Original ResearchArticle

BIOMOLECULAR CHARACTERIZATION OF LACTIC ACID BACTERIA (LACTOBACILLI AND COCCI) ISOLATED FROM NONNONKOUMOU, A ARTISANAL MILK CURD IN DALOA, CÔTE D'IVOIRE

Abstract

Nonnonkoumou is an artisanal sour milk consumed in Côte d'Ivoire, particularly in the town of Daloa. Its production is carried out under uncontrolled conditions and uses a variety of lactic acid bacteria. The main objective of this study was to characterize the cocci and lactobacilli present in the *nonnonkoumou*. The cocci had the highest loads going from 8.31 ± 0.14 log CFU / mL to 6.76 ± 0.01 log CFU / mL compared to the lactobacilli which had loads between 6.12 ± 0.30 log CFU / mL and 4.35 ± 0.42 log CFU / ml.This preponderance of cocci in all the *nonnonkoumou* samples taken in comparison to bacilli have been shown through the cocci / bacilli ratio which varied from 1.9 to 1.22.PCR followed by Amplified Ribosomal DNA Restriction Analysis (ARDRA) after enzymatic digestion with *Hae* III and *Hinf*Ihave revealed 4 *Hinf* I profiles and 3 *Hae* IIIfor lactobacilli and 2 *Hae* III profiles and 4 *Hinf*I profiles for cocci. This technique has revealed 4 species of lactobacilli isolated from *nonnonkoumou* which are *Lactobacillus reuteri*, *Lactobacillus paracasei*, *Lactobacillus plantarum* and *Fructobacillusdurionis* and 6 groups of cocci corresponding to 6 species. The species identified during this study made it possible to understand their technological role and their contribution to the health quality of nonnonkoumou.

Keywords: Milk, *nonnonkoumou*, fermentation, lactic acid bacteria, PCR-ARDRA

1- Introduction

Cow's milk plays an essential role in human food and remains the most consumed by the rural community and the other big cities, whereas it is very little available on the Ivorian market. So, national production of artisanal milk is low and covers only 17% of overall milk consumption in Côte d'Ivoire (Mirah-ddp, 2012). In addition, to improve milk production, the governmenthas installed small dairy farms, mainly in peri-urban areas, through development projects including the South Dairy Project. Milk represents a biological environment which is highly alterable microbially due to its high water content, its near-neutral pH and its richness

in biodegradable components (lactose, proteins and lipids). When collected under the right conditions, raw milk contains few germs (10^3 germs per mL). These are saprophytic germs and among them are lactic streptococci (*Lactococcus*) and lactobacilli. During milking and storage, milk can be contaminated by a varied flora consisting essentially of lactic acid bacteria belonging to the following genera: *Streptococcus*, *Lactococcus*, *Enterococcus*, *Leuconostocs*and *Lactobacillus* (Bekhouche, 2006).

In addition, since milk is a very perishable commodity, after milking, in the absence of conventional means of preservation such as heat or cold, local producers only resort to fermentation. The present fermentation of the artisanal milk is a spontaneous fermentation initiated by natural microorganisms that are found on raw materials, on the processing utensils/equipments, on the hands of producers and from the local atmosphere as natural starters (Jespersen *et al.*, 1994). Lactic ferments, containing one or more pure cultures in defined proportions of different lactic acid bacteria, are widely used in the food industry (Holzapfel, 2002). Homofermentative lactic bacteria mainly make up the ferments used to transform lactose into lactic acid. Lactic acid bacteria contribute, through their varied metabolism and enzymatic activities, to the production of volatile compounds which participate in the development of the aroma, flavor and texture of dairy products (Sansanwal, *et al.*, 2017)It has been recognized that LAB are capable of producing inhibitory substances such as organic acid, hydrogen peroxide, bacteriocin and diacetyl capable of preventing the proliferation of harmful germs (Mishra*et al.*, 1996). The production of CO2 by lactobacilli reduces the redox potential and inhibits aerobic germs such as mold (Desmazeaud, 1992). These lactic acid bacteria are thus at the origin of the manufacture of several traditional fermented milks such as *Kefir* in Eastern Europe, *Koumis* in Central Asia and also *Nonnonkoumou*in Côte d'Ivoire.Dairy products are prominent as natural healthy products that contain the most crucial elements of the balanced diet. In additions to nutritional benefits, milk plays a significant role in the control of chronic diseases, example blood pressure was being 'treated' with dairy products (Mohammed *et al.*, 2017).

