Use of Nisin-Producing Starter Cultures of *Lactococcus lactis subsp. lactis* on Cereal Based-Matrix to Optimize Preservative Factors over Fish Fermentation at 30°C Typical to Senegal

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Abstract

This study describes an application of bacteriocinogenic lactic acid bacteria to improve preservative factors over fish fermentation based on the most frequently used technique in Senegal. Traditional fish fermentation performed by immersion in salted sea water and incubation at 30°C for 24 to 48h to develop flavor, is characterized by a pH value around 7. The process led to the growth of Enterobacteriaceae to reach 9 log CFU/g. Four bacteriocinogenic lactic acid bacteria characterized in previous works were screened for decarboxylation activities. Only cultures of two *Lactoccus lactis subsp. lactis* (CWBI-B1410 and CWBI-B1426) producing nisin, a heat stable antibacterial peptide, did not produce histamine and tyrosine regarded as undesirable compounds on seafood products. The two lactococcal starter cultures grew well on a substrate based on millet, a local cereal. The AU/ml minimum inhibitory concentration of the antibacterial neutralized supernatant of the bacteriocinogenic starter cultures against *Listeria monocytogenes* was lower to those of many food poisoning or spoilage bacteria.

Then, pieces of lean (*Podamassys jubelini*) and fat (*Arius heudelottii*) fish inoculated with 10^4 CFU/g of *Listeriamonocytogenes*, were immersed in the millet flour based substrate seeded with 10^6 CFU/g pure cultures of the nisinogenic bacteria to evaluate the control of the fermentation at 30°C. *Listeria monocytogenes* contaminated fish samples immersed in the millet-based substrate without starter cultures addition were used as negative controls.

The starter culture fermentation gave the lowest fish pH reading. The pH was dropped and maintained to a final level lower to the minimum value for the growth of many Enterobacteriaceae genera reported in spoiled fish. The growth of *Listeria monocytogenes* was significantly inhibited by starter cultures compared to that in the negative controls.

These results indicate that cultures of the two nisin producers on the new formulated matrix can be used to improve fish fermentation in Senegal.

Keywords: Senegal; Fish fermentation; Nisinogenic starter cultures; Millet

Introduction

In Senegal (West Africa), spontaneous fermentation is a widespread method of food production from raw agricultural materials. In artisanal fisheries, it is used for preparation of indigenous seafood products across the country. Artisanal fisheries landings account for about 85% of the total catch in Senegal estimated at 350,000 t/year during the last decade, compared to only 15% for industrial fishing. About 30% of the artisanal landings products are transformed to different local seafood products [1].

“Guedj” fish is one of the three typical local seafood products in Senegal [2]. It is a naturally fermented, salted and sun dried fish. For its production, raw fish is fermented at ambient temperatures for 24 to 48h, then salted (30 to 40% NaCl, wt/wt) and sun dried. Spoilage bacteria and natural lactic acid bacteria (LAB) in the fish serve as starters for this spontaneous fermentation.

Guedj fish is found mainly in local markets across the Sahel and in Central Africa and it is appreciated for its taste and flavor. However, it is rarely sold in conventional or selective markets because of the inherent safety concern associated with the spontaneous fermentation process. Two traditional spontaneous fermentation procedures for guedj fish production are reported [2]. One is the practice in some Senegalese agrifood systems in the south of the country where fish are fermented at ambient temperature before being gutted, opened, thoroughly salted and then sun dried. The most frequently used technique of fermentation is mainly prevalent in the western and northern regions of Senegal. In this procedure, fish are first gutted, opened and fermented by immersion in a concentrated high salt solution (at least 30% NaCl wt/vol) made with sea or well water for 24 to 48h. After this first step, fish are dry salted and sun dried.

The two processes of fermentation are applied to a wide variety of fish species in artisanal fisheries. However, *Arius* sp. guedj fish are the most popular and commercialized products across the sub-Saharan countries. The production and commercialization of cured

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fish like guedj by many small enterprises, managed mainly by women, contributes significantly to the reduction of post-harvest losses of artisanal fisheries. It also contributes to the availability of animal protein food sources across the non-coastal rural regions lacking electricity or modern storage equipment. However, the serious microbial safety concerns with the products due to their poor hygienic conditions during processing limit their commercial competitiveness. The unsanitary conditions significantly contrast with seafood product safety standards [3]. Additionally, the excessive salt content of these fish products makes them unsuitable for consumption by people suffering from hypertension.

Acidic pH values are greater use in inhibiting microorganisms. It has been well established that most microorganisms grow best at pH value around 7.0 (6.6-7.5) whereas few grow below 4.0 [4]. The most frequently reported genera of bacteria on fresh and spoiled fish are Aeromonas, Pseudomonas, Shewanella, Vibrio. The genera of Bacillus, Escherichia, Enterococcus, Listeria and Clostridium are known to occur on fish products. Minimum pH for the growth of Aeromonas hydrophyla, Shewanella putrefaciens, Shigella flexneri, Clostridium botulinum G2 and Pseudomonas fragi is in the range 5-6, while for Escherichia coli O157:H7, Salmonella spp., Vibrio paraohemolyticus, Clostridium botulinum G1, Clostridium perfringens, Staphylococcus aureus and Listeria monocytogenes the value is between 4 and 4.9 [4]. When taking into account the technical and financial constraints in artisanal fisheries in Senegal, innovative technologies based on a significant acidification of fish are the most suitable for improving safety of indigenous fermented fish products. Some foods such as fish are better able to resist changes in pH than other. The food that tend to resist changes in pH are said to be buffered [4]. Contributing to the buffering capacity of fish is the various proteins. A promising low-cost approach for the improvement of fish fermentation at 30°C is possible by adapting the standard technique of using starter cultures to control the growth of non acidic bacteria such the most known genera of Enterobacteriaceae [5]. Applying starter culture fermentation to the first step of transformation at abusive temperature for guedj fish production could increase processing rates [6], improve control of undesirable microorganisms and limit the bacterial hazards.

