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RESEARCH ARTICLE

MOLECULAR CHARACTERIZATION OF LACTIC ACID BACTERIA INVOLVED IN TOGOLESE TRADITIONAL FERMENTED CEREAL FOODS

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ABSTRACT

Togolese population consumes more than twenty varieties of foodstuffs with a fermentation step in the production chain. The microorganisms involved in this production are mainly Lactic Acid Bacteria (LAB). To learn more about these bacteria and to develop strategies to ensure the safety, health and the conservation of these foods, this research was conducted. A total of 50 LAB strains were isolated from cereals fermented dough. These strains were purified by re-isolation on Man Rogosa Sharpe medium (MRS agar) and incubated anaerobically at 37°C for 48 hours followed by monitoring culture purity by the Gram stain and catalase assay. The 16S ribosomal DNA was amplified by PCR and sequenced to confirm the identity of the LAB strains regularly engaged in the manufacture of our traditional cereal products. They are *Lactobacillus, Lactococcus, Pediococcus* and *Streptococcus*. The genus *Lactobacillus* (56%) was dominant followed by *Pediococcus* (24%), *Lactococcus* (16%) and finally *Streptococcus* (4%) compared to the total number of strains.

INTRODUCTION

The lactic acid fermentation is a biological process in which a group of gram positive bacteria, growing under anaerobic conditions and using the carbon sources to produce lactic acid as a major organic acid is involved. In West Africa, the lactic acid fermentation has traditionally been developed for a wide range of raw materials consisting essentially of starch (≥80% of dry matter) (Mugula *et al*., 2003). Therefore, cassava and cereals such as maize, sorghum and millet are crushed and fermented to obtain alcoholic beverages and non-alcoholic products (paste and porridge) that are differently named according to the countries (Odunfa, 1988). A wide range of cereal based fermented foods exist including *ogi* and *mahew* in Benin, *kenkey* in Ghana, *injera* in Ethiopia, *poto-poto* in Congo, *ogi* and *kunu-zaaki* in Nigeria, *uji* and *togwa* in Tanzania, *kisra* in Sudan (Tomkins *et al*., 1988; Hounhouigan *et al*., 1994; Oyewole, 1997; Blandino *et al*., 2003) *éblima, Mawè, Emakumè* and *époma* in Togo. Lactic acid fermentation has many advantages such as: the reduction of the risk of growth of pathogenic microorganisms by acidification of the medium, the degradation of some anti-nutritional factors (phytates, α-galactosides), the development of specific organoleptic properties by synthesis of organic acids and aroma (Nout, 2009).

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Several studies (Kayodé *et al*., 2012; Merrifield *et al*., 2014; Sonsa-Ard *et al*., 2015) have highlighted the beneficial effects of lactic acid bacteria in particular reduction in the duration of the diarrhoea diseases, which is very important when one considers that in developing countries, diarrhoea is a significant cause of morbidity and mortality. Many studies have focused on the characterization of the microorganisms that are commonly used in the processing of these products (Halm *et al*., 1993; Hounhouigan *et al*., 1994; Vieira-Dalodé *et al*., 2007; Sawadogo-Lingani *et al*., 2010; Songré-Ouattara *et al*., 2010; Turpin *et al*., 2011; Ekwem, 2014; Obinna-Echem *et al*., 2014). Such researches have demonstrated that fermentation was natural and involved mixed cultures of lactic acid bacteria (LAB), yeasts and fungi. The LAB species identified included *Lactobacillus fermentum, L. plantarum, L. salivarius, L. delbrueckii, L. amylolyticus, L. reuteri, L. paraplantarum, Lactococcus lactis, Leuconostoc mesenteroides, Pediococcus acidilactici, Ped. pentosaceus, Streptococcus gallolyticus* and *Weissella confuse*. In most contexts of West Africa countries, local foods are consumed in the many different forms, which have the advantage of being known and appreciated by people and whose preparation methods are adapted to local constraints. The Togolese consumes more than twenty varieties of foodstuffs with a fermentation step in the production chain. However, these forms of consumption quality variables are often nutritionally inadequate and for certain types of consumers. It is important to improve traditional process in order to obtain products with added value. To develop strategies to ensure the safety, health and food preservation, our contribution have to improve the

traditional process of making fermented cereal-based foods in Togo by biotechnology and microbiological characterization of biogenic bacteria involved in the local production. Therefore, the present study aimed at documenting the LAB species involved in the Togolese traditionally foods such as *Mawè, Epoma, Emakumè, Kom, Egblin, Akpan* and *Elimawè* and to determine their genetic diversity using PCR, sequencing, and bioinformatics techniques. Through this, the study contributed to realize the first local collection of LAB strains with possible application in the food industry. So, once identified, these bacteria could be used as starter cultures to develop food ingredient with probiotic properties.

