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# Study of some Probiotic Properties of Lactic Acid Bacteria Isolated from Local Infant Flours Sold in Côte d'Ivoire

Research Article

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## Abstract

In Côte d'Ivoire, local infant formula does not always meet the standards recommended by the World Health Organization (WHO). It is therefore necessary to define an effective strategy to protect the health of infants.It is in this context that this study was carried out with the objective of determining some probiotic properties of lactic acid bacteria strains isolated from local infant flours with a view to incorporating them into local infant porridges. To do this, 50 strains of lactic acid bacteria were isolated from local infant flours of corn, millet and sorghum.The morphological, physiological and technological characteristics, the antibacterial activity, the resistance to antibiotics of the isolated strains were determined. Their identification was carried out by the study of biochemical characteristics and genotypic characterization by PCR method followed by digestion of PCR products. The results show that all the lactic acid bacteria strains isolated were Gram-positive cocci. 40% of these strains showed antimicrobial activity against all indicator microorganisms tested. All 50 strains tested showed good growth at all temperatures, pH and %NaCl tested.60 and 52% of these strains showed resistance to oxacillin and tircacillin + clavulanic acid respectively. These strains do not show proteolytic or lipolytic activity and do not produce exopoly saccharide. All these strains belong to the genera*Enterococcus*. Lactic acid bacteria isolated from local infant formula could be incorporated into local infant formula to maintain infant health.

**Keywords:** Local Infant Flour; Lactic Acid Bacteria; Antibacterial Activity; Probiotics.

## Introduction

Malnutrition in children is a public health problem worldwide and particularly in developing countries [1]. Malnutrition generally appears during the period corresponding to the introduction of complementary food to breast milk in infants [2]. Indeed, from the age of 6 months, breast milk becomes qualitatively and quantitatively insufficient for the infant whose nutritional needs are increasing [3]. It is therefore necessary to introduce in the diet of the young child, food supplements in liquid or semi-solid form to supplement the intake of breast milk [4]. In many developing countries, the complementary food is often introduced earlier or later than the recommended period and complementary foods are often nutritionally and healthily inadequate [4, 5].

In Sub-Saharan Africa, infant formula sold in pharmacies or supermarkets is generally not used because it is too expensive [6]. Local cereal-based porridges prepared at home or purchased ready-to-eat on the street are therefore the first and main foods given to infants to supplement breast milk.

However, these meals are generally difficult for infants to swallow and digest. Also, these locally produced infant formulas are usually contaminated with pathogens that can cause serious illness in infants [7]. The presence of these pathogens may be due to the environment associated with unhygienic handling during production, transportation, storage and inappropriate conditions of use. The presence of probiotic microorganisms could be an alternative to protect infants from health risks related to the con-

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sumption of infant formula.According to [8], probiotics are defined as live microorganisms that, when administered in adequate amounts, have a beneficial effect on the health of the host. Probiotics such as lactic acid bacteria (LAB) constitute the majority of the human intestinal flora and are present in the nasal, oral and vaginal mucosa, thus helping to protect us from pathogens. They naturally colonize several food matrices. These bacteria are tolerated by humans and animals, hence their GRAS status (Generally Recognized As Safe)[9]. These bacteria produce acidification, aroma, and enzymes in foods that improve digestibility and inhibit pathogenic or spoilage microorganisms.Studies have shown that lactic acid bacteria (LAB) isolated from flours could be used as probiotics to be incorporated into infant porridges to make them more digestible for infants [10, 11]. Considering the importance attributed to LAB in the pharmaceutical and agri-food industries, their valorization is therefore beneficial at the socio-economic, scientific and environmental levels. For all that, it is thus necessary to undertake a study for the characterization of LAB of the Ivorian infant flours because no study was still carried out on these flours in Côte d'Ivoire.

The main of this study was to select strains of lactic acid bacteria (LAB) from local infant flours with probiotic properties.The specific objectives of this study were to: (i) enumerate and isolate LAB found in flour samples collected; (ii) determine some physiological, biochemical and technological characteristics of these lactic bacteria strains; (iii) identify these strains of LAB by the molecular method.

## Materials and Methods

### Collection of Samples

Local infant flours made from corn, millet and sorghum were purchased from the community health centers of Abobo and Cocody and also prepared at the laboratory of the NANGUI ABROGOUA university.One sample corresponds to approximately 250 g of flour. A total of 30 samples (10 corn samples, 10 millet samples and 10 sorghum samples) were studied.