A starter culture can be defined as a microbial preparation of a large number of cells of at least one microorganism that is added to a raw material, to produce a fermented food by accelerating and guiding its fermentation process [7]. Starters also improve the sensory characteristics and microbiological quality of fermented foods [8]. Lactic acid bacteria (LAB) are important organisms in the food industry, particularly in traditional fermented foods. They are generally considered food grade organisms that are safe to consume [9] and have been also found to be the dominant microorganisms in many fermented fish products [10,11].

The primary role of LAB starter cultures is to ferment available carbohydrates (and produce organic acids), thereby decreasing the pH. The combination of low pH and organic acids (primarily lactic acid) is the main preservation factor in fermented food products. Some LAB strains produce, in addition to organic acids, specific antimicrobial compounds like bacteriocins, which enhance their technological potentialities of inhibiting spoilage and pathogenic bacteria [12], justifying the technique of targeting such bacteria to develop a new fermentation process to reduce spoilage and improve safety of the end products.

An important criterion for the development of a starter culture used on fish fermentation is to select safe microorganisms that are unable to decarboxylate the amino acids known to be precursors of toxic biogenic amines. Some investigations have dealt with the production of aromatic biogenic amines like histamine and tyramine by certain LAB strains. These two amines are regarded as undesirable compounds because of the potential toxic effects derived from their vasoactive and psychoactive properties. Histamine has been recognized as the causative agent of scombroid poisoning (histamine contamination) whereas tyramine has been related to food-induced migraines and hypertensive crisis in patients under anti depressive treatment with mono-amine oxidase inhibitor (MAOI) drugs. In addition, tyramine production has been correlated with the acidification of the growing medium [13-15], as it usually occurs during fermentation [16].

Approximate pH range value of most fish species is 6.6-6.8. The optimization of fish acidification can be achieved by its supplementation with carbohydrates compounds. Starter culture fish fermentation at high temperature was reported in Southeast Asia. The process in these countries is characterized by the addition of local cereal (rice) as a carbon source. The addition allows starter cultures to optimize acidification and control over the fermentation [17].

Preliminary work to improve fish fermentation by the application of lactic acid bacteria as starter culture at 30°C focused on the evaluation of the in situ antibacterial properties of a nisinogenic Lactococcus lactis subsp. lactis strain isolated from millet flour in Senegal [18]. Artisanal fish was subjected to the new process fermentation by adding glucose as a fermentable carbohydrate substrate source, which enhanced the in situ antibacterial effect of the starter culture by preventing or limiting undesirable bacteria growth.

Some research works have been done on the development of lactic acid bacteria pro-biotic products in using malted cereal substrates [19,20]. Millet (Pennisetum glaucum) and sorghum (Sorghum bicolor) are the most widespread cereals produced locally across the Sahel in Africa. The grains are used for preparation of a variety of staple food commodities in these countries and across Africa. Malting of the grains enhances the content of fermentable sugar, which improves the nutritional quality and digestibility. Ideally, these two cereals could be used as additional technological substrates for the development of starter cultures for fish fermentation in Senegal that could improve the main preservative factors for preventing or limiting growth of the most frequently Enterobacteriaceae genera and bacteriocin sensitive gram positive bacteria reported in spoiled fish stored at non conventional temperature.

The aim of the present study is to investigate four bacteriocinogenic LAB strains selected and characterized in previous international research works [21,22] which include a nisinogenic bacterial strain tested in a preliminary study of lactic acid bacteria application on fish incubated at high temperature , for identification of those suitable for use to optimize preservatives factors over fish fermentation at 30°C. The new process is based on the technique of immersion which is the most frequently applied in the localized agrifood system for guedj fish production. The fish are immersed in a local cereal based substrate, which enhanced the acidification and control over the fermentation [17].

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for decarboxylating activities for identifying those that did not produce histidine and tyrosine decarboxylating activity, (b) to determine the capacity of the negative histidine and tyrosine decarboxylate bacteria detected to grow well, develop in vitro antibacterial activity, and survive on malted millet flour based substrates incubated at 30°C, (c) and to preserve these antibacterial potentials when the fish are submitted to ferment at 30°C inside such starter cultures.

**Material and Methods**

**Origin and properties of Bacteria strains**

Pure cultures of bacteriocinogenic LAB were obtained from the bacterial collection of the “Centre Wallon de Biologie Industrielle” (CWBI), at the University of Liège, Gembloux Agro Bio Tech in Belgium. These bacteria were isolated from a diversity of food and characterized by polyphasic approach in previous works: *Enterococcus mundtii* CWBI_B1431 isolated from cheese, *Lactobacillus curvatus* CWBI_B28 isolated from meat, *Lactococcus lactis subsp. lactis* CWBI-B1410 isolated from milk flour and *Lactococcus lactis subsp. lactis* CWBI-B1426 isolated from fish. The cultures were found to display an antagonistic bactericidal activity against different food poisoning bacteria such as *Listeria monocytogenes* strains. This property justified the use of one of these pathogenic *bacterium* types as indicator, in the *in vitro* (in normal and cereal based fermented matrix) assessment of the antibacterial properties of LAB targeted for the development of starter cultures fish fermentation.

