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Diversity and dynamics of lactic acid bacteria during *Nonnonkoumou* (artisanal curdled milk) production in Daloa, Côte d'Ivoire

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Abstract

Defined as an artisanal curdled milk, *Nonnonkoumou* is a commodity appreciated by the Ivorian population, particularly that of the city of Daloa. In this food, there are a diversity of lactic acid bacteria (LAB) which play a very important role in fermented products. The objective of this work was therefore to identify the LAB isolated during the *Nonnonkoumou* production in order to get to know them better and use them for biotechnological purposes. LAB were isolated from fermenting raw milk used for *Nonnonkoumou* production at different fermentation times (T_0, T_{6H}, T_{12H}, T_{18H} and T_{24H}). These LAB were characterized after Rep-PCR followed by partial sequencing of the 16S rRNA gene. The loads of LAB from *Nonnonkoumou* samples increase of 1.27 ± 0.12 log CFU / mL at 7.11 ± 0.43 log CFU / mL between 0 and 12 hours of fermentation. The maximum value (8.56 ± 0.18 log CFU / mL) is reached at 18 h of fermentation. The results of the Rep-PCR profile of 179 isolates revealed 8 distinct groups of LAB in *Nonnonkoumou*. After amplification and partial sequencing of the 16S rRNA gene of LAB strains, the 179 LAB isolates were classified into 8 species: *Lactococcus lactis subsp cremoris* (0.56%), *Lactococcus Taiwanensis* (16.20%), *Weissella fabalis* (12.29%), *Wessiella confuse* (3.35%), *Lactiplantibacillus* (10.06%), *Weissella Hellenica* (18.43%), *Weissella paramesenteroides* (1.68%) and *Levilactobacillus brevis* (37.43%). Thus, the study of this dynamics has made it possible to observe the natural variations in the composition of the LAB communities.

Keywords: *Nonnonkoumou*; Milk; Lactic acid bacteria; Fermentation; Identification

1. Introduction

Nonnonkoumou is an artisanal curdled milk, consumed in Côte d'Ivoire particulary in Daloa city. Just like some traditional products such as *Rayeb* and *Jben* in North Africa, *Koumis* in Central Asia, *Nonnonkoumou* is a food made from raw cow's milk. Indeed, milk being a very perishable foodstuff, after milking, in the absence of conventional means of conservation such as heat or cold, local producers resort to fermentation. This fermentation is spontaneous and is therefore done in an artisanal way without the use of ferments. Raw cow's milk is heated for ten minutes and then cooled to room temperature. This milk is then bottled and fermented in a warm place wrapped in linens for a few hours (Assohoun *et al.,* 2020) [1]. Thus, the fermentation of this raw milk is initiated by a set of natural microorganisms. These microorganisms are found in the raw material, processing or packaging utensils, the local atmosphere and on the hands of producers as a natural inocula (Assohoun *et al.,* 2012) [2]. The cow's teat can also harbor microorganisms that participate in the spontaneous fermentation of raw cow's milk. The fermentation microorganisms of *Nonnonkoumou* consist mainly of lactic acid bacteria and yeasts and molds. Indeed, lactic acid bacteria are of great interest in the food

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industry (Mounkala, 2002) [3]. They are among the most important groups of microorganisms used in food fermentation (Lamontagne, 2002) [4]. Homofermentative lactic acid bacteria mainly make up the ferments used to transform lactose into lactic acid. They are also responsible, in part, for the taste and texture of fermented dairy products (Ngassam, 2007) [5]. In addition to the acidity they impart to these fermented products, they produce other metabolites such as hydrogen peroxide and also bacteriocins capable of preventing the proliferation of harmful germs. Assohoun-Djeni [1] and his collaborators have indeed shown in 2020 the presence of a high load of lactic acid bacteria in various samples of *Nonnonkoumou* taken from several producers in Daloa city as well as the disappearance of coliforms and Salmonella in all samples of *Nonnonkoumou* analyzed. Furthermore, few studies have been carried out on the lactic acid bacteria involved in the production of *Nonnonkoumou.* Also, the manufacturing processes of this curd product so much appreciated by Ivorians remain unknown and uncontrolled. Thus, given the importance of these lactic acid bacteria and the preponderant role they play in *Nonnonkoumou*, it would be interesting to characterize them in order to get to know them better and use them for biotechnological purposes. The general objective of this work is therefore to identify by biomolecular methods the lactic acid bacteria isolated during the production of *Nonnonkoumou*, in order to contribute to the improvement of traditional methods of milk fermentation.