*Nonnonkoumou*is an artisanal curd, consumed in Côte d'Ivoire and especially in the haut Sassandra region, particularly in Daloa. Its production is carried out under uncontrolled conditions (Assohoun-Djeni*et al*., 2020). The result is a heterogeneous finished product, with characteristics that vary from one producer to another. It is well known that the typicality of traditional dairy products is linked mainly to the microbes originating from the milk (Berthier *et al.*, 2001). The biodiversity of these microorganisms could therefore be considered as a

fundamental factor for the features and quality of these artisanal products (Morandi *et al.*, 2011).

However, to our knowledge, very few studies have been carried out on the aspects of making *nonnonkoumou*. In order to define and develop starter cultures for controlled fermentation during the production of *nonnonkoumou*, with greater stability in the quality and microbiological safety of this product, it is necessary that the lactic flora involved, be characterized. This characterization will then make it possible to study their technological role and their contribution to the sanitary quality of the product studied.Thus, this work is part of a contribution to the promotion of *nonnonkoumou*and the acquisition of scientific data on this product.

The objective of this work was to identify by biomolecular methods, the lactic acid bacteria (lactobacilli and cocci) isolated from *nonnonkoumou*.This identification will then make it possible to study their technological role and their contribution to the sanitary quality of the product studied.

2- Materials and methods

2-1- Sampling

The samples consisted mainly of fermented milk commonly called *nonnonkoumou*. The samples analyzed during this study were taken in 4 sites (Orly mosque, grandemosquée, Lobia ceinture1 and Lobia ceinture 2)from 4*nonnonkoumou*producers inDaloa town. It should be noted that the 4*nonnonkoumou*producers were chosen according to their availability and especially for their willingness to participate in this study. A total of 12 samples were taken and analyzed during the present study on the basis of 3 samples per producer. Each sample consisted of approximately 200 mL of *nonnonkoumou*. These sample were collected in sterile containers and transported immediately in an icebox directly to the laboratory for analyses.

2-2- Enumeration of lactobacilli and cocci

Preparation of stock solutions, inoculation of agar plates, cultivation and quantification of microorganisms were carried out according to Coulin*et al*., 2006. For all determinations, 10 ml of the sample of *nonnonkoumou* 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 *Lactobacillus* 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- Cocci / bacillus ratio

A count of cocci and bacilli was carried out according to Prescott *et al.*, 2003. Using a Pasteur pipette, a smear was made on a slide with a drop of a stock solution obtained by diluting each of the 12*nonnonkoumou*samples to the 10th. After drying, the smear was fixed by passing the slide over the flame of the Bunsen burner. The coloring was then carried out by pouring on the smear, a few drops of gentian violet for one minute. A water rinse is then carried out. The microscopic observation of the colored smear was made after adding a drop of immersion oil to the × 100 objective. A ratio was subsequently calculated by making the ratio of cocci to bacilli.

2-4- Bio molecular 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 (100) were purified twice on MRS agar for lactobacilli and M17 agar for cocci. 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 or nutritious broth in 30% (v/v) glycerol for preservation

2-4-2- DNA Extraction

The DNA of 30 cocci and 34 lactobacilli isolates was extracted accordingto Hassaine *et al.* (2008). The LAB strains stored at -80°C were multiplied for 24 hours at 30 ◦C in MRS broth (1.5 mL) for Lactobacilli and in nutritious broth (1.5 mL) for cocci.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 marbles0.5 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.

2-4-3- DNA amplification

The 16S rDNA gene was amplified by PCR with a thermal cycler (3 prime Base, Techne, UK). DNA fragments were amplified using the primersfD1 (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 10µL 5X PCR buff er for Taq polymerase (Promega), 1.5mM MgCl2, 200µM of each deoxynucleotide triphosphate (Promega), 0.4µM of each primer and 2U ofTaq Polymerase (Promega) and 5µL of template DNA. The termocyclerprogramm was as follows: 95◦C for 4 min; 30 cycles of 94°C for 30s, 56°C for 30sand 72°C for 1 m 30s; and a final extension step at 72°C for 10 min.After cycling, the PCR products were visualised by electrophoresis on a 0.7 % w/v agarose gel (40 min, 75V), by staining withethidium bromide (0.5µg/mL) and visualizing under UV light (DyNA Light UV Transilluminator, LabNet, UV light source wavelength 302nm).