### Enumeration and isolation of Lactic acid bacteriastrains isolated from corn, millet and sorghum local infant flours

The enumeration of lactic acid bacteria (LAB) was performed according to ISO 15214:1998 on MRS agar incubated at 30°C/ 72h in anaerobic conditions. The unit used is the Colony Forming Unit (CFU) per gram.50 LAB strains were isolated from the different samples (20 strains from corn flour; 15 strains from millet flour and 15 strains from sorghum flours), purified by successive replicates and preserved in cryotubes containing 30% glycerol and stored at -20°C.

### Phenotypical and biochemical characterization of lactic acid bacteria strains isolated from corn, millet and sorghum local infant flours

Plates were observed for the colonies with typical LAB morphology. These were isolated and further streaked again on fresh MRS agar at 37°C to check the purity. Colony morphology (form, size, shape, surface, texture, color, elevation, and margin) of the purified LAB isolates was evaluated as per the standard protocols. Phenotypical characterization was performed using Gram staining, endospore staining and motility evaluation. Research of catalase, oxidase, growth at different temperatures (30°C and 45°C), pH (2; 2.5; 4.8; 6.5 and 9.6), NaCl concentration (2%, 4% and 6.5%), thermoresistance test (63.5°C) were determined. The fermentation of sugars (glucose and lactose), the production of hydrogen sulfide and gas, the research of lysine decarboxylase, lysine deaminase and tryptophan deaminase, the use of Simmons citrate as the only carbon source, the production of urease and indole and the research of the fermentative type were determined [12-14].

### Research of antimicrobial activity of LAB strains

The antibacterial activity of LAB strains was tested by the well diffusion method on Mueller Hinton agaron the following microorganisms: Salmonella enteridis ; Staphylococcus aureus; Salmonella typhi; Bacillus subtilis; Klebsiella pneumoniae; Escherichia coli ; candida albicans; Aspergillus fumigatus and Penicillium chrysogenum. The diameter of the inhibition zones around the wells was measured with a graduated ruler [15].

### Antibiotic resistance of lactic acid bacteria strains

Antibiotic susceptibility testing of LAB strains was performed according to the method described by [15]. Antibiotic discs (oxacillin, penicillin, ticarcillin + clavulanic acid, piperacillin, chloramphenicol, erythromycin, lincomycin) were chosen based on their importance in different treatments in human. Inhibition diameters were measured and strains were classified as sensitive (S), intermediate (I) or resistant (R) according to the recommendations of the Antibiogram Committee of the French Society of Microbiology [16]. Strains with intermediate resistance were considered resistant.

### Study of some technological abilities of LAB strains

Proteolytic activity was determined by method of [17] usingPCA agar with 2% skim milk at 37°C/ 48h. Lipolytic activity was evaluated by method of [18] using MRS agar with 1% Tween 80 at 30°C/ 48h. Production of exopolysaccharides was determined by the method [19] using hypersaccharose agar at 37°C/ 48h.

### Molecular identification of LAB strains

It was performed according to the following steps: extraction of genomic DNA, amplification of DNA by PCR and electrophoresis of PCR products on agarose gel. Extraction of genomic DNA from LAB strains was performed according to [20]. For the amplification of the 16S rRNA gene-ITS region, forward EGE 1 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse L1 5'-CAAGGCATCCACCGT-3' primers were used [21].PCR reactions were performed in a total volume of 50 µl containing the Master Mix (deoxyribonucleoside triphosphate (dNTP), MgCl2, Taq polymerase), 10µM of each primer (EGE1 and EGE2), and 2µl of the DNA template. An initial denaturation step of 5 min at 94°C; 35 amplification cycles, each consisting of 30s denaturation at 94°C, 30s annealing at 56°C, and 1 min elongation at 72°C; and finally an extension step of 7 min at 72°C. Enzymatic digestion of PCR products was performed. The restriction enzymes used for the digestion of the amplicons of the 16S rDNA regions of lactic acid bacteria were Hae III and Dde I according to the method of

[22, 23]. Digestion products were separated on 0.7 % agarose gel containing 0.4 µg/mL ethidium bromide solution. After electrophoresis, gels were visualized in a gel documentation system.

### Statistical analysis

The bacteriological analyses of each sample were performed in duplicate. Analysis of variance (ANOVA) was used for statistical processing of the data using Statistica version 7.1 software. In case of significant difference between the studied parameters, the classification of means (homogeneous groups) was performed with Duncan's test. The significance level (α) is 0.05. Statistical differences with a probability value lower than $p<0.05$ are considered significant.