2. Materials and methods

2.1. Sampling

The samples analyzed during this study consisted of fermenting milk collected taken at different times (T_{0H}, T_{6H}, T_{12H}, T_{18H} and T_{24H}) during fermentation of milk. Indeed, 1 liter of milk is taken from 3 resellers and boiled for about 10 minutes. The milk is then cooled and then distributed in 5 plastic bottles and fermented in a container after packing in a cloth as the producers do. Every 6 hours, a bottle containing the fermenting milk is removed and the contents analyzed. Fermentation is thus carried out for 24 hours. In total, 15 samples were taken and analyzed during the present study at a rate of 3 samples per fermentation time. Each sample consisted of approximately 200 ml of *Nonnonkoumou*.

2.2. Enumeration of lactic Acid Bacteria

Preparation of stock solutions, inoculation of agar plates, and cultivation and quantification of microorganisms were carried out according to Coulin *et al*., 2006 [6]. For all determinations, 10 ml of the sample of *Nonnonkoumou* sample were homogenized in a stomacher with 90 ml of sterile diluent containing 0.85% NaCl and 0.1% peptone (Difco, Becton Dickinson, Sparks, MD, USA). Tenfold serial dilutions of stomacher fluid, ranging from 10^1 to 10^7, were prepared and spread-plated for the determination of microbial counts. So, Enumeration of was carried out using Man Rogosa and Sharpe (MRS) agar (Merck, Darmstadt, Germany), and Enumeration of cocci was carried out using M17 Agar (Scharlau, Sentmenat, Spain). These LAB were incubated in an anaerobic jar at 37 °C for 72 h.

2.3. Colony enumeration

Colony Forming Units per milliliter of sample (CFU/ g) were calculated according to standard NF/ISO 7218: 2007 using the following formula:

$$N = \frac{\sum c}{d(n_1 + 0{,}1n_2)v}$$

ΣC: Sum of characteristic colonies counted on all retained Petri dishes;
n_1: Number of Petri dishes retained at the first dilution;
n_2: Number of Petri dishes retained at the second dilution;
d: Dilution rate corresponding to the first dilution;
V: Inoculated volume (mL);
N: Number of microorganisms (CFU/g).

2.4. Biomolecular identification of LAB (Lactic Acid Bacteria) isolates

2.4.1. Isolates purification

Isolation of LAB was performed from *Nonnonkoumou* samples as follows: about 5 colonies were randomly selected among 20 by picking colonies from plates of highest dilutions showing growth. Collected isolates (266) were purified twice on MRS agar. Each isolate was then characterized for Gram stain, catalase activity by the 3% H_2O_2 method and

cytochrome oxidase production by Bactident Oxidase reagent (Merck). The purified LAB isolates with homogeneous cell morphology were stored in a -80°C deep freezer in MRS broth in 30% (v/v) glycerol for preservation.

2.4.2. DNA Extraction

The DNA of 179 lactic acid bacteria isolates was extracted according to Hassaine *et al.* (2008) [7]. The LAB strains stored at -80°C were multiplied for 24 hours at 30 ◦ C in MRS broth (1.5 mL). After centrifugation (13000 rpm, 10 min), the supernatant was removed and the pellet was resuspended in 200 µL of lysis buffer (2% 100X triton; 1% SDS; 10 mM NaCl; 10 mM tris-HCl, pH 8 and 1 Mm EDTA, ph 8), 0.30 g of glass marbles 0.5 mm in diameter and 200 µL of a chloroform solution. The mixture is homogenized using a vortex and then centrifuged at 13000 rpm for 5 minutes. Subsequently, the supernatant is collected and then added 20 µL of sodium acetate and 600 µL of absolute ethanol. The mixture was then left to precipitate for 1 h at 20 °C and then centrifuged at 12000 rpm for 10 min at room temperature. The DNA pellet thus obtained after removal of the supernatant is washed with 500 µl of 70% ethanol. The DNA is finally obtained after a final centrifugation at 13000 rpm for 2 min, dried in an oven at 37 ° C for 30 minutes. The amount of DNA obtained was quantified by measuring it in an UV spectrum (260nm) and its integrity was visualized by agarose gel electrophoresis to 0.7% w/v, by staining with ethidium bromide and visualizing under UV light.

