Application of culture dependent methods and culture-independent methods (DGGE analysis) to study Lactic acid bacteria ecology of Ivorian fermented fish *Adjuevan*

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The occurrence of lactic acid bacteria flora (LAB) was investigated on Ivorian fermented fish *Adjuevan* products produced with sea fish at different salt concentration (10, 15, 20, 25, 30%) according two traditional methods. LAB biodiversity was investigated using traditional culture-dependent method and culture-independent method (DGGE).

LAB isolates were *Lactobacillus fermentum* 54%, *Leuconostoc lactis subsp lactis* 27%, *Pediococcus pentosaceus* 19% according method 1 and *Pediococcus pentosaceus* 61%, *Lactococcus garviae* 32%, *Streptococcus difficilis* 7% for method 2. The results of culture-independent method using DGGE patterns and sequencing of DNA bands revealed a higher number of lactic acid bacteria species even if the identification of several lactic acid bacteria species were not possible by traditional microbiological procedures. LAB were *Lactobacillus delbrueckii subsp bulgaricus, Lactobacillus helveticus, Leuconostoc lactis, Lactococcus raffinolactis.* Microflora was influence by salt percentage and more by the method of fermentation used. The molecular method DGGE which used three primers Lac1, Lac2GC and Lac3 to study LAB biodiversity directly in the fermented fish matrix, have allowed us to get a more complete picture of the dominant lactic acid bacteria diversity in these fermented product *Adjuevan.*

Keywords: *Adjuevan*; Fermented fish; PCR-DGGE, Ivory Coast.

Introduction

Ivory Coast fermented fish products are mostly produced according two main traditional methods at ambient temperature (28–30°C). Therefore, some differences exist in the production methods and in the names used for the final products for the various area and languages. The fermented fish product *Adjuevan* is typically composed of fish and sea salt and is mainly produced by women in the center and south of Ivory Coast. Fermented fish *Adjuevan* which is a traditional product will retain more attention in the last few years, largely because of consumer appreciation. Like in other fermented products, Lactic acid bacteria (LAB) are found as dominant microorganisms in many fermented products [1–3].

Primary role of LAB is to ferment available carbohydrates and thereby cause a decrease in pH by producing organic acids [4, 5]. These acids do not only contribute to the taste, aroma and texture of the product but also lower product's pH which is one of the key factors to ensure quality and safety [5–8]. Previous researches have already been done with dependent--method of microbiology to analyze Lactic acid bacteria communities of some fermented fish [5, 9, 10]. Previous work on *Adjuevan* focused on Lactic microflora using dependent-method of microbiology showed *Lactobacillus fermentum* as dominant Lactic acid bacteria [11]. Only easily cultivable microorganisms can be detected by classical microbiological methods based on plate counts. Isolation and biochemical identification were criticized because of the need of selective enrichments. So identification of fermented fish *Adjuevan* LAB by traditional phenotypic methods, such as morphological and biochemical characteristics using carbohydrate fermentation patterns, was considered to give relatively poor reproducibility and low taxonomic resolution. Today, culture-independent methods are particularly attractive as they offer a good and rapid strategy for Lactic acid bacteria detection and represent a valid

alternative to classical microbiological analyses. On the other hand, it has been demonstrated that microbiological patterns obtained after DGGE (Denaturant Gradient Gel Electrophoresis) analysis were strictly connected to numerically dominant species [12, 13] and the detection limit of this technique was highly matrix dependent. Cocolin, et al. [14] demonstrated that during wine fermentation, PCR-DGGE did not detect yeast species at <10^3 cells/mL. Hence, accurate identification should combine conventional identification methods, based on microbiological and biochemical features, along with genotypic methods [15]. DGGE method has been applied in recent years to many fermented product like cheese [16] and yogurt [17]. Montet, et al. [18] determined by using DGGE method the fish origin by using 16S rDNA or 26 rDNA fingerprinting of microbial communities However, we believe that this is the first paper that introduces the analysis of Lactic acid bacteria communities in salted and fermented fish using the molecular method PCR-DGGE.

In the present study, we describe the application of culture dependent (traditional methods after direct isolation procedure) and culture-independent (DGGE analysis) methods to study Lactic acid bacteria ecology of fermented fish produced with different percentage of salt. This study was done to show the applicability of the DGGE method to the Lactic acid bacteria communities directly in the matrix of fermented and salted food.