## Results and Discussion

### Lactic acid bacteria load, pH and acidity level of three types of flours

pH, acidity and LAB loads of the flours collected from community health centers of Abobo and Cocody are between 4.8 and 6.7; 2 and 6 meq/100 g, and $5.5x10^5$ and $7.2x10^5$ CFU/g respectively. As for the flours prepared in the laboratory, the pH, acidity and LAB loadsare between 5.32 and 6.32; 9 and 11 meq/100g; and 2.5x106 and $4.7x10^6$ CFU/g respectively (table 1). There are significant differences depending on the sampling site and the type of flour. pH of the flours is slightly acidic. According to [24], flours that have an acidic pH are better preserved against microorganism attacks. Thus, maize, millet, and sorghum flours could be preserved for a long time.The high acidity level and the high LAB load of the flours could be due to the metabolism of the endogenous microbial flora (fungi, molds, yeasts and bacteria) present in the fermentation medium. Indeed, lactic acid bacteria are microorganisms whose metabolism is exclusively fermentative and leads to the production of lactic acid [25].

### Physiological and biochemical characteristics of lactic acid bacteria strains isolated from corn, millet and sorghum local infant flours

Macroscopic observation of LAB isolated from maize, millet and sorghum flours on MRS medium revealed small (1 to 2 mm diameter), circular, bulging and white colonies. Microscopic observation after Gram staining showed that all 50 isolates tested were Gram-positive cocci clustered in chains or clusters. All 50 strains of LAB tested are catalase and oxidase negative. These strains grew well at all temperatures tested (30, 45, and 63.5°C) and were able to grow at pH 4.8, 6.5 and 9.6 while no growth was observed at pH 2 and 2.5. All strains were also able to grow in the presence of 2, 4 and 6.5% NaCl (Table 2).According to the biochemical characteristics studied, the 50 strains of lactic acid bacteria isolated from maize, millet and sorghum flours can be grouped into 4 groups (Table 3). The resistance of lactic bacteria strains to low pH is an important criterion in the search for bacteria with probiotic abilities. This parameter is directly related to the pH of the stomach, which is acidic. Thus, a bacterium that is not resistant to low pH would not be able to survive during the passage through the digestive tract. These results corroborate those of [26] who reported that the majority of lactic acid bacteria possess a mechanism of tolerance in an acidic environment and are able to survive lethal acid concentrations. Indeed, the increase in the specific rate of glucose consumption under acidic conditions would favor a greater energy supply, thus allowing lactic acid bacteria to better resist [27]. According to the general scheme of differentiation of genera belonging to lactic acid bacteria [28], all 50 strains isolated from the different types of infant formula belong to the genus Enterococcus.

**Table 1. LAB load, pH and acidity level of corn, millet and sorghum local infant flours.**

| Types of flours | Source | pH | Acidity (meq/ 100g) | LAB loads (CFU/g) |
|---|---|---|---|---|
| Corn | Abobo | $4.94 \pm 0.08^{b}$ | $4 \pm 1.73^{c}$ | $(6,8\pm0,7) \times 10^{5a}$ |
| | Cocody | $4.8 \pm 0.02^{b}$ | $2.66 \pm 0.57^{b}$ | $(7,2\pm0,5) \times 10^{5a}$ |
| | LUNA | $5.79 \pm 0.01^{a}$ | $9 \pm 1.73^{a}$ | $(4,7\pm0,9) \times 10^{6c}$ |
| Millet | Abobo | $4.94 \pm 0.05^{b}$ | $5 \pm 1^{c}$ | $(5,5\pm0,4) \times 10^{5b}$ |
| | Cocody | $6.7 \pm 0.01^{e}$ | $2.66 \pm 0.77^{b}$ | $(6,0\pm0,4) \times 10^{5a}$ |
| | LUNA | $5.32 \pm 0.03^{c}$ | $9.33 \pm 0.6^{a}$ | $(2,5\pm0,8) \times 10^{6d}$ |
| Sorghum | Abobo | $6.31 \pm 0.04^{d}$ | $3 \pm 0.01^{b}$ | $(6,3\pm0,6) \times 10^{5a}$ |
| | Cocody | $6.61 \pm 0.01^{e}$ | $2 \pm 0.01^{b}$ | $(6,5\pm0,6) \times 10^{5a}$ |
| | LUNA | $6.32\pm 0.04^{d}$ | $9.1 \pm 1.15^{a}$ | $(3,0\pm0,7) \times 106^{cd}$ |

Values with the same letter on a column are not significantly different for $p>0.05$.