**Culture conditions and inoculate preparation procedures**

A drop of defrosted stock culture of the different bacteria strains was streaked on MRS (LAB) or 863 (*Listeria monocytogenes*) media. Plates were incubated at 30°C for 48 h and cell forming colonies were sub-cultured onto the same respective media. Plates were incubated for 48 h at 30°C to enable development of pure colonies of the different bacteria. After incubation, stock plates were maintained at 4°C and used to prepare different inoculates in subsequent microbiological tests. Colonies of the revitalized lactic acid bacteria or *Listeria monocytogenes* strains were removed from the culture plates and sub-cultured in 10 ml of MRS or 863. Broths were incubated for 20 h at 30°C. The different 20 h sub-cultured broths of the bacteria were separately centrifuged (Sigma2-4, Sigma Laborzentrifugen GmbH, Osterodeam Harz, Germany) at 2,280 x g for 10 min. The cells were washed twice in 0.1% sterile NaCl water and resuspended in 10 ml 0.1% sterile NaCl. The suspensions were used as inoculates in the different applications of the starter cultures and indicator bacteria performed on standard and local cereal based substrates or on fish matrix.

**Screening of histidine and tyrosine decarboxylase activity of LAB**

The improved decarboxylation media proposed by Rover-Cid and Holzapfel were used to detect decarboxylating LAB strains. The media consist of a basic composition (peptone, yeast or meat extract, salt and glucose) to which histidine or tyrosine as well as 0.06% (w/v) of bromocresol purple was added. Freshly grown LAB strains were streaked onto improved decarboxylation agar. Broth was formulated with and without (control) histidine and tyrosine added at 1% (w/v), under aerobic and anaerobic conditions. Plates and tubes inoculated with pure culture of the LAB were incubated for 4 days at 30°C. A positive reaction was recorded when a purple color occurred (histamine production) or when tyrosine precipitate disappeared around the colonies or in the decarboxylase broth (tyramine production), respectively.

**Preparation and constituents of the millet-based fermentation matrix**

Melting can be defined as the germination of grain in moist air under controlled conditions. During germination process reserves in the seed (starch, proteins and lipids) are used for the stems and roots to appear on external porosity by nutrient release and the external porosity on starch access by amylase enzyme. This biological process results in mobilization of hydrolytic enzymes such as amylases and proteases which are essential for the solubilization of starch and proteins in the cereal grains and the increase of sugar content for fermentation [23,24]. Several research works demonstrated a significant increase of total sugar in millet flour obtained from germinated seed.

Pearl millet (*Pennisetum glaucum*) seeds were purchased from a local market in Dakar and cleaned thoroughly with distilled water, then soaked in distilled water for 12 h at room temperature at a ratio of 1: 2.5 (w/v) with a change of water at regular 4 hour intervals. The soaked seeds were subjected to germination at 30°C for 24 hours and the resulting sprouts were dried at 50°C for 48h. A dried sample of germinated seeds was ground into fine powder using an electric grinding blender (Lab Mixer Waring VWR). A millet based substrate was prepared using germinated pearl millet flour supplemented with sodium chloride and distilled water at a ratio of [(2 + 0.5): 100] (w/v). The resulting slurry was filtered and the millet based fluid was collected. The pH of the solution was measured before aliquoting 10 ml into 15 ml glass tubes. The pH of MRS broth was adjusted to the pH of the millet-based solution with HCl solution (0.01 N). Both standard and cereal based media were sterilized at 121°C for 20 min. Additional millet based substrate was prepared using the sterilized millet solution described above. One ml of the solution was substituted with the same volume of commercial pasteurized skimmed milk in sterile condition under flow. The normal medium (MRS) was used as a reference fermentation matrix for the assessment of the growth and antibacterial properties of the bacteriocinogenic LAB on the millet based substrates.

**Characterization of technological capacities of LAB strains on millet based substrates**

The bacteriocinogenic LAB strains which demonstrated a negative decarboxylating activity on histidine and tyrosine were assayed for some important technological features on the two millet-based substrates (with and without skimmed milk supplementation). To determine acidifying and antibacterial properties activities, or acid tolerance, bacteriocinogenic bacteria strains were revitalized in 10 ml of the sterilized millet-based substrates and in the same volume of standard MRS broth used as positive control, by 20 h culture at 30°C. Tubes containing 10 ml of the different sterile matrix lightly salted millet substrate, lightly salted millet substrate supplemented with skimmed milk at the ratio 1:9 (v/v) and MRS broth were inoculated (1%/w/v) with revitalized pure LAB strains and incubated at 30°C for 20 h.

For the acidifying capacity test, the pH was measured with a pH-meter (HI 9124 HI 9125 Portable Waterproof pH meter) before incubation and after 20 h of incubation, in considering that the final pH should be below 4.6 which is lower to the minimum value for the growth of many frequently reported undesirable bacteria on fresh or spoiled fish [25].

For the antibacterial potentiality, neutralized cell-free supernatants were prepared from the 20 h LAB cultures, pasteurized and subjected to the well diffusion assay [26] in using *Listeria monocytogenes* as indicator. For acid tolerance assessment, the surviving cell number of
lactic acid bacteria in the 20 h cultures at 30°C was determined by a standard plating method on MRS Agar.