2.5. DNA amplification

2.5.1. Repetitive sequence-based PCR

LAB was further differentiated by rep-PCR as described by Ouoba *et al.* (2012) [8]. The primer (GTG)5 (5′-GTGGTGGTGGTGGTG-3′) was used to amplify repetitive palindromic sequences of isolates and PCR reactions were carried out as follows: initial denaturation at 94°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 53.1°C for 30 seconds and elongation at 65°C for 8 minutes. PCR were ended with a final extension at 65°C for 16 minutes and amplified products cooled at 4°C. DNA fragments were separated by electrophoresis on 0.8% agarose gel for 3h at 80 V. DNA profiles were examined and bacteria showing the same profile were clustered in the same group. About five (5) isolates from each group were randomly chosen for sequencing.

2.5.2. 16S rRNA gene sequencing

The 16S rDNA gene was amplified by PCR with a thermal cycler (3 prime Base, Techne, UK). DNA fragments were amplified using the primers fD1 (5'-AGAGTTTGATCTGGCTCAG-3 ') and rD1 (5'TAAGGAGGTGATCCAGCC-3'), primers specific for 16S rRNA of LAB (Weisburg *et al.,* 1991). Each PCR tube (50µL) contained a reaction mix of 10 µL 5X PCR buffer for Taq polymerase (Promega), 1.5 mM MgCl2, 200 µM of each deoxynucleotide triphosphate (Promega), 0.4 µM of each primer and 2U of Taq Polymerase (Promega) and 5 µL of template DNA. The termocycle programme was as follows: 95°C for 4 minutes; 30 cycles of 94°C for 30 seconds, 56°C for 30 seconds and 72°C for 1 minute 30 seconds; and a final extension step at 72°C for 10 minutes and the product was cooled at 4°C. After cycling, the PCR products were visualised by electrophoresis on a 0,7 % w/v agarose gel (40 minutes, 75V), by staining with ethidium bromide (0.5µg/mL) and visualising under UV light (DyNA Light UV Transilluminator, LabNet, UV light source wavelength 302 nm). The resulting sequences were assembled into a unique contig with BioEdit sequence alignment software and then submitted to the NCBI database (NCBI, Bethesda, USA, http://www.ncbi.nlm.nih.gov/) for representation of sequence and similarity searches in the GenBank database.

2.5.3. Microbial diversity, dynamics and statistical data analysis

Gels obtained from Rep-PCR were analyzed with UPGMA (Unweighted pair group method with arithmetic mean) using the Dice coefficient with GelJ software version 2.2, to cluster lactic acid bacteria. Sequencing data were analyzed by Chromas Lite 2.0 (Technelysium) software where all raw sequence files were treated by deleting the beginning and the end of raw sequence; if necessary. Chromas Lite 2.0 was also used to blast treated sequence in the nucleotide database of the National Centre for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/) using blastn (Altschul *et al.*, 1990) [9] and obtain identity of isolates. Phylogenetic tree was built with Mega 7 (Version 7.0.26 1993-2019) with the neighbour-joining method, using as references, LAB species 16S rRNA gene sequences downloaded from NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The evolutionary distances were computed using the maximum composite likelihood method (Nzeteu *et al*., 2018) [10]. One-way ANOVA was performed with XLSTAT 2016.02.27444 (Addinsoft 1995-2018) to determine significant differences (at $p \leq 0.05$) between biochemical parameters throughout the tapping process.

3. Results

3.1. Enumeration of lactic acid bacteria

The change in the average lactic acid bacteria (LAB) load of *Nonnonkoumou* samples taken at different fermentation times is shown in **Fig. 1**. Indeed, at T0h (start of fermentation) the lactic acid bacteria load is 1.27 ± 0.12 log CFU / mL. This load increases to reach its maximum value (8.56 ± 0.18 log CFU / mL) at 18 h of fermentation. The lactic acid bacteria load decreases slightly to reach the value of 8.26 ± 0.47 log CFU / mL at 24 h of fermentation.

