Use of a Nisin-Producing Starter Culture of
Lactococcus lactis subsp. lactis To Improve Traditional Fish
Fermentation in Senegal

MICHEL BAKAR DIOP,1,2* ROBIN DUBOIS-DAUPHIN,1 JACQUELINE DESTAIN,1 EMMANUEL TINE,2
AND PHILIPPE THONART1

1Centre Wallon de Biologie Industrielle, Gembloux Agricultural University, 2 passage des Déportés, B 5030 Gembloux, Belgium; and 2Laboratoire de
Microbiologie Appliquée et Génie industriel, Université Cheikh Anta DIOP, Ecole Supérieure Polytechnique, BP 5085 Dakar, Sénégal

ABSTRACT

Lactococcus lactis subsp. lactis strain CWBI B1410, which produces various antibacterial compounds including organic acids and nisin, was used as a starter culture to improve the traditional Senegalese fish fermentation in which fish are mostly transformed to guedj by spontaneous fermentation for 24 to 48 h at ambient temperatures near 30°C followed by salting (with NaCl) and sun drying. Assays were performed on lean (Podamasys jubelini) and fat fish (Arius heudelotii) purchased at a local market. The total viable microbial counts in raw fillets of P. jubelini and A. heudelotii were 5.78 and 5.39 log CFU/g, respectively. Populations of enteric bacteria (which can include pathogenic bacteria) in P. jubelini and A. heudelotii were 4.08 and 4.12 log CFU/g, respectively. Spontaneous fermentation of raw fillets at 30°C led to the proliferation of enteric bacteria to 9 log CFU/g after 24 h in fermented P. jubelini and A. heudelotii fillets with pH values of 6.83 and 7.50, respectively. When raw fish fillets were supplemented with glucose (1%, wt/wt) and inoculated with Lactococcus lactis (106 CFU/g), the pH decreased to about 4.60 after 10 h at 30°C, and nisin activity was detected in juice from the fillets. Traditionally fermented fillets of P. jubelini and A. heudelotii contained enteric bacteria at higher levels of 4 and 2 log CFU/g, respectively, than did fillets of the same fish supplemented with glucose and fermented with the starter culture. These data suggest that this new fish fermentation strategy combined with salting and drying can be used to enhance the safety of guedj.

Senegal is a West African nation known for the importance of its marine fishery products, with an annual fish harvest averaging 403,911 tons during the last decade. Industrial fisheries contribute only about 15% of the total seafood production compared with 85% contributed by artisanal fisheries (6, 8). Traditional fish production has some inherent food safety concerns considering the high susceptibility of seafood to bacterial spoilage (3).

In artisanal fisheries, fish species are stored either at ambient temperatures (around 30°C) or in small quantities of ice (6). Overall, about 30 to 40% of the total catch is cured for domestic consumption and export. The most common traditional fish products are kethiakh (roasted, salted, and dried Ethmalosa or Sardinella), guedj (fermented, salted, and dried fish species such as Podamasys jubelini and Arius heudelotii), and tambadiang (whole dried Ethmalosa). These fish products are important animal protein sources for populations in sub-Saharan countries (6, 10, 17).

In Senegal, no starter cultures are used during the traditional transformation by fermentation for guedj. This fermentation is performed at ambient temperatures for 24 to 48 h, then salt (30 to 40% NaCl, wt/wt) is added to the fish, which are then sun dried. Spoilage bacteria and natural lactic acid bacteria (LAB) in the fish serve as starters for spontaneous fermentation. Unlike traditional methods of fish fermentation in southeast Asia (2, 24), there is no external fermentable carbohydrate supply during spontaneous fermentation of fish in Senegal. The fact that most fish muscle tissue contains very low levels of carbohydrates (<0.5%) with only small amounts of lactic acid produced postmortem has important consequences for microbial spoilage of naturally fermented fish products. Under these high pH conditions, spoilage bacteria including Enterobacteriaceae and Shewanella putrefaciens can proliferate (14, 15).