Materials and methods

A total of 60 samples of fresh fish *Galeoides decadactylus* were collected at Abobo-Doume fish marketplace in Abidjan (Ivory Coast), from which 30 were salted and fermented into *Adjuevan* according method 1 and 30 other were fermented according method 2.

Methods of production of fermented fish *Adjuevan*

All these fermentations were done in the laboratories of the university in Abidjan on fish sample collected on the market. A sample consisted of 5 fresh fish (300–500 g) putted in the same basket of fermentation with different concentrations of salt (10%, 15%, 20%, 25%, 30% (w/w)). For method 1, entire fishes were salted, then wrapped in sterile plastic containers and arranged in different coolers. They were left to ferment for 5 days at ambient temperature (30°C) followed by drying in a ventilated dryer (at 30°C with minimal ventilation) for 24 h. For method 2, fishes open in the middle and eviscerated, after been salted, were deposited on a sterile plastic surface then placed in sterile trays and put in the dryer. Fermentation was done simultaneously as well as drying for 10 days at 30°C followed by ventilation for 24 h the last day. Fermentation experiments were done in triplicate. Samples were taken per method at the starting and the end of the fermentation process. They were put in aseptic sterile tubes, stored in a cooler filled with ice and transported to laboratory for analysis.

Microbiological analysis

Lactic acid bacteria isolation

Ten grams of fermented fish "Adjuevan" were aseptically weighed and transferred into sterile 50 mL bottles containing 25 mL Peptone Physiological Saline at 0.8% NaCl (Merck, Germany), 0.1% neutral peptone (Oxoid, Basingstoke, UK). Then, bottles were vortexed for 15 min. Appropriated serial dilutions (10^{-1} to 10^{-6}) were plated in duplicate. Lactic acid bacteria were incubated anaerobically on Petri dish of Man Rogosa and Sharpe Agar (MRS) (Biomerieux, France) at 30°C for 48h. All isolates were purified and primarily confirmed as LAB based on their microscopic and biochemical characterizations by performing the Gram's stain, catalase test, and O–F glucose fermentative test. All LAB isolates were stored at -20°C in 10% glycerol with MRS broth until identification by molecular method PCR-DGGE.

Bacterial total DNA extraction

DNA extraction from bacteria present in samples of fermented fish was based on the methods of Ampe, et al. [12] and Leesing [19] modified and optimised by Le Nguyen, et al. [20]. Samples of 8 g were taken at the level of skin, abdomen and head and were homogenized for 30 min in 16 mL sterile peptone water pH 7.0 (Dickinson, France) with a vortex (Genie 2 SI-A256, USA). Two Eppendorf tubes of 2 mL containing the homogenate were then centrifuged at 10,000g for 10 min. 100 µL of TE Lysis buffer (10 mM Tris–HCl, 1mM EDTA, pH 8.0, Promega France). 100 µL of lysozyme solution (25 mg/mL, Eurobio, France) and 50 µL of proteinase K solution (20 mg/mL, Eurobio, France) were added and the mixture was incubated at 42°C for 20 min after 1 min vortex. Then 50 µL of 20% SDS (Sodium Dodecyl Sulphate, Sigma, France) were added to each tube, then incubated at 42°C for 10 min. 300 µL of MATAB (mixed alkyltrimethyl ammonium bromide, Sigma) were added to each tube, then incubated at 65°C for 10 min. The lysates were then purified twice by repeated extraction with 700 µL of phenol/chloroform/isoamyl alcohol (25/24/1, v/v/v, Carlo Erba, France) and the tubes were vortexed for 1 min and then centrifuged at 10 000 × *g* for 10 min. The aqueous layer was transferred to an Eppendorff vial and the residual phenol was removed by extraction with 600 µL of chloroform/isoamyl alcohol (24/1 v/v) and centrifuged at 10000 × *g* for 10 min. The aqueous phase was collected and DNA was stabilized with 30 µL of sodium acetate (3 M, pH 5), followed by precipitation by adding equal

volume of ice cold isopropanol, and stored at −20°C for 12 h (overnight). After centrifugation at 10 000 × *g* for 10 min, the supernatant was eliminated, DNA pellets were washed with 500 μL of 70% ethanol and tubes were centrifuged at 10 000 × *g* for 10 min. DNA was precipitated with isopropanol, washed with 70% ethanol and then air dried at room temperature. DNA was resuspended in 50 μL ultra pure water and stored at −20°C until analysis.