**Table 2. Physiological and biochemical characteristics of lactic acid bacteria strains isolated from corn, millet and sorghum local infant flours.**

| Total LAB (50) | CAT | OX | T30 | T45 | T63.5 | pH2 | pH2.5 | pH4.8 | pH6.5 | pH9.6 | $NaCl_2$ | $NaCl_4$ | NaCl6.5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LAB of Corn (20) | - | - | + | + | + | - | - | + | + | + | + | + | + |
| LAB of millet (15) | - | - | + | + | + | - | - | + | + | + | + | + | + |
| LAB of sorghum (20) | - | - | + | + | + | - | - | + | + | + | + | + | + |

**Antimicrobial activity of lactic acid bacteria strains isolated from corn, millet and sorghum local infant flours**

Table 4 shows the frequency of lactic acid bacteria strains with inhibitory activity on microorganisms. 40% of LAB showed antimicrobial activity on all microorganisms tested. All LAB (100%) were active against Salmonella enteridis, Staphylococcus gallinarum and Candida albicans. More than half of LABshowed inhibitory activity against the rest of the microorganisms.The antimicrobial properties of LAB strains could be due to the combined effect of different metabolic factors of LAB such as pH and organic acid production and also hydrogen peroxide (H2O2) accumulation [29] (Strus et al., 2005). Other products such as carbon dioxide, diacyl, bacteriocins and reuterin (intermediate metabolite of fermentation) are involved in the antimicrobial phenomenon of lactic strains (Merzoug, 2010).Many studies have also reported an inhibitory effect of lactic acid bacteria against pathogens such as E. coli and S. aureus (Benmechernene et al., 2013; Chang et al., 2016).

**Antibiotic resistance of lactic acid bacterial strains isolated from corn, millet and sorghum local infant flours**

The percentages of resistance observed for the antibiotics tested range from 6% to 52% (Table 5). LAB strains are more resistant to the antibiotics oxacillin and tircacillin + clavulanic acid. Natural resistance of lactic acid bacteria to a wide range of antibiotics has been observed by Botes et al (2008). The work of Temmerman et al (2003) showed that 68.4% of lactic acid bacteria have resistance to one or more antibiotics.

**Lipolytic and proteolytic activities and production of exopolysaccharides of lactic acid bacteria strains isolated from corn, millet and sorghum local infant flours**

All strains of LAB isolated from the different types of local infant flours show no lipolytic and proteolytic activity and do not produce exopolysaccharides. Theseactivities are important because itscontribute to improve texture, consistency, rheology and flavor of products (Fox, 1989; Ortiz de Apodaka et al., 1993; DurluÖzkaya et al.,2007).

**Species of lactic acid bacteria strains isolated from corn, millet and sorghum local infant flours**

According to the general scheme of differentiation of genera belonging to lactic acid bacteria all 50 strains isolated from the different types of infant formula belong to the genus Enterococcus (Carr et al., 2002)(Figure 1).

Amplification of the 16 S region of the rDNA after agarose gel electrophoresis was positive for all isolates. The amplicons generated by the 50 isolates all have the same size around 1500 bp. This method confirmed that all isolated strains were lactic acid bacteria. Enzymatic hydrolysis was used to obtain the RLFL profile of the amplicons. The amplicons of the strains were digested with the restriction enzymes HaeIII and Dde I. While all amplicons had (1500 pb) the same sizes after amplification this is not

**Table 3. Group of LAB strains isolated from corn, millet and sorghum local infant flours.**

| | Lysine desaminase | Lysine decarboxylase | Simmon'sCitrate | Glucose | Lactose | Gaz | H2S | Urease | Indole | TDA | $CO_2$ | Number of Strains | % |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Gpe A | + | - | - | + | + | - | - | - | - | - | - | 40 | 80 |
| Gpe B | + | - | - | + | + | - | - | + | - | - | - | 7 | 14 |
| Gpe C | - | - | + | + | + | - | - | - | - | - | - | 2 | 4 |
| Gpe D | - | + | - | + | + | - | - | - | - | - | - | 1 | 2 |
| Total LAB | | | | | | | | | | | | 50 | 100 |