LAB have been widely used to protect foods from the proliferation of spoilage and pathogenic bacteria (25, 26). Among the defense mechanisms of these bacteria are the production of organic acids, hydrogen peroxide, and bacteriocins (5, 22). Bacteriocins are ribosomally synthesized peptides that have activity against other bacteria, usually closely related species (11). Nisin, a bacteriocin known in three natural variants (A, Z, and Q) (16, 23, 29), has been extensively used during the past 40 years to prevent spoilage of various foods, including lightly preserved fish products (5, 9, 28). Lactococcus lactis subsp. lactis strain CWBI B1410, isolated from traditional fermented millet flour, produces various antimicrobial substances, including organic acids, hydrogen peroxide, and nisin A. This nisin-producing strain showed broad inhibition against various gram-positive spoilage and/or pathogenic bacteria, including Bacillus cereus CWBI B1432 and Staphylococcus epider-

* Author for correspondence. Tel: (221) 33 825 08 79; Fax: (221) 33 825 55 94; E-mail: diopmb@yahoo.fr.
midis CWBI B1433, which were isolated from traditionally handled P. jubelini fish in Senegal (7).

The aim of the current study was to assess the growth of LAB (generally considered as safe) and enteric bacteria (that can include pathogenic bacteria) populations in naturally fermented fish commodities under conditions similar to those used for guedj production in Senegalese artisanal fisheries. L. lactis subsp. lactis CWBI B1410 also was assessed as a starter culture to improve the traditional Senegalese fish fermentation. The ability of strain CWBI B1410 to produce organic acids (for control of enteric bacteria) and nisin (for control of potential spoilage and pathogenic gram-positive bacteria) during fermentation at 30°C also was evaluated.

MATERIALS AND METHODS

Bacterial strains and media of cultivation. L. lactis subsp. lactis CWBI B1410 was used as the starter culture, and S. epidermidis CWBI B1433 was used as an indicator strain to assess nisin production by the starter culture in fish. These bacterial strains (obtained from Centre Wallon de Biologie Industrielle, Gembloux Agricultural University, Gembloux, Belgium) were maintained on glass beads at −80°C and first propagated on solid media (de Man Rogosa and Sharpe [MRS] for L. lactis and plate count agar [PCA] supplemented with 0.5% NaCl (wt/vol) for S. epidermidis) before being subcultured in MRS broth and nutritive broth to prepare the inoculum.

L. lactis CWBI B1410 inoculum preparation. Three or four isolated colonies of L. lactis were inoculated into 30 ml of MRS broth, and the culture was incubated at 30°C for 16 h and then centrifuged (Sigma 2-4, Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) at 2,280 × g for 20 min. The pellet was washed twice in 5 ml of 0.85% NaCl and then suspended in 20 ml of 0.85% NaCl to obtain a population of about 10⁶ CFU/ml.

Fish source, preparation, filleting, and analyses. Sumpat grunts (P. jubelini) and smooth mouth sea catfish (A. heudelotii) 40 to 50 cm in length and 700 to 900 g were purchased at the Soumbedioune landing and marketing site in Dakar, Senegal. Fish were scaled and eviscerated on site under the prevailing fish handling conditions. These fish were generally gutted on site by fish operators at the local markets. This business activity enhances the risk of cross-contamination (6). Gutted fish were brought to the laboratory within 15 min in plastic bags purchased on site and were rinsed in drinkable water. Filleting was done under sterile conditions. Fish fillets of the two species with the skin attached (each 0.6 cm thick and 150 g) were examined for chemical composition and microbiological characteristics. Growth of enteric bacteria and LAB (including the starter culture) and the concentrations of organic acids and nisin produced by these bacteria during storage of these raw fish fillets at 30°C were determined. The data presented in this study are the mean ± standard deviation from duplicate samples and two trials performed at different times.