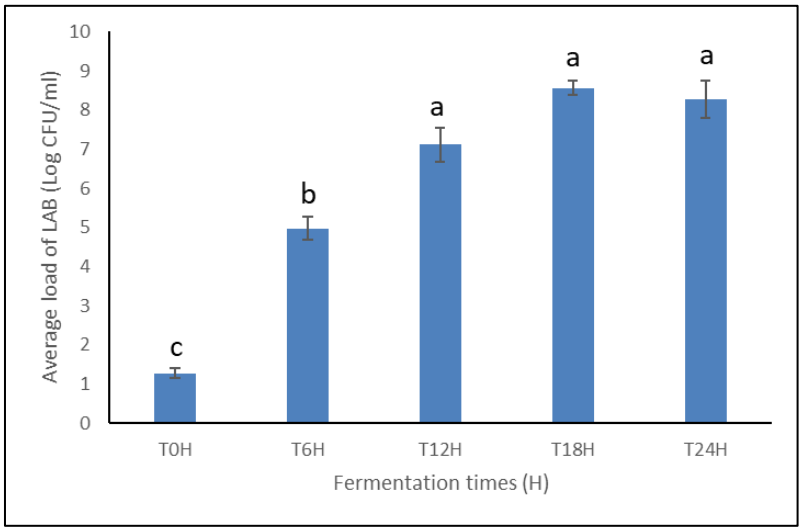

Figure 1 Lactic acid bacteria populations in milk during *Nonnonkoumou* production

3.2. LAB species diversity

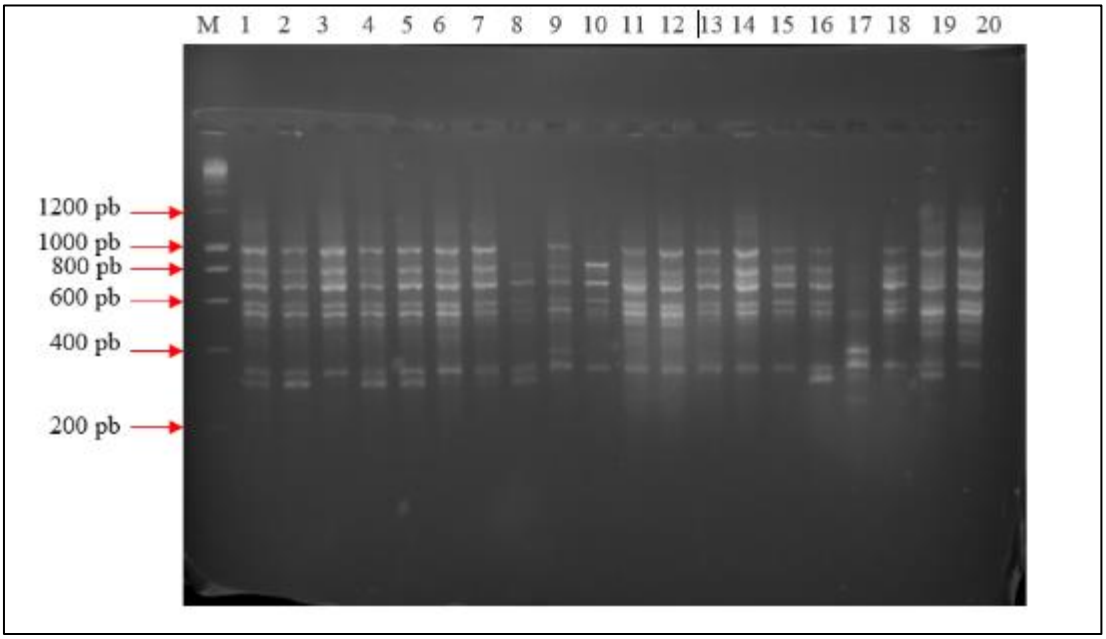

Figure 2 Rep PCR profile of lactic acid bacteria isolated from *Nonnonkoumou*. Lane M represent marker 200 bp Ladder. Lanes 1 to 20 represents the profile of LAB strains isolated from *Nonnonkoumou*