The specific criteria used for assaying millet based substrate as immersion matrix and fermentable carbon source are: its ability to both enable the bacteriocinogenic LAB strains to drop the pH on a final level similar or below of the minimum value for inhibition of the growth of the most frequently reported foodborne bacteria on fresh or spoiled fish such as without significantly affecting the survival of the starter cultures, and to secrete bacteriocin-like substance. This was demonstrated by the presence of an antibacterial activity of the pasteurized neutralized cell-free supernatants on culture of the pathogenic *Listeria monocytogenes* used as indicator bacterium.

**Determination of the antibacterial activities in the cell-free culture supernatants**

Five (5) ml of the 20h bacteriocinogenic bacteria cultures on the different media (millet based substrate types and the standard medium) were separately centrifuged (Sigma2-4, Sigma Laborzentrifugen GmbH, Osterodeam Harz, Germany) at 2,280 x g for 10 min. The pH of the different supernatants was adjusted to 6.5 with 5 N of NaOH. The neutralized cell free culture supernatants were pasteurized by heat incubation at 80°C for 10 min. The presence of inhibitory activity in the pasteurized neutralized culture supernatants was determined by a well diffusion assay using *Listeria monocytogenes* as the indicator. The minimum inhibitory concentration (MIC) of the positive solution was determined by critical dilution using the same indicator bacterium. The MIC was defined as the reciprocal of the highest dilution showing a halo of inhibition. It was expressed in arbitrary units per milliliter (AU/ml).

**Starter cultures and spontaneous fermentation of fish in millet based substrate**

Lean sumpt grunt (*Podamassys jubelini*) and fat smooth mouth sea catfish (*Arius heudelotii*), widely used in the localized agrifood systems for gazed fish production in Senegal, were purchased at a local market and used for assaying the novel fish fermentation processing. The fish were scaled, eviscerated, rinsed in potable water in the laboratory, and frozen at -18°C for further use for fermentation. The frozen fish were defrosted, filleted and cut into small pieces using a pair of kitchen scissors. For each fish, a total of 130 g of small pieces were divided into 13 portions of 10 g each which were placed in the bottom of 13 sterile, disposable 50 ml Falcon culture tubes. Three sets of culture tubes were divided into 13 portions of 10 g each which were placed in the bottom of 13 sterile, disposable 50 ml Falcon culture tubes. Sterilized millet based substrate was poured on the fish pieces at a ratio of 1:1 (wt/vol), immersing the flesh. One culture tube preparation was reserved for measuring the initial pH and the viable cell population of natural lactic acid bacteria. The remaining preparations were divided into three replicate assays of starter fish fermentation (fish inoculated with *Listeria monocytogenes*) fermention (fish inoculated only with *Listeria monocytogenes*) and pure cultures of CWBI-B1410 or CWBI-B1426 fermentation, and separate starter culture fermentation with the nosinogenic CWBI-B1410 and CWBI-B1426 in the presence of the *Listeria monocytogenes* bacterium was randomly removed from the incubator at ten [27] hour intervals and measured for pH and viable cell population (LAB and *Listeria monocytogenes*) during 48 h.

Ten grams samples of the three different fermenting fish flesh treatments in the 10 ml cereal-based substrates were collected in a sterile plastic Stomacher bag of 1L (BA6141/CLR, Seward, and Worthington, UK). One hundred eighty (180) ml of sterile peptone water were added to make a tenfold dilution. Five (5) ml of the dilutions were used for fish pH measurement using 3151 WTW pH meter (WTW GMBH, Germany) calibrated with buffers at pH 4.0 and 7.0. The rest of the suspensions were serially diluted tenfold in sterile peptone (1%) water and 0.1 ml aliquot dilutions were pour plated on MRS agar supplemented with Polymixin B (100 UI/ml) and Palcam agar for viable cell populations of LAB and *Listeria monocytogenes* enumeration counts, respectively.

**Statistical analysis**

A one-way analysis of variance (ANOVA) was applied to the results of acidifying capacity and *Listeria monocytogenes* cell population of fish subjected to ferment in the millet based substrate with and without revitalized CWBI-B1410 and CWBI-B1426 culture, using the Student–Newman–Keuls test, for comparison of the means (P<0.05) obtained after 24h and 48h of incubation at 30°C. For this purpose, the STATA software package ver.12.0 (STATA, Texas, USA) was used. The significance of the starter culture use on fish pH drop, and *Listeria monocytogenes* growth inhibition during the incubation time was assessed. The data (pH drop level and *Listeria monocytogenes* viable cell number) obtained for two replicate assays of spontaneous fermentation (fish inoculated only with *Listeria monocytogenes*) and two replicate assays of starter fish fermentation (fish inoculated with both *Listeria monocytogenes* and pure cultures of CWBI-B1410 or CWBI-B1426) were analyzed.

**Results and Discussion**

**Bacteriocinogenic starter cultures identified as suitable for use on fish**

Only the two nisinogenic bacterial strains B-1410 and B-1426 did not show a purple color occurring and a precipitate disappear around their colonies or in the decarboxylase broth within the four bacteriocinogenic lactic acid bacteria screened for decarboxylating activity (data not shown). This result means that the two nisinogenic bacterial strains (B-1410 and B-1426) did not produce histamine and tyrosine. The two negative histidine and tyrosine decarboxylate nisinogenic lactic acid bacterial strains were originally isolated from Senegalese traditional foods.