Chemical composition analyses of the two tested fish commodities. One hundred fifty grams of both raw fish fillet types were dried separately at 100°C for 24 h and then weighed to determine the water content. The dried material obtained was minced to estimate the chemical composition. Methods used were the Kjeldahl assay to determine proteins with a 6.25 conversion factor (21), the Folch assay for lipids (12), and overnight calcination at 550°C for ash (1).

Microbial analyses of raw fish fillets. Eight grams of unskinned flesh was removed from the two types of fish fillets under sterile conditions using sterilized scissors. The samples were suspended in 72 ml of sterile 0.8% saline in a sterile sealed plastic bag (BA 6141/CLR, Seward, Worthington, UK) and homogenized in a stomacher (Blender 80, Seward) for 2 min. The resulting homogenate was 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/liter 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 (Scharlab Chemie, Barcelona, Spain) for quantifying total viable bacteria, LAB, staphylococci, Escherichia coli, and enteric bacteria, respectively. Colonies developing on the plates were counted after 48 h of incubation at 30°C (total viable bacteria and LAB) or 37°C (E. coli, staphylococci, and enteric bacteria).

Fish fermentation procedures. Fish fillets (150 g) of each species were fermented in separate sterile plastic bags (BA 6141/CLR) from which the resulting fish juice was later evaluated for nisin production by L. lactis. Three different fish fermentation procedures were tested.

In the first method, resembling the traditional procedure used for guedj production in Senegal, the two species of raw fish fillets were held at 30°C for 24 h to ferment (spontaneous fermentation).

In the second method, the fish fillets were inoculated with the L. lactis starter culture at 10⁴ CFU/g by spreading 1.5 ml of this bacterial suspension on the fish surface. These fillets were then held at 30°C for 24 h to ferment.

In the third method, raw fish fillets of the two species were treated with glucose (1% glucose powder, wt/wt, spread over the fillets), inoculated with the L. lactis starter culture (10⁵ CFU/g), and then held at 30°C for 24 h to ferment.

Assessment of microbial levels and pH of fermented fish fillets. For each fish fermentation method, the in situ growth of enteric bacteria and LAB was assessed by direct plating using the previously described media after 10 and 24 h of fermentation. The pH of these same fish flesh suspensions was determined (Eutech Instruments, Singapore) immediately after assessment of the microbial population.

Evaluation of fermented fish juice for nisin activity. Nisin activity against S. epidermidis was assessed in the fermented fish juice using the well diffusion technique (7) after 10 and 24 h of fermentation at 30°C. In this procedure, 500-μl samples of fish juice from both fish species fermented with L. lactis with or without added glucose were centrifuged at 17,000 × g for 15 min (Eppendorf AG, Hamburg, Germany). The supernatants obtained were then pasteurized for 10 min at 80°C (this treatment has no effect on the biological activity of nisin (7)) and serially diluted in phosphate buffer (50 mM, pH 6.3), and 50-μl aliquots were loaded into separate wells cut in the agar medium seeded with 110 μl of an overnight culture of S. epidermidis. 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 (7). Inhibitory activity of the fish juice was expressed in arbitrary units (AU) per milliliter, defined as the reciprocal of the highest dilution showing a definite...
TABLE 1. Chemical composition of Podamasys jubelini and Arius heudelotii muscle

<table>
<thead>
<tr>
<th>Constituents</th>
<th>P. jubelini</th>
<th>A. heudelotii</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>77.10</td>
<td>74.2</td>
</tr>
<tr>
<td>Proteins</td>
<td>20.58</td>
<td>16.8</td>
</tr>
<tr>
<td>Lipids</td>
<td>0.70</td>
<td>7.50</td>
</tr>
<tr>
<td>Ash</td>
<td>1.05</td>
<td>1.20</td>
</tr>
<tr>
<td>Carbohydrates&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.52</td>
<td>0.30</td>
</tr>
</tbody>
</table>

<sup>a</sup> Carbohydrates were estimated using the formula 100 – (water + proteins + lipids + ash).

Inhibition zone (27). Juice from both naturally fermented fish species tested against *S. epidermidis* served as a negative control.