2-4-4- ARDRA (Amplified Ribosomal DNA Restriction Analysis)

For the molecular characterization of the cocci and lactobacilli isolates, amplified ribosomal DNA restriction analysis based on 16S region of rDNA was used. The result of 16S rRNA gene amplification were separated by using two restriction enzymes: *Haemophilusaegyptius (Hae* III*)*and *Haemophilus Influenzae (Hinf* I) (Promega) (Attchelouwa*et al.,* 2018).In order to achieve complete digestion, restriction mixes (20µL of final volume) were carried out for 4 h

at 37°C. Each reaction tube contained 2µL of 10X incubationbuffer,0.2µLofbovineserumalbumin,6Uofthe respective restriction enzyme, 2.5µL of bidistilled water and 15µL of PCR product. The resulting digestion products were visualized under UV-light (LabNetTransilluminator, UV light source wavelength 302nm), after agarose gel electrophoresis 2% w/v (90 min, 75V) by staining with ethidium bromide (0.5µg/mL). Restriction patterns identical to the sequenced strains led to the identification of the corresponding species (Kim & Chun, 2005).

2-5- Statistical analysis

All trials were repeated four times. The different sample treatments were compared by performing one-way analysis of variance on the replicates at a 95% level of significance using the statistica (99th Ed, Alabama, USA) statistical program. Unless otherwise stated, significant results refer to $P < 0.05$. This software was also used to calculate mean values and standard deviations of the trials.

3- Results

3-1- Enumeration oflactobacilli

The *nonnonkoumou*samples from Lobia ceinture 1 site have the highest lactobacilli loads (6.12 ± 0.30 log CFU / mL).The samples taken from Orly mosqué and Lobia ceinture 2 sites have substantially equal loads of 5.07 ± 0.20 log CFU / mL and 5.00 ± 0.19 log CFU / mL, respectively. The lowest average lactobacillus loads (4.35 ± 0.42 log CFU / mL) were observed in the *nonnonkoumou*samples taken at the Grande mosquée site (**Figure 1**). The lactobacilli load of *nonnonkoumou* taken from the 4 sites in the Daloacity are significantly different ($P < 0.05$).

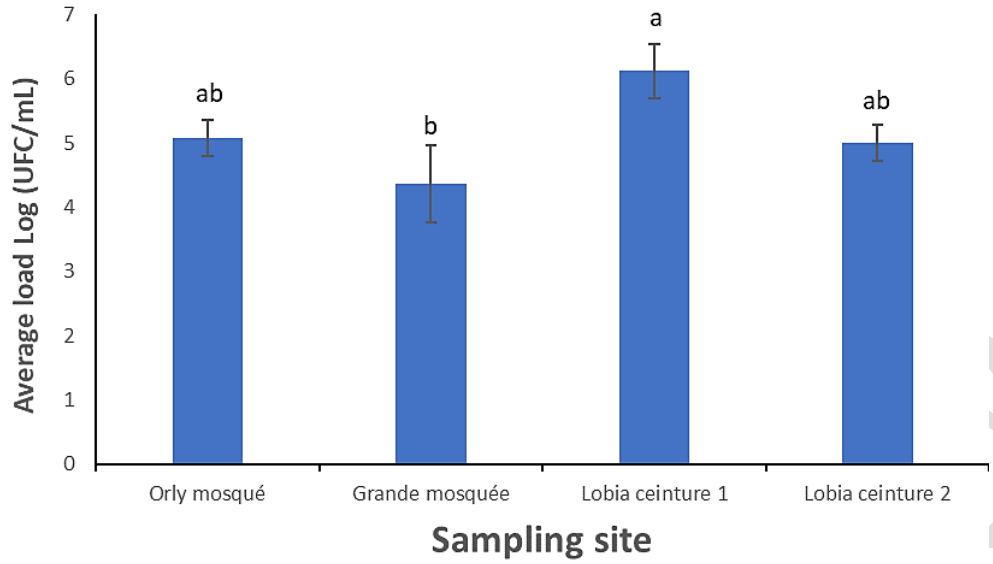

Figure 1 :Lactobacilli populations in *nonnonkoumou*samples

Values at each time point are the means of our replicates ± SD (error bars). LAB For the same type of germ, histograms bearing the same alphabetical letter are not statistically different (P> 0.05) (Tukey, HSD).