PCR-Denaturing Gradient Gel Electrophoresis (DGGE) analysis

The variable region of 16S DNA of bacteria flora from fermented fish was amplified using three specific primers Lac1 (5'AGCAGTAGGGAATCTTCCA3'), Lac2GC (5'CGCCCGGGGCGCGCCCCGGGCGGCCCGG GGGCACCGGGGGATT YCACCGCTACACATG3') and Lac3 (5'AGCAGTAGGGAATCTTCGG 3') that were used to amplified the V3 variable region of 16S DNA of several strains of lactic acid bacteria [21]. 40-pb GC-clamp (Sigma, France) was added to the forward primer in order to insure that DNA fragment will remain partial double-stranded and that the region screened is in the lowest melting domain [22]. Each mixture (final volume 50 μL) contained about 100 ng of template DNA, 0.2 μM of each primer, all the deoxyribonucleotide triphosphate (dNTPs) at 200 μM, 1.5 mM MgCl2, 5 μL of 10× of reaction Taq buffer, $MgCl_2$- free (Promega); and 0,5 U of Taq polymerase (Promega, France). The amplification was carried out as follow: an initial denaturation at 94°C for 1 min and 10 touchdown cycles of denaturation at 94°C for 1 min, then annealing at 65°C (with the temperature decreasing 1°C per cycle) for 1 min, and extension at 72°C for 3 min, followed by 20 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 3 min. Aliquots (5μL) of PCR products were analysed first by conventional electrophoresis in 2% (w/v) agarose gel with TAE 1x buffer (40 mM Tris-HCL pH 7.4, 20 mM sodium acetate, 1.0 mM Na_2-EDTA), stained with ethidium bromide (Sigma, France) 25 μg/mL in TAE 1x and quantified by using a standard (DNA mass ladder 100 bp, Promega). PCR products were analysed by Denaturing Gradient Gel Electrophoresis (DGGE) by using a Bio-Rad Dcode TM universal mutation detection system (Bio-Rad, USA), using the procedure first described by Muyzer, et al. [23] and improved by Leesing (2005). Samples containing approximately equal amounts of PCR amplicons were loaded into 8% (w/v) polyacrylamide gels (acrylamide/ *N*,*N*-methylene bisacrylamide, 37/5, Promega, France) in 1× TAE buffer (40 mM Tris–HCl, pH 7.4, 20 mM sodium acetate, 1.0 mM Na_2-EDTA). All electrophoresis were performed at 60°C, using a denaturing gradient in the range 30–60% (100% corresponded to 7 M urea and 40% v/v formamide; Promega, France). The gels were electrophoresed at 20 V for 10 min and then at 80 V for 12 h. After electrophoresis, the gels were stained for 30 min with ethidium bromide and rinsed for 10 min in distilled water and then photographed on a UV transilluminator with the Gel Smart 7.3 system (Clara Vision, Les Ulis, France). For Lactic acids bacteria strain isolates, PCR product after verification on acrylamide gel, were send immediately for sequencing to GATC Biotech (Germany).

Image and statistical analysis

Individual lanes of gel images were straightened and aligned using ImageQuant TL software v. 2003 (Amersham Biosciences, USA). Banding patterns were standardized with two reference patterns included in all gels: *Escherichia coli* DNA and *Lactobacillus plantarum* DNA. This software permitted the identification of the band's relative positions compared with standard patterns. In DGGE analysis, the generated banding pattern is considered as an image of all of the major bacterial species in the populations. An individual discrete band refers to a unique 'sequence type' or phylotype [23, 24], which is treated as a discrete bacterial population. This was confirmed by Kowalchuk and De Boer [25], who showed that co-migrating bands generally corresponded to identical sequences. The DGGE fingerprints were manually scored by the presence and absence of co-migrating bands, independent of intensity. Pairwise community similarities were quantified using the Dice similarity coefficient (*S*D) [26]:

$$SD = 2NC/Na + Nb$$

Where *N*a represents the number of bands detected in sample A, *N*b the number of bands in sample B, and *N*c the number of bands common to both samples. The similarity index was expressed within a range of 0 (completely dissimilar) to 100 (perfect similarity). Significant differences of bacterial communities of fermented fish were determined by factorial correspondence analysis, using the first two variances that described most of the variation in the data set.