Postfermentation salting. Postfermentation salting was performed only on completely fermented fish fillets containing relatively low populations of enteric bacteria. Fish fillets (150 g) were submerged in 1-liter sterilized glass jars containing 30 ml of a previously pasteurized (80°C for 10 min) 35% NaCl solution, held for 11 h at 10°C, and assessed for microbial populations.

RESULTS AND DISCUSSION

Proximate analysis of fish fillets and microbial populations during storage at 30°C. The chemical composition of both fish fillet species is presented in Table 1. These two fish commodities are important sources of protein. The muscle of *A. heudelotii* also is high in lipids, making this fish a more important source of lipids than is *P. jubelini*.

The microbial populations in the two fish species were similar, with *P. jubelini* and *A. heudelotii* yielding total viable microbial counts of 5.78 and 5.39 log CFU/g, respectively (Fig. 1). These levels were barely within the limit of acceptability (6 log CFU/g) defined for seafood destined for human consumption (18) and similar to those reported by Diei-Ouadi (6) and Gram (14). Populations of enteric bacteria in *P. jubelini* and *A. heudelotii* were 4.08 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 (18), whereas those of *E. coli* were above the limit defined for these bacteria (1.20 log CFU/g) (18) (Fig. 1). These data indicate that the technologies of fish handling and preparation prevailing in the local landing and marketing sites are not adequate to meet hygienic requirements defined for fish products (3) and can lead to the propagation of enteric bacteria as the main bacterial population in “fresh” fish products.

*Enterobacteriaceae* and LAB in the fillets of both fish types transformed by spontaneous fermentation at 30°C for 24 h increased rapidly and reached 9 and 7 log CFU/g, respectively (Table 2). The spontaneously fermented fillets of *P. jubelini* and *A. heudelotii* had relatively high pH values of 6.83 and 7.50, respectively (Table 2). No inhibitory activity was detected against *S. epidermidis* in the juice from naturally fermented fillets of the two fish species. However, putrid odors developed after 5 to 6 h of incubation at 30°C and became very pronounced after 24 h. The H<sub>2</sub>S-producing strains (black colonies on Hektoen agar) grew to levels of $3 \times 10^6$ and $3 \times 10^7$ CFU/g in the processed lean (*P. jubelini*) and fat (*A. heudelotii*) fish fillets, respectively. Because enteric bacteria and the H<sub>2</sub>S-producing strains can include some pathogens, these data suggest that the traditional Senegalese fish fermentation used for *guedj* is not sufficient to ensure the biosafety of these fish products and by-products.

Predominance of *Enterobacteriaceae* in naturally fermented fish fillets can be explained by a combination of factors, including the slightly acidic pH of the fish flesh and the low carbohydrate content that limits the decrease in pH associated with production of lactic acid from fermentable carbohydrates. Because both types of fish muscle are high in protein, release of ammonia from bacterial deamination of amino acids contributes to the increase in pH in naturally fermented fish products and explains the proliferation of enteric bacteria in these products. Therefore, a novel fish fermentation strategy using *L. lactis* as a starter culture was assessed to enhance the control of enteric bacteria during storage of both types of fish fillets at 30°C, similar to ambient temperatures used for *guedj* production in Senegal.

**Influence of *L. lactis* as a starter culture to inhibit enteric bacteria growth in fillets supplemented with glucose.** *L. lactis* had a higher growth rate and higher metabolic activity (synthesis of nisin and organic acids) in the glucose-supplemented fishes. *L. lactis* decreased the pH of fillets supplemented with glucose to about 4.60, whereas in fillets not supplemented with glucose the pH decrease was similar to that described for spontaneously fermented fish (Table 2). For starter-fermented fillets of *P. jubelini* and *A. heudelotii*, those fillets supplemented with glucose had lower levels of enteric bacteria (4 and 2 log CFU/g, respectively) than fillets of the same fish not supplemented glucose, which had enteric bacteria levels similar to those found in traditionally processed fillets (Tables 3 and 4). The putrid odors were not very perceptible in fillets supplemented with glucose, indicating the inhibition of enteric bacteria in these fishes. The homofermentative degradation of glucose by the *L. lactis* starter, which involves the release...
TABLE 2. Changes in microbial populations and pH in fillets of Podamasys jubelini and Arius heudelotii naturally fermented at 30°C