They have been tested on malted millet flour based substrates
to evaluate their in vitro capacity of growing and developing an antibacterial potentiality that could enable control of undesirable spoilage and poisoning bacteria.

**Growth capacity of nisinogenic starter cultures on millet-based liquid matrix**

The capacities of these two bacteria to lower the pH of the local cereal based matrix, to survive in the resulting low final pH, and to secrete heat stable nisin-like antibacterial compounds was determined by comparison with their potentialities on MRS broth used as a reference.

After sterilization of the millet based substrate, sedimentation of solids (possibly protein mixtures) was observed in the millet based media as described by Charalamopoulos et al. for malt, wheat and barley based media. The pH of the different two millet based matrix types and the reference medium seeded with revitalized *Lactococcus lactis subsp. lactis* CWBI-B1410 and viable bacteria before and after 20h incubation at 30°C are presented in the Table 1.

The pH dropped from 6.33 ± 0.07 to 4.44 ± 0.06, 3.41 ± 0.01 and 4.21 ± 0.12, after 20h of incubation respectively of the normal medium (MRS), lightly salted millet flour and this medium supplemented with skimmed milk, seeded with the nisinogenic bacterium. The pH drop level reached in the cultures of the bacterium on the two millet based substrate types were below 4.6 which is required to inhibit pathogenic and spoilage bacteria. The final pH level was lower than that obtained for the reference medium, indicating a good acidifying capacity of the tested bacteria.

The lactic acid bacteria cell population estimated at 6.5 ± 0.07 log (CFU.ml⁻¹) before incubation were determined at 9.5 ± 0.71, 6.40 ± 0.71 for the reference medium, indicating a good acidifying capacity of the indicator bacterium. The results are presented in Table 3.

The pH drop after 20h of incubation at 30°C similar to the typical high temperature of Senegal guedj fish production. The millet based pre-mixture substrate was formulated with malted millet flour, sodium chloride and glucose in distilled water at a ratio of (2 + 0.5 + 1):100 (w/v). This preparation was sterilized and supplemented with commercially pasteurized skimmed milk at a ratio of 1:9 (v/v) for use as the laboratory test technological immersion matrix and carbon source to improve the acidifying capacity of fish and in situ control growth of a nisin sensitive pathogenic bacteria during fermentation at the range of temperature typical to Senegal. The *Listeria monocytogenes* strain tested for in vitro determination of the nisin-like inhibitory activity of the pasteurized neutralized cell-free supernatants of the cultures of CWBI-B1410 and CWBI-B1426 on millet based matrix has been also used as indicator to measure the in situ inhibitory capacity by these bacteria on pieces of lean sumpat grunt (*Podamassys jubellini*) and fat smooth catfish (*Arius heudelottii*) fillets. The two fish species are widely transformed to guedj in Senegal.

The optimization of preservative factors over fish fermentation at high temperature is mainly based on a double antibacterial potential of the nisinogenic starter cultures: lowering the pH to a value lower than minimum for growth of a wide range of bacteria, combined with specific additional nisin-like bactericidal activity. The first type of antagonism is performed against both Gram-negative (such as many undesirable genera of Enterobacteriaceae), and Gram positive bacteria. The complementary inhibition can be effective only against certain Gram-positive bacteria for which a specific sensitivity has been determined. Minimal inhibitory concentration (MIC) of the neutralized cell-free culture of CWBI-B1410 and CWBI-B1426 against some strains of *Listeria monocytogenes*, *Bacillus coagulans* and *Bacillus subtilis* were similar and reached respectively 80, 320 and 5120 AU/ml.

The minimum pH for growth of the genus *Listeria* is 4.1 [4]. It is lower than the final pH reached for the cultures of CWBI-B1410 and CWBI-B1426 on the millet based matrix (Tables 1 and 2). The
substrate at the ratio 1:1 (v/w), seeded with 10^6-7 CFU/g revitalized pure bacterium, were immersed in the newly developed millet based sensitive pathogenic strain, tested as indicator *Listeria monocytogenes*.

would mean a high probability of preventing the proliferation of other resistant bacterium on fish inoculated with nisinogenic starter cultures effective inhibition of the growth of this relatively less acidic and/or more sensitive to nisin-like antibacterial activity.

A potential new technique of fish fermentation at 30°C in millet-based substrate.

The pieces of fish flesh inoculated with 10^4-5 CFU/g of a nisin sensitive pathogenic *Listeria monocytogenes* strain, tested as indicator bacterium, were immersed in the newly developed millet based substrate at the ratio 1:1 (v/w), seeded with 10^6 CFU/g revitalized pure culture of the nisinogenic *Lactococcus lactis* subsp. *lactis* CWBI-B1410 or CWBI-B1426 strains. *Listeria monocytogenes* contaminated fish samples immersed in the millet flour based substrate without starter culture addition were used as negative controls.

**Inhibitory potential by the starter cultures over fish fermentation on millet-based matrix**

The evolution of the pH, LAB and *Listeria monocytogenes* cell population during incubation at 30°C for 48h of the fish inoculated only with the pathogenic bacterium, and those seeded with both revitalized culture of the nisinogenic CWBI-B1410 strain and *Listeria monocytogenes* living culture, are presented in the Figure 1.