Statistical analyses: The results of isolate were statistically evaluated by one way analysis of variance (ANOVA) and Bonferroni test (Dunn) with the software Statistica. Statistical differences with $p < 0.05$ were considered significant.

Purification and sequencing

Strips were then cut from the gel and the amplicons were purified using a Wizard PCR purification kit (Preps DNA Purification System, Promega, France) followed by a new PCR but with primers without GC clamp. The PCR products were then sent to GATC Biotech

(Germany) where they were submitted to a second DNA purification followed by sequencing. Sequences of 16S DNAr were compared using the data bank Site NBCI (National Center for Biotechnology Information databases) with the BLAST program *http://blast.ncbi.nlm.nih.gov/Blast.cgi.* Sequence comparisons of the strains were performed using the software ClustalW2 (software available on EMBL-EBI site) *htt://www.ebi.ac.uk/Tools/clustalw2/index.html.*

Results and discussions

A range of samples were analyzed by DGGE and compared with some selected reference species (Fig. 1). PCR- DGGE patterns of samples of method 1 revealed 3 to 5 bands of Lactic acid bacteria DNA and 2–4 bands in samples of method 2 (Table 1). These results showed the participation of few species of Lactic acid bacteria in the fish fermentation. This could be due to the small amount of carbohydrate (< 0.5%) in the fish [27]. According to method 1 and due to anaerobic fermentation condition, typical LAB mainly corresponded to hetero and homo-fermentative strains belonging to the genus Lactobacillus, Leuconostoc, Pediococcus. Five different dominant species such as *Lactobacillus fermentum, Lactobacillus delbrekii subsp bulgaricus, Lactobacillus helveticus, Leuconostoc lactis subsp lactis and Pediococcus*

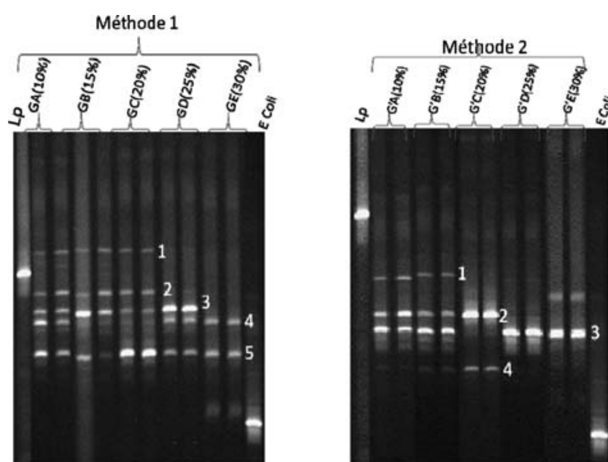

Fig. 1. PCR- DGGE of 16s rDNA profiles of Lactic acid bacteria from five samples.

Table 1. Identification of lactic acid bacteria from samples of *Adjuevan* by PCR-DGGE.

Methods	PositionN°	Strains Closest relative	%Identity	Genbank accession no.
Method1	1	*Lactobacillus fermentum*	99	HQ697614
	2	*Lactobacillus delbrekii subsp bulgaricus*	100	CP00156
	3	*Leuconostoc lactis subsp lactis*	98	JF297358
	4	*Lactobacillus helveticus*	99	HM218568
	5	*Pediococcus pentosaceus*	100	EU121678
Method2	1	*Leuconostoc lactis*	99	EU091451
	2	*Pediococcus pentosaceus*	98	EU121678
	3	*Lactococcus raffinolactis*	99	HM218821
	4	*Lactococcus garviae*	100	EU121675

pentosaceus were founded. Then some bands disappeared when the percentage of salt increased such as *Lactobacillus fermentum* and *Lactobacillus delbrekii subsp bulgaricus* absent in sample with 25 and 30% (w/w) of salt or *Lactobacillus helveticus* absent in sample at 30% (w/w) of salt. However *Leuconostoc lactis subsp lactis; Pediococcus pentosaceus* were present in all the samples. For the second method, *Leuconostoc lactis* was found in samples with 10 and 15% of salt, *Pediococcus pentosaceus* and *Lactococcus garviae* in samples with 10, 15 and 20% of salt and *Lactococcus raffinolactis* in all samples. Species were different in function of the method of fermentation except the single specie *Pediococcus pentosaceus* present in all samples regardless of the method of fermentation used. The influence of salt on the lactic bacteria flora was observed since 20% of salt whatever the methods used. All these results confirmed that the influence of salt on the species of lactic acid bacteria varied with the method of fermentation used for *Adjuevan* production.