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>LAB (log CFU/g)</th>
<th>pH</th>
<th>Enteric bacteria (log CFU/g)</th>
<th>LAB (log CFU/g)</th>
<th>pH</th>
<th>Enteric bacteria (log CFU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.22 ± 0.31</td>
<td>6.04 ± 0.08</td>
<td>4.01 ± 0.29</td>
<td>3.54 ± 0.13</td>
<td>6.30 ± 0.02</td>
<td>4.12 ± 0.12</td>
</tr>
<tr>
<td>10</td>
<td>7.15 ± 0.06</td>
<td>6.20 ± 0.02</td>
<td>8.14 ± 0.14</td>
<td>6.95 ± 0.30</td>
<td>7.13 ± 0.20</td>
<td>8.35 ± 0.35</td>
</tr>
<tr>
<td>24</td>
<td>7.52 ± 0.74</td>
<td>6.83 ± 0.19</td>
<td>8.55 ± 0.47</td>
<td>7.18 ± 0.26</td>
<td>7.50 ± 0.19</td>
<td>8.98 ± 0.30</td>
</tr>
</tbody>
</table>

*Bold values indicate high levels of the population of enteric bacteria in the naturally fermented fillets (high potential for microbiological risk).*

TABLE 3. Changes in microbial populations, pH, and nisin activity in fish juice during starter culture (L. lactis CWBI B1410) fermentation of Podamasys jubelini at 30°C

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>LAB (log CFU/g)</th>
<th>pH</th>
<th>Enteric bacteria (log CFU/g)</th>
<th>LAB (log CFU/g)</th>
<th>pH</th>
<th>Enteric bacteria (log CFU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.30 ± 0.42</td>
<td>6.04 ± 0.08</td>
<td>4.01 ± 0.29</td>
<td>7.30 ± 0.42</td>
<td>6.04 ± 0.10</td>
<td>4.01 ± 0.30</td>
</tr>
<tr>
<td>10</td>
<td>9.61 ± 0.61</td>
<td>6.15 ± 0.02</td>
<td>6.65 ± 0.64</td>
<td>10.24 ± 0.36</td>
<td>4.57 ± 0.62</td>
<td>5.99 ± 0.41</td>
</tr>
<tr>
<td>24</td>
<td>9.63 ± 0.32</td>
<td>6.71 ± 0.19</td>
<td>8.98 ± 0.14</td>
<td>10.19 ± 0.52</td>
<td>4.68 ± 0.63</td>
<td>4.40 ± 0.62</td>
</tr>
</tbody>
</table>

*Bold values indicate the correlation between decreasing pH of fish to an acidic value and the reduction of levels of enteric bacteria (potential mean for enhancing guedj safety). Nisin inhibitory activity (evaluated using Staphylococcus epidermis CWBI B1433 as the indicator strain) was detected only in juice from fish supplemented with glucose. Inhibitory levels were 1,280 AU/ml after 10 h and 480 ± 226.3 AU/ml after 24 h.*

Supplementation of the fish fillets with glucose enhanced nisin production by the L. lactis starter. Residual antibacterial activity in juice from the fillets of the two fish species supplemented with glucose was 1,280 AU/ml after 10 h of incubation at 30°C, whereas no activity was detected in fillets not supplemented with glucose. The inhibitory concentrations of nisin in juice from glucose-supplemented starter-fermented fillets of both fish types were similar to those measured in the neutralized supernatant of L. lactis grown in MRS broth (7). These concentrations were effective against various pathogenic gram-positive bacteria such as Listeria monocytogenes and B. cereus strains (7). This ability of the starter culture to produce nisin in fish treated with glucose and fermented could be applied to the control of pathogenic acidophilic gram-positive bacteria that can proliferate during guedj production. The antibacterial activity of nisin in the juice from the two fish species decreased by the end of fermentation (Tables 3 and 4). This decline may be due to degradation of the bacteriocin (the nisin secreted by the starter) by proteolytic enzymes present in the muscle of the two fish types (13, 20).