MATERIALS AND METHODS

Biological material

Fifty LAB strains (Table 1) were isolated from traditional Togolese fermented foods prepared from maize, sorghum or millet at Microbiology and Quality Control of Foodstuffs Laboratory (University of Lomé). Each isolated colony was checked for purity by streaking on Man Rogosa Sharpe medium and incubated anaerobically at 37°C for 24 to 72 hours followed by monitoring with Gram stain and catalase DNA extractionTotal cellular DNA extraction was performed using QIAamp DNA Mini kit (QIAgen, France) on a 16 hours culture of strains (incubated at 30°C) in 5 ml of MRS broth following the manufacturer's instructions.

Table 1. Origin of the bacteria strains

Foods	Number of isolates
Fermented maize dough "Mawè"	10
Fermented sorghum dough "Epoma"	10
Fermented dough cooked maize "Emakumè"	05
Fermented dough baked and packaged maize "Kom"	05
Fermented dough baked and packaged maize "Egblin"	05
Fermented dough baked and packaged maize "Akpan"	05
Millet fermented dough "Elimawè"	10
Total	50

Table 2. Primers used in PCR reaction

Position of Primers	Sequence of oligonucleotides (5′→3′)
fD1 16S rRNA gene, forward (positions 8 to 27)	AGAGTTTGATCCTGGCTC AG 56
rD1 16S rRNA gene, reverse (positions 1525 to 1542)	TAAGGAGGTGATCCAGCC 56

Amplification of 16S rDNA genes of LAB by PCR (Polymerase Chain Reaction)

DNA amplification was conducted in a PTC-100 thermocycler (MJ ResearchInc.., Watertown MA, USA). Oligonucleotides primers pairs used in this study were obtained from Invitrogen (Invitrogen, Cergy Pontoise, France) and are listed in Table 2. A volume of 50µl was used: 30 µl of Taq DNA polymerase (MP Biomedicals, Strasbourg, France), 4 µl of primer, 8 µl of sterile water and 4 µl of DNA extract. The amplification profile was 94°C for 5 min, 94°C for 1 min, 56 °C for1 min 15 s, 72 °C for 1 min15 s and 72 °C pour 10min, 35 cycles.

Electrophoresis

The presence of PCR products was determined by gel electrophoresis in 1.5% agarose gel (MP Biomedicals, Strasbourg, France) containing ethidium bromide. Electrophoresis in Tris-borate-EDTA was performed at 150 volts for 30 min. The visualization was made by UV illumination Image MasterVDS-CL (Amersham. Biotechnology Pharmacia, Orsay, France). The length of PCR products was evaluated by gel electrophoresis method using a Mass Ruler™ DNA ladder, ready to use 80-10.000 bp (Ferment as Life Science, Vilnius, Lithuania). Sequencing The nucleotide sequence of the amplified 16S rDNA was determined using an ABI370 automated sequencer the TaqDye-Deoxy ™ terminator cycle sequencing method (Genome Express Company, Meylan, France), using the same primers (Table 2) used for PCR reactions.

Strains identification

The possible errors of amplification reaction are avoided by classifying the two DNA failed with a whole of 5 internal primers (not published) of the 16S rDNA. Resulting sequences in total five (05) are assembled into uniquecontig with BioEdit sequence alignment (Hall, 1999). The contig sequences are then submitted to the National Center for Biotechnology Information (NCBI, Bethesda, USA). The computer program CLUSTALW (Thompson et al., 1994) is used for the alignment of the base sequence and the program 2 Local Alignment Search Tool (BLAST) for the representation of sequences and their similarity search in the database GenBank. Phylogeny and molecular evolution analyzes were performed using version 4.0.MEGA (Tamura et al., 2007).

RESULTS

Genotypic characterization of lactic acid bacteria

Genomic DNA of fifty strains were amplified with specific primer pairs for their identification. The running electrophoresis show that strains of lactic acid bacteria involved in the traditional fermented cereal foodsbelong to the genera lactobacillus, lactococcus and Pediococcus. We meet sporadically *Streptococcus*. The results of identification of 50 isolates of lactic acid bacteria at species level are shown in Table 3.

Distribution of lactic acid bacteria species

The results of the distribution of lactic acid bacteria according to the genera are presented in table 4. The number of species identified shows that *Lactobacillus fermentum* is most involved in food fermentation in Togo. It is followed by *Lactococcus graviaeae*, *Pediococcus pentosaceus and Pediococcus acidilacti*. Strain of *Streptococcus vertibularis* was found only in millet fermented dough.

DISCUSSION

The natural fermentation of cereals is unpredictable. Spontaneous fermentation typically results from the competitive activities of different microorganisms whereby strains best adapted and with the highest growth rate will dominate during particular stages of the process. Among the bacteria associated with food fermentation, LAB are of predominant importance.