The identification of 65 strains of Lactic acid bacteria isolated per production method, by the dependant--method gave just three species for method 1such as *Lactobacillus fermentum* 54%, *Leuconostoc lactis subsp lactis* 27%, *Pediococcus pentosaceus* 19% then *Pediococcus pentosaceus* 61%, *Lactococcus garviae* 32% and *Streptococcus difficilis* 7% for method 2. *Pediococcus pentosaceus* 61% and *Lactobacillus fermentum* 54% were the dominant species according respectively to method 1 and method 2 as in other fermented fish [5, 11]. *Streptococcus difficilis* was not found using the molecular method DGGE. This strain is not a dominant fermentation strain but a contaminant probably present at a low limit load not detectable by PCR- DGGE in the product. The microbiological patterns obtained after DGGE analyses were strictly connected to the numerically dominant species [12].

Cluster analysis permitted to compare the levels of similarities of bacterial communities according to fermentation methods. It showed a gradual similarity between the samples according to the method of fermentation and salt percentage. This similarity decreases with increasing of the percentage of salt. Similarity of 18% was observed between samples of a method 1. In between samples of the second method, it was observed 26% of similarity. But a similarity (37%) was found between the two methods (Fig. 2). All these results showed that the effect of salt on lactic acid bacteria flora depended of method used.

Lactic acid bacteria identification obtained by the culture-independent method PCR-DGGE carried out in this study allowed us to describe the fermented fish *Adjuevan* biodiversity. In particular, *Lactobacillus delbrekii* subsp *bulgaricus*; *Lactobacillus helveticus*, *Leuconostoc lactis*, *Lactococcus raffinolactis* were only found with

Table 2. Identification of lactic acid bacteria isolated from samples of *Adjuevan*.

Methods	Strains Closest relative	Isolate %	%Identity	Genbank accession no.
Method1	*Lactobacillus fermentum*	54%	99	HQ697614
	Leuconostoc lactis subsp lactis	27%	100	JF297358
	Pediococcus pentosaceus	19%	98	EU121678
Method2	*Pediococcus pentosaceus*	61%	99	EU121678
	Lactococcus garviae	32%	98	EU121675
	Streptococcus difficilis	7%	100	GU426062

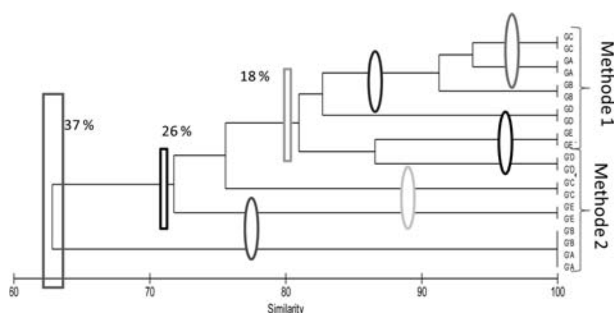

Fig. 2. Cluster analysis of 16s rDNA banding profiles of bacteria from five samples of *Adjuevan*. GA (10%); GB (15%); GC (20%); GD (25%); GE (30%) are five samples of fermented fish prepared with different percentages of salt.

DGGE method. But DGGE could confirm only the dominant Lactic acid bacteria population because of its lack of sensibility.

Therefore, the combined use of culture-dependent and independent approaches was so useful for the description of the biodiversity of fermented fish *Adjuevan*. Indeed, the analysis of 16S rDNA by DGGE using three primers (Lac1, Lac2GC and Lac3) and the available database has allowed us to identify and differentiate Lactic acid bacteria species without their isolation.

Conclusion

We thus conclude that PCR-DGGE was a valid analytical technique to study Lactic acid bacteria biodiversity in salted fermented fish *Adjuevan*. But a more complete picture of the ecological distribution of Lactic acid bacteria in salted fish needed the combination of culture--dependent microbiological methods and culture independent method DGGE. The influence of salt on the lactic flora of *Adjuevan* was mainly dependent of the fermentation method used.

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