A total number of 179 isolates out of 266, positive for Gram staining, negative for catalase and oxidase were definitively considered as LAB after amplification and sequencing of their 16S rRNA gene using the NCBI GenBank database. The Rep-PCR profiles of the isolates observed on agarose gel consisted of several bands arranged according to their molecular weight (Fig.2). Banding patterns were clustered using the unweight pair-group method with arithmetic

averages (UPGMA) algorithm, with correlation levels expressed as percentage values of Dice correlation coefficient of similarity. The clustering of these 179 isolates allowed obtaining eight (8) unique phylogenetic profiles assigned to eight different groups of LAB involved in *Nonnonkoumou* production. These groups are composed of 1 isolate for group 1 (0.55%), 29 isolates for group 2 (16.20%), 22 isolates for group 3 (12.29%), 6 isolates for group 4 (3.35%), 18 isolates for group 5 (10.06%), 33 isolates for group 6 (8.43%), 3 isolates for group 7 (1.68%) and 67 isolates for group 8 (37, 43%) (Fig. 3, Table I).

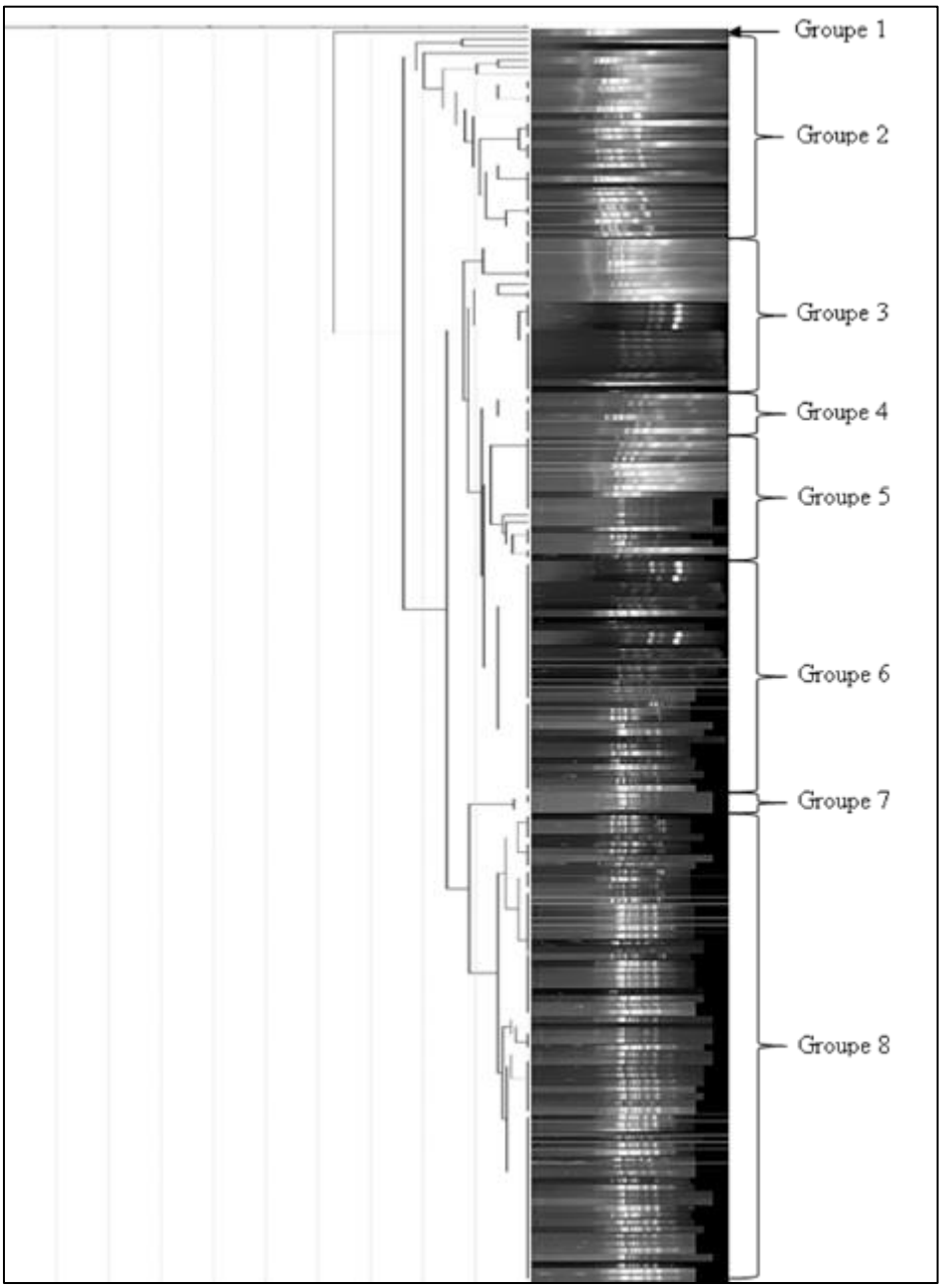

Figure 3 Dendrogram of cluster analysis of (GTG)5-PCR fingerprints from 179 lactic acid bacteria isolated from milk during *Nonnonkoumou* production

Table 1 Proportion of isolates by group

Groups	Isolates number	Proportion (%)
1	1	0.56
2	29	16.20
3	22	12.29
4	6	3.35
5	18	10.06
6	33	18.43
7	3	1.68
8	67	37.43

3.3. Dynamic and identification of LAB species

A total of 8 LAB groups (179 isolates) were detected in *Nonnonkoumou* after the Rep-PCR. So, these LAB isolates were identified after amplification and partial sequencing of their 16S rRNA gene, using the NCBI GenBank database. As seen in **Table 2**, the identification allowed to classsify the 179 LAB isolates into 8 species. *Lactococcus lactis subsp cremoris* (group 1) represented 