These results indicated that the use of L. lactis as a starter culture combined with supplementation of the muscle of both fish with fermentable carbohydrates (glucose) can be a useful strategy for enhancing the biosafety of fermented fish products in Senegal. A L. lactis culture in the presence of glucose was particularly effective in controlling the growth of enteric bacteria in fermented fish muscle low in lipids.

The use of NaCl as an additional barrier in these starter-fermented fish fillets enhanced the control of enteric bacteria by the starter. The decrease in counts of enteric bacteria in salted fermented fillets of P. jubelini and A. heudelotii was about 1 and 0.5 log CFU/g greater, respectively, than that resulting from the biopreservative effects of the starter.

TABLE 4. Changes in microbial populations, pH, and nisin activity in fish juice during starter culture (L. lactis CWBI B1410) fermentation of Arius heudelotii at 30°C

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>LAB (log CFU/g)</th>
<th>pH</th>
<th>Enteric bacteria (log CFU/g)</th>
<th>LAB (log CFU/g)</th>
<th>pH</th>
<th>Enteric bacteria (log CFU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>7.30 ± 0.40</td>
<td>7.03 ± 0.20</td>
<td>4.12 ± 0.12</td>
<td>7.30 ± 0.40</td>
<td>7.03 ± 0.20</td>
<td>4.12 ± 0.12</td>
</tr>
<tr>
<td>10</td>
<td>9.40 ± 0.22</td>
<td>7.41 ± 0.10</td>
<td>8.30 ± 0.50</td>
<td>10.30 ± 0.51</td>
<td>4.60 ± 0.50</td>
<td>6.40 ± 0.50</td>
</tr>
<tr>
<td>24</td>
<td>9.43 ± 0.13</td>
<td>7.50 ± 0.62</td>
<td>9.30 ± 0.23</td>
<td>10.30 ± 0.72</td>
<td>4.90 ± 0.32</td>
<td>7.40 ± 0.10</td>
</tr>
</tbody>
</table>

*Bold values indicate the correlation between decreasing pH of fish to an acidic value and the reduction of levels of enteric bacteria (potential mean for enhancing guedj safety). Nisin inhibitory activity (evaluated using Staphylococcus epidermis CWBI B1433 as the indicator strain) was detected only in juice from fish supplemented with glucose. Inhibitory levels were 1,280 AU/ml after 10 h and 480 ± 226.3 AU/ml after 24 h.*
culture alone at 30 °C (data not shown). The salted fish fillets contained about 7% (wt/wt) NaCl, which is considerably lower than the 35 to 40% NaCl typically found in traditionally processed gudé. Fish salting is traditionally performed at ambient temperatures by immersing the fresh or naturally fermented product in seawater supplemented with dry salt (17). This procedure negatively impacts the dietetic quality of the product because of the high NaCl levels and the sanitary quality of the product because of possible cross-contamination from seawater, indicating the need for the procedural changes described in this study. Storage at 10 °C enhances the control of psychrotrophic acidopholic and halophilic spoilage bacteria in fish (19). After salting, the fermented fish products must be dried under the sun for 2 to 5 days to extend their shelf life (17).

The use of a starter culture of \textit{L. lactis} subsp. \textit{lactis} strain that produces various antibacterial compounds combined with fermentable carbohydrate supplementation (1%, wt/wt) of fish muscle tissue enhanced the acidification and in situ nisin secretion in the fermented fish, resulting in inhibition of enteric bacteria in product. These data suggest that this novel fish fermentation method combined with salting and drying can be used to enhance the safety of gudé in Senegal.

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**REFERENCES**