The initial pH of the lean and fat fish was 6.22 ± 0.25 and 6.79 ± 0.15, and dropped after 10h of incubation at 30°C to 4.51 ± 0.085 and 4.55 ± 0.078 respectively, when they were treated with the nisinogenic CWBI-B1410 bacterium strain (Figure 1). The two level drops in pH of the starter fermented fish in the millet based substrate are below the

| Time               | MRS                  | MS                    | MSSM                  |
|--------------------|----------------------|-----------------------|-----------------------|
|                    | pH                   | Lactic acid bacteria  | pH                   |
|                    | log10 (CFU/g)        | cell population      | log10 (CFU/g)        |
| Before incubation  | 6.33 ± 0.03          | 6.50 ± 0.70           | 6.33 ± 0.03          |
| After 20h of incubation | 4.44 ± 0.06          | 9.50 ± 0.71           | 3.41 ± 0.01          |

a (MRS): Man, Rogosa and Sharpe broth used as reference
b (MS): Millet flour and salt (NaCl) in distilled water at the ratio (2 ± 0.5): 100 (w/v)!
c (MSSM): Millet flour and salt (NaCl) in distilled water at the ratio (2+0.5): 100 (w/v)!

**Table 1:** Level of pH and lactic acid bacteria cell population in standard medium a and millet flour based substrates b, c seeded with revitalized nisinogenic *Lactococcus lactis* subsp. *lactis* CWBI-B1410 before and after 20h of incubation at 30°C. The data are the mean ± standard deviation from duplicate trials performed on different samples at different times.

| Time               | MRS                  | MS                    | MSSM                  |
|--------------------|----------------------|-----------------------|-----------------------|
|                    | pH                   | Lactic acid bacteria  | pH                   |
|                    | log10 (CFU/g)        | cell population      | log10 (CFU/g)        |
| Before incubation  | 6.33 ± 0.03          | 6.60 ± 0.50           | 6.33 ± 0.03          |
| After 20h of incubation | 4.38 ± 0.01          | 9.60 ± 0.65           | 3.58 ± 0.18          |

a (MRS): Man, Rogosa and Sharpe broth used as reference
b (MS): Millet flour and salt (NaCl) in distilled water at the ratio (2 ± 0.5): 100 (w/v)!
c (MSSM): Millet flour and salt (NaCl) in distilled water at the ratio (2 ± 0.5): 100 (w/v)!

**Table 2:** Level of pH and lactic acid bacteria cell population in standard medium a and millet flour based substrates b, c seeded with revitalized nisinogenic *Lactococcus lactis* subsp. *lactis* CWBI-B1426 before and after 20h of incubation at 30°C. The data are the mean ± standard deviation from duplicate trials performed on different samples at different times.

| Niogenic LAB strains seeded in the matrix | Antibacterial Activity in the 20 h pasteurized neutralized cell-free culture supernatants for the different matrix (AU/ml) |
|-----------------------------------------|-------------------------------------------------------------------------------------------------------------------|
| *Lactococcus lactis* subsp. *lactis* CWBI-B1410 | MRS | MS | MSSM |
| 80 ± 00 | 0 | 40 ± 00 |
| *Lactococcus lactis* subsp. *lactis* CWBI-B1426 | 60 ± 28 | 0 | 40 ± 00 |

a (MRS): Man, Rogosa and Sharpe broth used as reference
b (MS): Millet flour and salt (NaCl) in distilled water at the ratio (2 ± 0.5): 100 (w/v)!
c (MSSM): Millet flour and salt (NaCl) in distilled water at the ratio (2 ± 0.5): 100 (w/v)!

*Nisin is a heat stable antibacterial peptide produced by some Lactococcus lactis strains (10, 25, 26), and the antibacterial activity was assessed by critical dilution in using a Listeria monocytogenes strain as indicator bacterium*

*0* means lack of inhibitory action of the neutralized culture supernatant.

**Table 3:** Anti-*Listeria monocytogenes* activity in the heated neutralized cell-free supernatants of 20h at 30°C incubated standard medium a and millet based substrates b, c seeded with revitalized nisinogenic *Lactococcus lactis* subsp. *lactis* CWBI-B1410 or *Lactococcus lactis* subsp. *lactis* CWBI-B1426. The data are the mean ± standard deviation from duplicate trials performed on different samples at different times.
minimal value considered sufficient to inhibit potential neutrophilic spoilage bacteria such as Enterobacteriaceae. The low pH levels were maintained after 24 h and 48 h of incubation at 30°C.

The starter culture fermentation gave the lowest fish pH reading at each incubation sampling time (10 h, 24 h and 48 h). The pH levels at the different sampling time were significantly different (P<0.05) to those obtained for the controls at 24 h and 48 h of incubation for both the lean and fatty fish subjected to ferment in the newly formulated local cereal based matrix. Similar results were obtained when the fish were treated with the nisinogenic Lactococcus lactis subsp. lactis CWBI-B1426 (data not shown). The difference of the pH level reached between spontaneous and starter culture fermentation of fish on the millet based substrate was positive indicating a higher capacity of acidification in the presence of nisinogenic bacteria starters (CWBI-B1410 or CWBI-B1426).