3-2- Enumeration of cocci

The cocci load of *nonnonkoumou* taken from the 4 sites in the Daloacity are significantly different (P < 0.05). The *nonnonkoumou*samples from Orly mosqué site have the highest cocci loads (8.31 ± 0.14 log CFU / mL).The samples taken from Grande mosquée and Lobia ceinture 2 sites have substantially equal loads of 7.97 ± 0.26 log CFU / mL and 7.07 ± 0.30 log CFU / mL, respectively. The lowest average lactobacillus loads (6.76 ± 0.01 log CFU / mL) were observed in the *nonnonkoumou*samples taken at the lobia ceinture 1 (**Figure 2**).

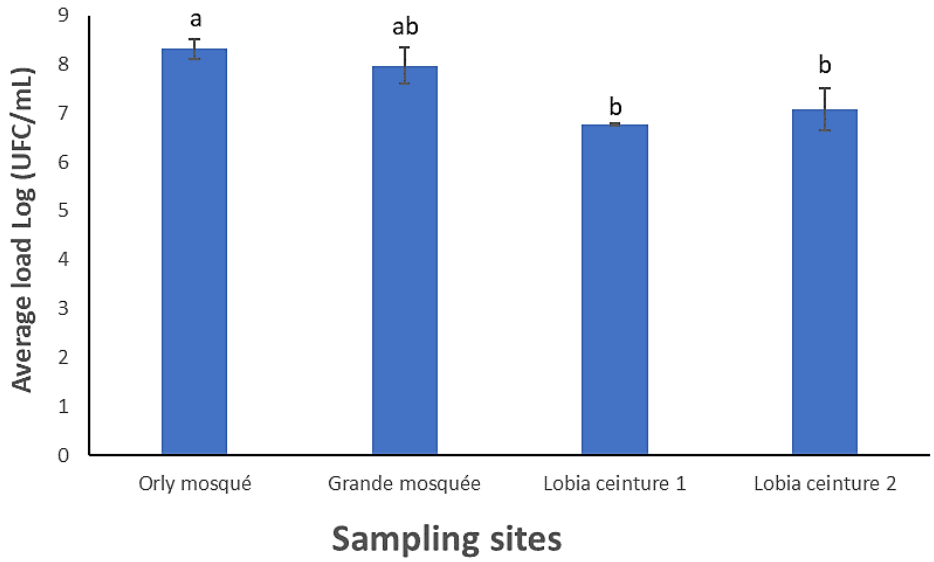

Figure2 :Cocci populations in *nonnonkoumou*samples

Values at each time point are the means of our replicates ± SD (error bars). LAB For the same type of germ, histograms bearing the same alphabetical letter are not statistically different (P> 0.05) (Tukey, HSD).

3-3- Cocci / bacillus ratio

The average loads of cocci are higher than those of bacilli as evidenced by the cocci/bacilli ratio**(Table I**). These ratios vary between 1.22, obtained from the samples taken from the Grande mosquée site and 4 for the samples from the Lobia ceinture 2 site. The samples from the Orly mosqué and Lobia ceinture 1 sites have respective cocci / bacilli ratios of 1.9 and 1.5.

Table I:Cocci / bacillus ratio

Sampling Site	**Cocci / bacillus ratio**
Orly mosqué	1.9
Lobia ceinture 1	1.5
Lobia ceinture 2	4
Grande mosquée	1.22

3-4- Identification of LAB isolates by ARDRA

An about 1.5-kb portion of the 16S rRNA gene from 34 cultures of lactobacilli and 30 cultures of cocci was amplified using the primers fD1 and rD1. The purified PCR products were then digested with the*Hae*III and *Hinf* I restriction enzyme.Following digestion, different banding patterns were obtained for each of the restriction enzymes tested.

3-3-1- Lactobacilli identification

In total 34 lactobacilli isolates were digested with *Hinf*I. Six (6) of Lactobacilli isolates were determined to be *Lactobacillus paracasei*, 4 isolates were determined to be *fructobacillusdurionis*and 1 is determined to be *Lactobacillus plantarum*. The restriction fragment patterns of 23 isolates were the same and have good correlation with the pattern of *Lactobacillus reuteri* reference strain (**Figure 3)**. The molecule weight varies from 500 bp - 1400 bp for these different profiles (**table II**).