**Table 4. Frequency of lactic acid bacteria strains with inhibitory activity on microorganisms.**

| | Klebsiella pneumoniae | Salmonella enteridis | Salmonella typhi | Escherichia coli | Bacillus subtilis | Staphylococcus aureus | Staphylococcus gallinarum | Candida albicans | Aspergillus fumigatus | Penicillium chrysogenum | All strains |
|---|---|---|---|---|---|---|---|---|---|---|---|
| LAB of Corn | 13 (65%) | 20 (100) | 11 (55) | 14 (70) | 16 (80) | 18 (90) | 20 (100) | 20 (100) | 17 (85) | 18 (90) | 6 (30) |
| LAB of millet | 7(46,7) | 15 (100) | 8 (53,3) | 10 (66,66) | 15 (100) | 14 (93,3) | 15 (100) | 15 (100) | 12 (80) | 13 (86,7) | 7 (46,7) |
| LAB of sorghum | 11 (73,3) | 15 (100) | 7 (46,7) | 12 (80) | 11 (73,3) | 15 (100) | 15 (100) | 15 (100) | 14 (93,3) | 15 (100) | 7 (46,7) |
| Total N (%) | 31 (62) | 50 (100) | 26 (52) | 36 (72) | 42 (84) | 47 (94) | 50 (100) | 50 (100) | 43 (86) | 46 (92) | 20 (40) |

**Table 5. Prevalence of lactic acid bacteria strains resistant to the different antibiotics tested.**

| | C | E | OX | P | L | PIP | TCC |
|---|---|---|---|---|---|---|---|
| LAB of Corn | 0 (0) | 1 (5) | 13 (65) | 2 (10) | 7 (35) | 4 (20) | 8 (40) |
| LAB of millet | 0 (0) | 3 (20) | 9 (60) | 4 (26,7) | 2 (13,3) | 4 (26,6) | 9 (60) |
| LAB of sorghum | 3 (20) | 2 (13,3) | 8 (53,3) | 4 (26,7) | 2 (13,3) | 3 (20) | 9 (60) |
| Total N (%) | 3 (6) | 6 (12) | 30 (60) | 10 (20) | 11 (22) | 11 (22) | 26 (52) |

C = Chloramphénicol; E = Erythromycin ; OX = Oxacillin ; P = Pénicillin ; L = Lincomycin ; PIP = Pipéracillin; TCC = Tircacillin + clavulanic acid

**Figure 1. Scheme of differentiation between lactic acid bacteria.**

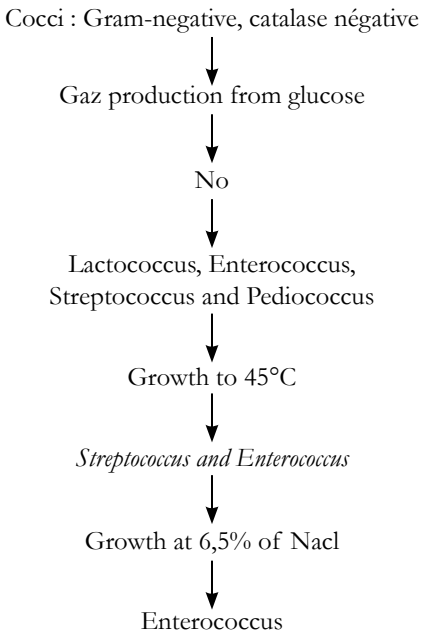


**Table 6. Enzymatic digestion profile and identification of lactic acid bacteria isolated from corn, millet and sorghum local infant flours.**

| Profile | 16S rDNA size | Restriction fragment | | Species |
|---|---|---|---|---|
| | | Hae III | Dde I | |
| I | 1500 | 630+490+350 | 590+ 490+330 | *Enterococcus* sp |

the case for the digested fragments. The profile determined consists of three restriction fragments after digestion with Hae III (630+490+350) and three fragments after digestion with Dde I (590+ 490+330). This profile is characteristic of the species Enterococcus sp (Table 6). However the species could not be identified. Other authors such as Jayaro et al (1992) and Scheidegger et al (2009) determined Enterococcus species after digestion with the restriction enzymes Hae III and Dde I.

## Conclusion

Lactic acid bacteria are probiotics that have a beneficial effect on the health of the consumer.The 50 strains of lactic acid bacteria isolated from the different flours showed good growth at all temperatures and percentages of NaCl tested. These LABs were also able to grow at pH 4.8, 6.5 and 9.6. 40% of these strains had antimicrobial activity on the tested microorganisms. LAB strains are more resistant to the antibiotics oxacillin and tircacillin + clavulanic acid.The general scheme of differentiation of the genera belonging to the lactic acid bacteria and the molecular methods showed that all the strains studied belong to the Enterococcus genera. The first probiotic tests on lactic acid bacteria isolated from different local infant flours (corn, millet and sorghum) are positive and show that these LABs could be used to preserve the health of infants.

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