The pathogenic bacteria seeded in the fish grew significantly in the spontaneous fermented fish compared to that in the starter fermentation (Figure 1). The Listeria monocytogenes cell population of the spontaneous fermented lean and fatty fish reached or was about 8 log_{10} (CFU/g), while in samples treated with the nisinogenic Lactococcus lactis subsp. lactis CWBI- B1410 the initial level was reduced below 2 log_{10} (CFU/g). The surviving pathogenic bacteria level on the fish immersed in the culture of CWBI-B1410 on the millet based matrix were significantly different than control (P<0.05) after 24 h for both the lean and fatty fish. The differences observed were negative indicating an inhibition of the growth of the pathogenic bacterium by the starter culture. Similar significant differences in the Listeria monocytogenes surviving population were obtained (P<0.05) after 24 h of incubation at 30°C between control samples and the starter fermented fish, when culture of Lactococcus lactis subsp. lactis CWBI-B1426 on the millet based substrate are used as technological immersion matrix.

The lactic acid bacteria cell population of the control sample and CWBI-B1410 starter fermented lean fish reached 8.05 ± 0.21 and 10.01 ± 0.15 log_{10} (CFU/g), respectively, after 24 h of incubation at 30°C. Similar results were obtained for the fatty fish, indicating good growth of the starter cultures on fish immersed in the millet based substrate (Figure 1). The levels of lactic acid bacteria populations on fish fermented in using cultures of CWBI-B1426 as immersion matrix were reached about those described for CWBI-B1410 strain (data not shown).

Fish are able to resist change in pH; they are said to be buffered. However, when considering only the starter culture treated lean or fatty fish, the low pH level reached and maintained during the 48h of incubation mean and improvement of the preservative factor in comparison to the neutrophilic pH level characterizing the traditional process. The growth of the relatively acidic and low sensitive nisin indicator bacterium was also inhibited for 48 h in the lean and fat starter culture fermented fish. The 48 h of incubation for fermentation to develop flavor is the most frequently used in de localized agrifood system for guedj production. These results mean that the fermentation of the fish on nisinogenic starter cultures in the millet-based substrate can be performed for 48h at 30°C with prevention of limitation of neutrophilic and/or high sensitive more nisin sensitive bacteria.

The technologies of fish fermentation prevailing in the localized agrifood system for guedj fish production in Senegal are not adequate to meet hygienic requirements defined for fish products [29]. The spontaneous fermentation applied is characterized by high final pH level reaching 7, which lead to the propagation of undesirable bacteria such as enteric bacteria. These bacteria have been found at final level at 9 log_{10} CFU/g. The uncontrolled salty sea water used for immersion the raw fish for the spontaneous fermentation is a source of cross contamination and hazards.

In these unsatisfactory hygienic conditions of fish fermentation at high temperature in the microenterprises, lowering the pH to reach the range 4.6-4 which is below the minimum for growth of the most frequently reported spoilage and poisoning bacteria in fish products could enable to limit the bacterial contamination or hazard. The combination of the acidification with in situ secretion of antibacterial compounds by using starter cultures of bacteriocinogenetic lactic acid bacteria will have the advantage of extending the control to some pathogenic gram positive bacteria and improve the safety of the end products. The two nisinogenic Lactococcus lactis subsp. lactis strains tested in this study did not produce histamine and tyrosine regarded as undesirable compounds in seafood products because of their toxic effects derived from their vase-active and psychoactive properties. The can be used to improve fish fermentation at the typical high temperature levels typical to Senegal.

The preliminary starter culture fish fermentation work based on guedj in using CWBI B-1410 strain on fish pieces supplemented with 1% (wt/wt) demonstrated a potential to significantly lower the pH and control Enterobacteriaceae. But this strategy can be difficultly implemented in the localized agrifood systems for guedj fish production in Senegal because of the potential constraint of pure glucose availability. The challenge for implementation of the new procedure of using starter cultures of suitable nisinogenic bacteria is to found fermentable carbon source available across the country. The matrix containing the carbon source must enable to maintain the final low pH targeted for at least 48h of fermentation at 30°C applied during guedj fish flavor development.

This study showed that the two nisinogenic bacterial strains, CWBI-B1410 and CWBI B-1426 grow and survive well on millet flour substrate supplemented with skimmed milk that contributes to optimize control of the pH drop level and the antibacterial activity during the incubation at 30°C.

The decisive growth and post-fermentation survival limiting factor of lactic acid bacteria starter cultures in fermented products is pH. The drop of pH during the bacteria growth is due to the production of organic acids, particularly lactic acid from fermentable carbohydrates and the final level reached is depending to the buffering capacity of the media or the fermentation matrix.

In the group of lactic acid bacteria, Lactococcus sp strains are known to display a significantly lower acid tolerance than Lactobacillus which can survive to pH values ranging between 1 and 4 [28,29]. The levels of pH reached in using the millet-based substrate supplemented with milk were under the minimal considered enough to inhibit undesirable non acidophilic bacterial group such as enteric bacteria [30]. The final pH level did not significantly affect the survival of the nisinogenic Lactococcus lactis subsp. lactis strains. Addition of milk on cereal-based substrates enhances the buffering capacity [31,32], resulting in maintain of the final pH in the ranges preventing spoilage bacteria growth without affecting significantly the starter cultures surviving.

The findings of the present work indicate that the nisinogenic bacteria can develop a nisin like inhibitory antibacterial activity of millet based substrate and the immersion of fish in the newly developed substrate seeded with the pure culture of the two nisinogenic bacteria strains enable their acidification. The final pH of fish reached was...
below the minimum known for inhibition of many bacterial genera of Enterobacteriaceae and other bacterial groups frequently characterized in fresh and spoiled. The low final pH that is the main preservative factor over the process is better stabilized when the fish is directly immersed in the millet-based substrate than in the preliminary starter fermentation work performed by addition at (1% w/w) of glucose concentrations. J Food Prot 65: 333-338.