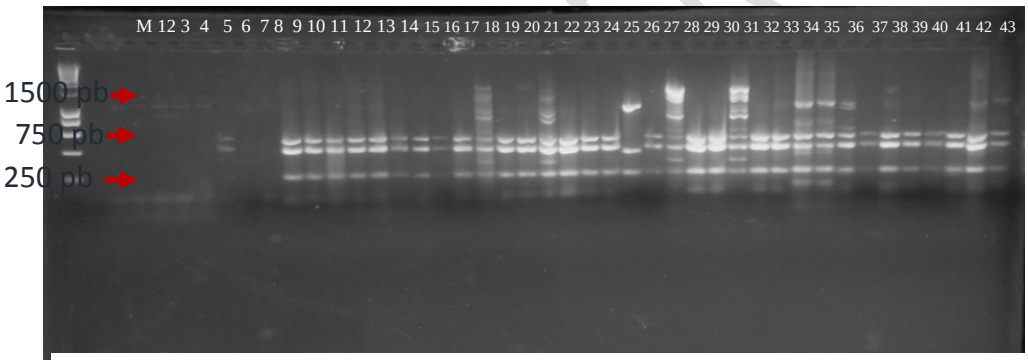

Figure 3: ARDRA patterns of lactobacilli isolated from *nonnonkoumou* after digestion with *Hinf I*. Lanes 1-9 represents the profile of reference strains. Lane 10-18, 20, 21, 23-25, 27, 29, 30, 32, 33, 37, 39-41 correspond to profile 1. Lanes 34, 35, 36, 42, 43, correspond to profile 2. Lanes 19,22, 28, 31 correspond to profile 3. Lane 26correspond to profile 4. Lane M representmarker 250 bp Ladder.

Table II: Lactobacilli species isolated from *nonnonkoumou*after enzymatic digestion by *Hinf*I

Profile	Restriction fragment (bp)	Species
	Hinf I	
I	750+500	*Lactobacillus reuteri*
II	500+750+900	*Lactobacillus paracasei*
III	500+900	*Lactobacillus plantarum*
IV	500+750+800+900+1300+1400	*Fructobacillusdurionis*

Hae III*:* Restrictive enzyme produced by*Haemophilusaegyptius*

*Hinf*I:Restrictive enzyme produced by*Haemophilus Influenzae*

Furthermore, the restriction of the amplified fragment of the 16S rDNA gene with *Hae*III generated three diff erent profiles. The first profile consists of 2 DNA bandwhich is typical of *Lactobacillus reuteri*and the second consists of 6 DNA bandwhich is typical of*Fructobacillusdurionis*.As for profile 3, it could not be digested with the *Hae* III enzyme and therefore retained its size of 1500 bp (**Figure 4)**. The molecule weight for the first 2 other profiles varies from 500 bp - 1400 bp (**table III**).