0.56% of isolates, *Lactococcus Taiwanensis* (group 2), *Weissella fabalis* (group 3), *Wessiella confuse* (group 4), *Lactiplantibacillus*, (group 5), *Weissella Hellenica*, (group 6), *Weissella paramesenteroides* (group 7) and *Levilactobacillus brevis*. (group 8) represented 16.20%, 12.29%, 3.35%, 10.06%, 18.43%, 1.68% and 37.43% of the isolates respectively. Throughout the *Nonnonkoumou* production process, there are a succession of LAB species, marked by the predominance of three main species which are *Weissela fabalis*, *Weissella Hellenica* and *Levilactobacillus brevis*. Indeed, these 3 species were detected at all times of fermentation during *Nonnonkoumou* production. The highest number of isolates enumerated was obtained for most groups at T_{12h}. Only group 8 showed a maximum number of isolates (38) at T_{24h}. This group corresponding to *Levilactobacillus brevis* specie. It should be noted that at T_{0h}, only groups 2, 3, 6, and 8, corresponding to *Lactococcus Taiwanensis*, *Weissella fabalis*, *Weissella Hellenica*, and *Levilactobacillus brevis* species, were detected. Moreover, the *Lactococcus lactis subsp cremoris* specie corresponding to group 1 was found only at T_{12h}. This species was absent at all other fermentation times (T_{0h}, T_{6h}, T_{18h} and T_{24h}) **(Fig. 4).**

Table 2 Biodiversity of LAB identified based on BLAST comparison in GenBank, of the sequencing of the 16S rRNA gene

Groups	N° (%) of identified LAB	Percentage of homology Species	Species
1	1 (0.56)	94.92	*Lactococcus lactis subsp cremoris*
2	29 (16.20)	99,86	*Lactococcus Taiwanensis*
3	22 (12.29%)	99,40	*Weissella fabalis*
4	6 (3.35%)	99.9	*Wessiella confuse*
5	18 (10.06)	99.9	*Lactiplantibacillus*
6	33 (18.43)	100	*Weissella Hellenica*
7	3 (1.68)	99.60	*Weissella paramesenteroides*
8	67 (37.43)	99.9	*Levilactobacillus brevis*

