and smooth catfish immersed on pasteurized salted [NaCl added at 14% (w/v)] water, NCFCS from LMG 6890 and CWBI-B1410 is presented in the Figure 5. The microbial profile of samples treated with the salted water or NCFCS which did not show *in vitro* antibacterial potential were similar. It declined of 1 log10 CFU/g followed by an increase to reach 6 log10 CFU/g, considered as the limit of acceptability, within 7 days of storage at 10°C. In contrast the total viable microbial of samples treated with salted NCFCS from CWBI-B1410 that demonstrated *in vitro* antibacterial activities against different gram positive bacteria, was maintained below 6 log10 CFU/g during 12 days at least following the nature of the fish, in particular the fat content. Similar trends of improving the spoilage bacteria control were obtained when the assays have been performed in using NCFCS from *Lactocbacillus curvatus* strains (Figure 6).

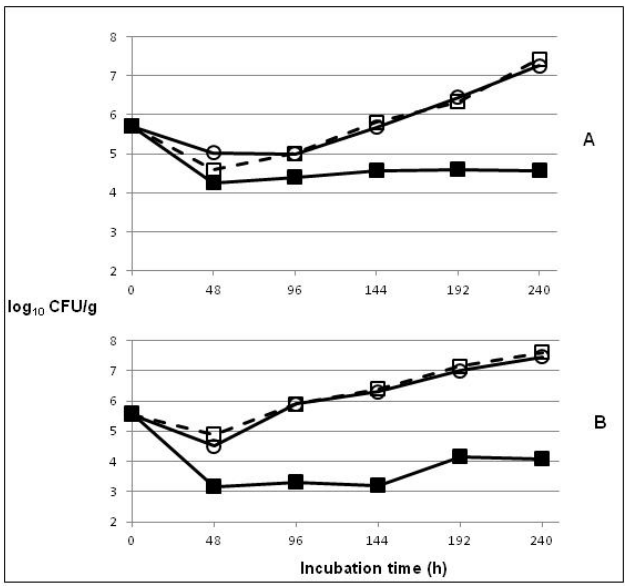

Figure 6: Evolution of total viable microbial of fillets from artisanally handled *Podamassys jubelini* (A) and *Arius heudelotii* (B) fish immersed in pasteurized 14% (w/v) salted (sodium chloride) water (○), NCFCS from *Lactobacillus curvatus* LMG 21688 (□) and NCFCS from *Lactobacillus curvatus* CWBI-B28 (▪) in combination with 10°C refrigeration for improving spoilage bacteria control. The NCFCS from CWBI-B28 demonstrated an *in vitro* antibacterial potential contrarily to that from LMG 21688.

An overview of the bacterial changes in the fish immersed in salted antibacterial NCFCS of the two bacteriocinogenic LAB combined with 10°C refrigeration, by enteric bacteria and LAB level determination during the course of preservation is presented in the Figures 7 and 8.

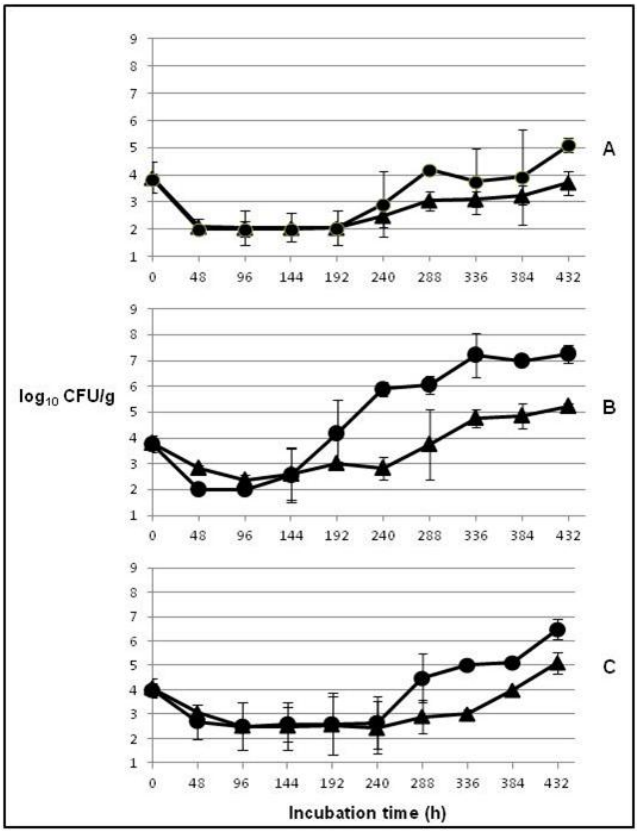

Figure 7: Evolution of enteric bacteria (▲) and lactic acid bacteria (●) present in fillets from artisanally handled *Podamassys jubelini* (A), *Polydactylis quadrifilis* (B) and *Arius heudelotii* (C) immersed in 14% (w/v) salted (sodium chloride) antibacterial NCFCS from CWBI-B1410 combined with 10°C refrigeration for improving spoilage bacteria control.