Table 3. Lactic acid bacteria identified in the Togolese traditional fermented cereal foods

Source	Identification ADNr16S	%I.D
Fermented maize dough "Mawè"	Lactococcus gravieae	100%
	Lactobacillus fermentum	99%
Fermented sorghum dough "Epomawè"	Lactobacillus fermentum	100%
Fermented dough cooked maize "Emakumè"	Pediococcus pentosaceus	100%
	Lactobacillus fermentum	99%
Fermented dough baked and packaged maize"Kom"	Pediococcus acidilacti	99%
	Pediococcus pentosaceus	99%
Fermented dough cooked maize"Egblin"	Lactobacillus fermentum	99%
	Pediococcus acidilacti	99%
Fermented dough baked and packaged maize"Akpan"	Lactobacillus fermentum	100%
Millet fermented dough "Elimawè"	Lactococcus gravieae	99%
	Streptococcus vetibularis	100%
	Lactobacillus fermentum	100%

%I.D: percentage of identification

Table 4. Frequency of lactic acid bacteria involves in fermented food in Togo

Genera	Specie	Number of strains	Percentage
Lactobacillus	*Lactobacillus fermentum*	28	56%
Pediococcus	*Pediococcus pentosaceus*	07	24%
	Pediococcus acidilacti	05	
Lactococcus	*Lactococcus graviaeae*	08	16%
Streptococcus	*Streptococcus vertibularis*	02	4%

The LAB are known to be associated with many natural and man-made environments. It was reported elsewhere (Damelin *et al*., 1995) that samples from plant material showed the greatest diversity of LAB. A total of 50 lactic acid bacteria strains were isolated during the traditional fermentation process and were characterized by molecular methods. Lactic acid bacteria types identified included members of the genera *lactobacillus, lactococcus, Pediococcus* and *Streptococcus*. *Lactobacillus fermentum*, *Lactococcus graviaeae*, *Pediococcus pentosaceus, Pediococcus acidilacti* and *Streptococcus vertibularis* have been isolated from several indigenous fermented foods. The dominant lactic acid bacteria identified in the present work to be responsible for cereals fermentation was *L. fermentum* which accounted more than 50% of the lactic acid bacteria population. This result is in agreement with the work of Halm *et al*. (1993) who found fermentation of whole maize meal in kenkey production to be dominated by a group of obligatory heterofermentative lactobacilli consistent with *L. fermentum* and *Lactobacillis reuteri* in their patterns of carbohydrate fermentation. Hayford and Jespersen (1999) later confirmed the dominant species to be *L. fermentum* using molecular characterization.

It is therefore not surprising that *L. fermentum* has been found in the present work to be responsible for the fermentation of cereals. *L. fermentum* have been reported to dominate in the intermediate and final stages of the fermentation of fufu and to produce the flavor typical of the product (Adegoke and Babaola, 1988). In Benin, Hounhouigan *et al*. (1993) also reported *L. fermentum* to be the dominant lactic acid bacteria responsible for the fermentation of maize into mawè. *Lactobacillus* were predominant, constituting 56% while *Pediococcus* constituted 24%. Nche *et al*. (1994) reported that *Pediococcus* species predominate in the latter stage of corn dough fermentation. The presence of *P. pentosaceus* and *P. acidilactici* which were identified in the lactic acid bacteria composition in fermented cereals dough in the current work have also been reported in kenkey by Halm *et al*. (1993).

Their presence can be linked to production of propionic acid which both Plahar and Leung (1982) and Halm *et al*. (1993) have reported to be one of the main organic acids present in kenkey. These organisms may also ferment lactic acid and do so as a primary end-product of CHO catabolism. Only 16% of the isolated strains were identified as *Lactococcus graviaeae*. *Lactococcus* may contribute to the development of flavor quality attributes of fermented products but their lower percentage in fura could be explained by their complex nutritional requirements. *Lactococcus* have also been shown to exhibit a weak competitive ability during the fermentation of milk (Wood and Holzapfel, 1995).

Olsen *et al*. (1995) showed that about half of all *L. plantarum* and practically all *L. fermentum* isolates inhibited all other Gram positive and Gram negative bacteria and explained the elimination of these organisms during the initial stages of kenkey production. The present work has shown that *L. fermentum* was the predominant microbial species responsible for the fermentation of cereals dough. The *Lactobacillus* constitutes an important group of organisms particularly in the food processing industry. The reasons for the wide spread use of *Lactobacillus* in the preparation of foods and other fermentation processes are (i) production of desired flavour or physical property such as appearance and texture in food, (ii) retardation of spoilage and reduction of contamination through the production of antimicrobial substances, (iii) enhancement of nutritional values of foods e.g. by providing vitamins, amino acids and (iv) beneficial effects on human health (N'tcha *et al*., 2016).

Conclusion

In general, the genotypic analysis showed that a wide variety of lactic acid bacteria's genera, and species are associated with traditional fermented products. These microorganisms spontaneously come from raw materials, the environment, processing equipment's and persons involved in the production.

Further work is required to establish their technological roles and contribution to product quality and safety. It is suggested to investigate bacteriocins which constitute the essential metabolites for optimization bioreactor's production. The development of starter culture from these organisms is important for the potential production of fermented foods on a commercial, small industrial scale, and for the improvement of its acceptability, microbiological stability and hygienic safety.

Conflict of Interest

The authors declare no conflict of interest.

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