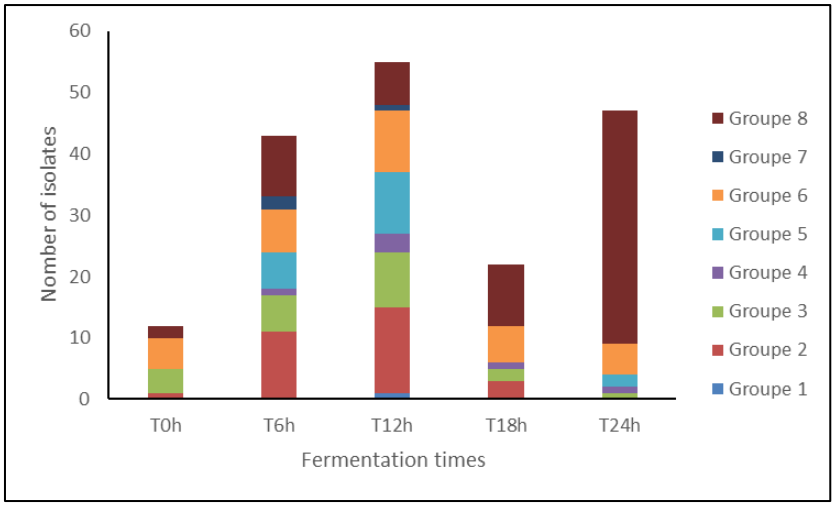

Figure 4 Abundance of lactic acid bacteria species involved in *Nonnonkoumou* production

4. Discussion

Artisanal curdled milk, commonly called *Nonnonkoumou* in the Malinké language, is consumed in Côte d'Ivoire, particularly in Daloa city (Assohoun-Djeni *et al.*, 2020) [2]. Raw milk is likely to contain a diverse and varied microbial flora. This milk undergoes spontaneous lactic fermentation who improves the final quality of dairy products (Ouazzani *et al.,* 2014) [11]. So, the fermentative microflora of raw milk, composed mainly of lactic acid bacteria plays an important role during milk fermentation. These Lactic acid bacteria produce several aromatic compounds, enzymes and other compounds that have a profound effect on the texture and taste of dairy products (Ngassam, 2007) [5]. The results of enumeration obtained have shown an abundance of LAB in artisanal curd milk (*Nonnonkoumou*). As shown in **Fig. 1**, the LAB count in milk taken at different fermentation times during the production of *Nonnonkoumou* increased and exceeded the minimum bacteria populations (106 cfu/mL), required for probiotic foods to possess health claims. LAB are also recognized for their fermentative ability and thus enhancing food safety, improving organoleptic attributes, enriching nutrients and increasing health benefits. Milk fermentation process has been relied on the activity of LAB, which play a crucial role in converting milk as raw material to fermented milk products (Widyastuti *et al*., 2014) [12]. The most important properties of lactic acid bacteria in the fermentation are their ability to acidify milk and generate flavor and texture, converting milk proteins through their proteolytic activities (Mäyrä & Bigret, 2004) [13]. The proteolytic system of lactic acid bacteria is important for the growth of the other microorganisms. These bacteria thus involved in the use of casein and contributes to the development of the organoleptic properties of fermented dairy products (Moulay *et al*., 2013; Yamina *et al.*, 2013) [14, 15]. The abundance and maintenance of lactic acid bacteria in *Nonnonkoumou* would surely also be linked on one hand to their capacity to tolerate high concentrations of organic acids and therefore to low pH, and on the other part, to their ability to use the substrates present in the fermentation medium for their development.

Repetitive sequence based polymerase chain reaction (rep-PCR) technique has been devised for the characterization of bacteria and also widely employed to distinguish species, strains, and serotypes among others. This technique used three specific primers, designated BOX, enterobacterial repetitive intergenic consensus (ERIC), and repetitive extragenic palindromic (REP) designed to match the conserved sequences distributed in diverse bacterial genomes. These primers amplify the genomic regions between repeating sequences and have been shown to be extremely useful in the study of microbial diversity (Rampadarath, 2016) [16].