10. Daeschel MA (1989) Antimicrobial substances from lactic acid bacteria for use as food preservatives. Food Technol 43: 164-167.

11. Saithong P, Panthavee W, Boonyaratanakornkit M, Sikkhamondhol C (2010) Use of a starter culture of lactic acid bacteria in plaa-som, a Thai fermented fish. J Biosci Bioengin 110: 553-557.

12. Marcobal A, Martin-Alvarez PJ, Moreno-Arribas MV, Munz R (2006) A multifactorial design for studying factors influencing growth and dry matter production of the lactic acid bacterium Lactobacillus curvatus. Bioprocess and Biosystem Engineering 29: 263-269.

13. Connolly PL, Plissonneau, Onno B, Pilet MF, Prévost H, et al. (1998) Fermentation and spoilage of som-fak, a Thai salted fish product. Trop Sci 38: 105-112.

14. Bover-Cid S, Hugas V, Izquierdo-Pulido V, Vidal-Carou M (2001) Amino acid decarboxylase activity of Lactobacillus curvatus 27: 263-264.

15. Masson F, Lebert R, Tatot R, Montel MC (1997) Effects of physico-chemical factors influencing tyramine production by Carnobacterium divergens. J Appl Microbiol 83: 36-42.

16. Bover-Cid S, Hugas V, Izquierdo-Pulido V, Vidal-Carou M (2001) Amino acid decarboxylase activity of bacteria isolated from fermented pork sausages. J Food Microbiol 66: 185-189.

17. Bourgeois CM, Larpent JP (1996) Microbiologie alimentaire, Aliments fermentés et fermentations alimentaires, Technique et Documentation, Lavoisier, Paris.

18. Diop MB, Dubois-Dauphin R, Dortu C, Tine E, Destain J, et al. (2008) In vitro

These results indicate that the use of starter cultures of the nisinogenic bacterial strains on millet based substrate immersed fish could enable to limit bacterial spoilage over fish transformation at high temperature to improve safety new guedj-like products.

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detection and characterization of bacteriocin-like inhibitory activity of lactic acid bacteria (LAB) isolated from Senegalese local food products. Afr J Microbiol Res 2: 206-216.

19. Arora S, Sudesh J, Khetarpaul N (2011) Effect of germination and probiotic fermentation on nutrient profile of pearl millet based food blends. British Food J 113: 470-481.

20. Salovaara H (1996) The time of cereal-based functional foods is here: introducing Yosa R, a vellie. Proceedings of the 26th Nordic Cereal Congress, Haugesund, Norway.

21. Aguilar-Galvez A, Dubois-Dauphin R, Ghalfi H, Campos D, Thonart P (2009) Description of two Enterococcus strains isolated from traditional Peruvian artisanal produced cheeses with a bacteriocin-like inhibitory activity. Biotechnol Agron Soc Environ 13: 349-356.

22. Ghalfi H, Allaoui V, Destain J, Benkerroum N, Thonart P (2006) Bacteriocin activity by Lactobacillus curvatus CWBI-B28 to inactivate Listeria monocytogenes in cold-smoked salmon during 4°C storage. J Food Prot 69: 1066-1071.

23. Ndiaye C, Xu SY, Ngom PM, NDoye BS (2008) Malting germination effect on rheological properties and cooking time of millet (P. typhoides) and shorgum (S. bicolor) flours and Rolled flour products (arraw). Am J Food Technol 3: 373-383.

24. Usai T, Nyanum BC, Mutonhodza B (2013) Malt Quality Parameters of Finger Millet for Brewing Commercial Opaque Beer. Int J Sci Research (JSR) 2: 2319-5690-764.

25. Owen JD, Mendosa V (1985) Enzymatically hydrolysed and bacterially fermented fishery products. J Food Technol 20: 2730-293.

26. Barefoot SF, Klaenhammer TR (1983) Detection and activity of lacticin B, a bacteriocin produced by Lactobacillus acidophilus. Appl Environ Microbiol 45: 1808-1815.

27. Cai Y, Ng LK, Farber JM (1997) Isolation and characterization of nisin-producing Lactococcus lactis subsp. lactis from bean sprouts. J Appl Microbiol 93: 498-507.

28. Aymerich T, Martin B, Garriga M, Vidal-Carou MC, Bover-Cid S, et al. (2006) Safety properties and molecular strain typing of lactic acid bacteria from slightly fermented sausages. J Appl Microbiol 100: 40-49.

29. Charalampopoulos D, Pandiella SS, Webb C (2003) Evaluation of the effect of malt, wheat and barley extracts on the viability of potentially probiotic lactic acid bacteria under acidic conditions. J Food Microbiol 82: 133-141.

30. Faye T, Tamburello A, Vegardur GE, Skeie S (2012) Survival of lactic acid bacteria from fermented milks in an in vitro digestion model exploiting sequential incubation in human gastric and duodenum juice. J Dairy Sci 95: 558-566.

31. Kailasapathy K, Chin J (2000) Survival and therapeutic potential of probiotic organisms with reference to Lactobacillus acidophilus and Bifidobacterium species. Immunol Cell Biol 78: 80-88.

32. Zárate G, Chaia AP, González S, Oliver G (2000) Viability and h-galactosidase activity of dairy propionibacteria subjected to digestion by artificial gastric and intestinal fluids. J Food Prot 63: 1214-1221.