The new fish preservation system by combining preservative action of moderately salted NCFCS from CWBI-B1410 or CWBI-B28 bacteriocinogenic strains in combination with 10°C refrigeration allowing to maintain enteric bacteria population that can include pathogenic gram negative strains at their initial levels in the different fish reaching in some case 4 log10 CFU/g for at least 12 days of incubation. These results indicate a significant preservative potential by the new approach as alternative to the traditional salt overuse combined with 30°C incubation during which these undesirable bacteria present in the products reached 6.75 log10 CFU/g with in 48hr in despite of the abusive salt addition making the products non-competitive.

The LAB became the main bacteria in the fish immersed in the NCFCS compared to the level of enteric bacteria. LAB are generally considered as GRAS except some strains inside *Enterococcus* sp. which can cause infections in humans [32-34]. These results indicate that the use of the antibacterial cell free culture supernatants from the two bacteriocinogenic LAB strains (CWBI-B1410 and CWBI-B28) as biopreservatives, combined with 10°C refrigeration and moderate salting as additional stress factors, can be the basis of a new fish processing adapted from fermentation immersion procedure for guedj,

allowing optimization of spoilage bacteria control. The new procedure has the advantage to limit the total viable microbial in the fish compared the high level determined for the starter cultures fermentation at 30°C [19,20] and can enable flavor development.

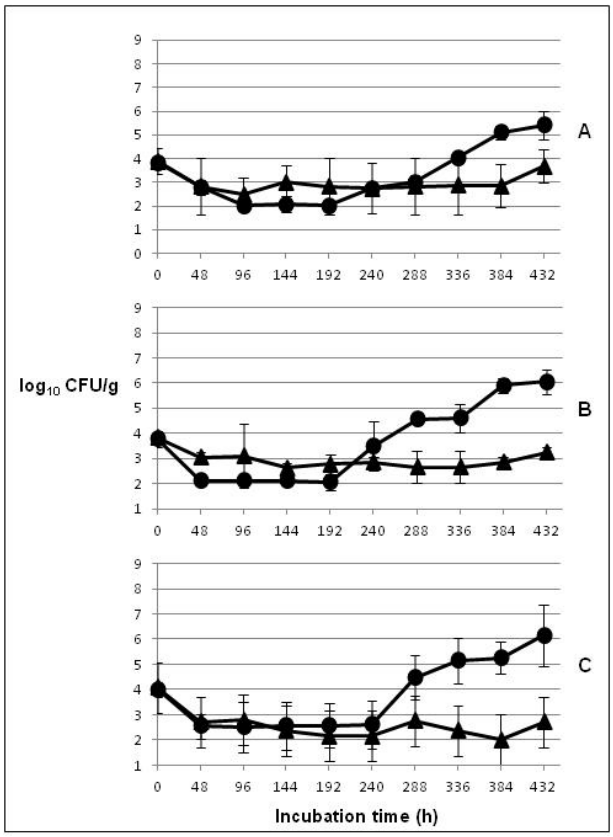

Figure 8: Evolution of enteric bacteria (▲) and lactic acid bacteria (●) present in fillets from artisanally handled *Podamassys jubelini* (A), *Polydactylis quadrifilis* (B) and *Arius heudelotii* (C) immersed in 14% (w/v) salted (sodium chloride) antibacterial NCFCS from *Lactobacillus curvatus* CWBI-B28 in combination with 10°C refrigeration for improving spoilage bacteria control.

Conclusion

Marine resources from artisanal production contain high levels of microorganisms. The storage of such fish at 10°C is more efficient than over salting by immersion procedure at 30°C in the control of the microorganisms in the fish, in particular, enteric bacteria that can include pathogenic strains. The combination of antibacterial potentiality of NCFCS from CWBI-B1410 and CWBI-B28 with significantly lower levels of sodium chloride compared to the traditional guedj procedures, and 10°C incubation is more efficient for the control of spoilage bacteria present in the fish. These results indicate that these new strategies can be a means for promoting production of moderately salted indigenous fish.

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