The results of the Rep-PCR profile of the 179 isolates reveal 8 distinct groups of LAB involved in the manufacture of *Nonnonkoumou*. The diversity of the Rep-PCR profiles produced demonstrates that there is a bacterial diversity involved in the fermentation of milk. Group 8 is the predominant group with a proportion of 37.43% (**Table 1**). These results are similar to those of Aouadhi *et al.* (2014) [17] whose Rep-PCR profiles revealed a remarkable diversity of 9 groups of lactic acid bacteria. Milk in healthy udder cells is thought to be sterile (Tolle, 1980) [18] but thereafter becomes colonized by microorganisms. The presence of these different groups of lactic acid bacteria could be explained by an initial contamination of the milk. These microorganisms could come from a variety of sources, including, milking equipment, air, water, feed, grass, soil and even from the teat of the cow or the milker of the cow (Quigley *et al*., 2013) [19]. Native microorganisms, which depend on raw milk, the environment and technological conditions, are considered to play a major role in determining the typical characteristics of traditional fermented dairy products. These

microorganisms have the capacity to lower the pH of a mixture by producing acid from sugars, which makes it possible to develop the desired organoleptic properties (Corsetti and Settani, 2007) [20].

The diversity of bacterial strains in *Nonnonkoumou*, their development and succession during the fermentation process are influenced by many factors such as artisanal and / or traditional technology and environmental conditions during fermentation. The results of the Rep-PCR and the UPGMA dendrogram established made it possible to highlight the development and succession of LABs isolated from *Nonnonkoumou* during the entire production process (Fig.3). Thus, the study of the dynamics of bacterial communities over time has made it possible to observe the natural variations in the composition of the communities. A total of 8 LAB groups (179 isolates) were detected in *Nonnonkoumou* after the Rep-PCR. So, after amplification and partial sequencing of their 16S rRNA gene, using the NCBI GenBank database, the identification allowed to classsify the 179 LAB isolates into 8 species: *Lactococcus lactis subsp cremoris* (group 1) represented 0.56% of isolates, *Lactococcus Taiwanensis* (group 2), *Weissella fabalis* (group 3), *Wessiella confuse* (group 4), *Lactiplantibacillus*, (group 5), *Weissella Hellenica*, (group 6), *Weissella paramesenteroides* (group 7) and *Levilactobacillus brevis* (group 8) represented 16.20%, 12.29%, 3.35%, 10.06%, 18.43%, 1.68% and 37.43% of the isolates respectively (Table II). *Weissella fabalis*, *Weissella Hellenica* and *Levilactobacillus brevis* species were detected at all sampling times, reflecting a strong implication of these isolates in the production of fermented milk (Fig. 4). These species could promote the acidification of milk and generate flavor and texture, by converting milk proteins through their proteolytic activities (Widyastuti *et al.,* 2014) [12]. Above T12h the majority of groups showed a declining number of isolates. This result suggests that from T12h, a limiting factor appears which is at the origin of the decline in certain bacterial populations. Indeed, according to Groenenboom *et al.* (2019) [21], during fermentation there is bacterial selection as nutrient levels decrease from a high level while the environment becomes harsher, due to a drop in pH. Only group 8 corresponding to *Levilactobacillus brevis* species showed a maximum number of isolates of 38 at T24h suggesting that this group is most resistant to the environmental conditions obtained during the 24 hours of fermentation and is even favored by these conditions (Fig.4).

5. Conclusion

The objective of this work was therefore to identify the lactic acid bacteria isolated during the *Nonnonkoumou* production by using biomolecular methods. Analysis of fermenting milk samples collected during this production showed that this curdmilk contains a high load of lactic acid bacteria, with a maximum value of 8.56 ± 0.18 log CFU/mL at 18 hours of fermentation. The Rep-PCR technique used to amplify the 266 lactic acid bacteria, 179 of which tested positive by PCR, revealed a diversity of lactic acid bacteria. After amplification and partial sequencing of the 16S rRNA gene of LAB strains, the identification allowed to classsify the 179 LAB isolates into 8 species. *Lactococcus lactis subsp cremoris*, *Lactococcus Taiwanensis*, *Weissella fabalis*, *Wessiella confuse*, *Lactiplantibacillus*, *Weissella Hellenica*, *Weissella paramesenteroides* and *Levilactobacillus brevis*. The diversity of the Rep-PCR profiles produced demonstrates the diversity of lactic acid bacteria involved in the *Nonnonkoumou* production. *Weissella fabalis*, *Weissella Hellenica* and *Levilactobacillus brevis* species were detected at all sampling times, reflecting a strong implication of these isolates in the production of fermented milk.

Compliance with ethical standards

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Disclosure of conflict of interest

No, there is no conflict of interest

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