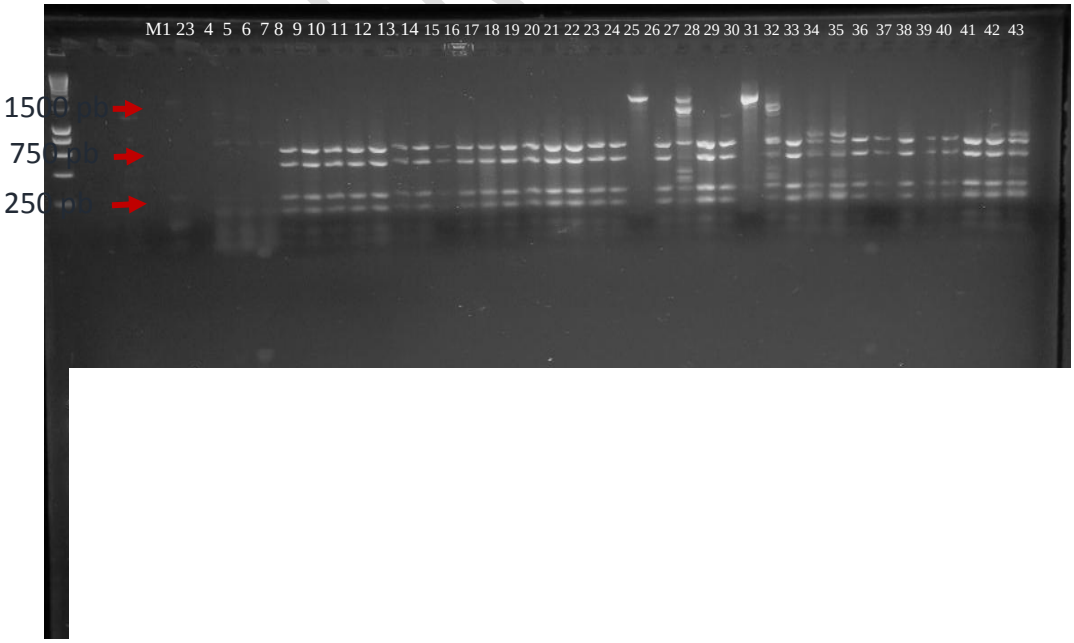

Table III: Lactobacilli species isolated from *nonnonkoumou*after enzymatic digestion by*Hae* III

Profile	Restriction fragment (bp)	Species
	Hae III	
I	750+500	*Lactobacillus reuteri*
II	500+750+800+900+1300+1400	*Fructobacillusdurionis*
III	1500	nd

Hae III*:* Restrictive enzyme produced by*Haemophilusaegyptius*

*Hinf*I:Restrictive enzyme produced by*Haemophilus Influenzae*

nd: non identified

3-3-3- Cocci identification

The restriction of the amplified fragment of the 16S rDNA gene with *Hae* III generated two diff erent profiles. The first profile consists of 2 DNA band and the second consists of 3 DNA band(**Figure 5)**. The molecule weight for the first 2 other profiles varies from 250 bp - 600 bp (**table IV**).

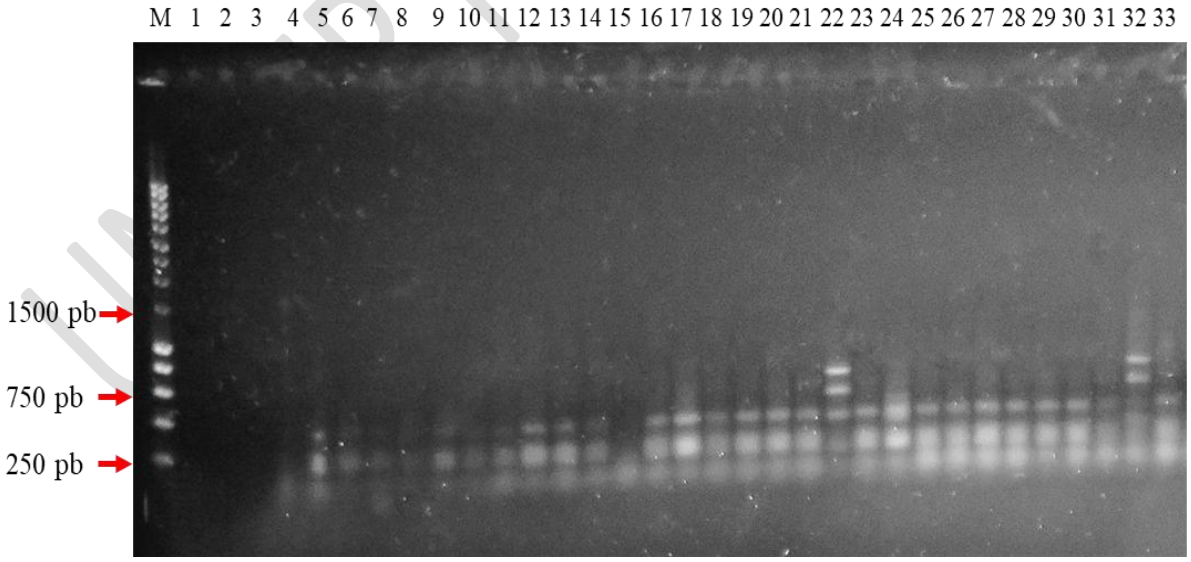

Fig 5: ARDRA patterns of cocci isolated from *nonnonkoumou* after digestion with *Hae* III. Lane M representmarker 250 bp Ladder.Lanes 4-21, 23-31, 33 correspond to profile 1. Lanes 22, 32 correspond to profile 2.

Furthermore, the restriction of the amplified fragment of the 16S rDNA gene with *Hinf*I generated four diff erent profiles. The first and the second profile consists of 2 DNA band. The third consists of 1 DNA band. As for profile 4, it could not be digested with the *Hinf*I enzyme and therefore retained its size of 1500 bp (**Figure 6)**. The molecule weight for the first 3 other profiles varies from 450 bp - 1300 bp (**table IV**).

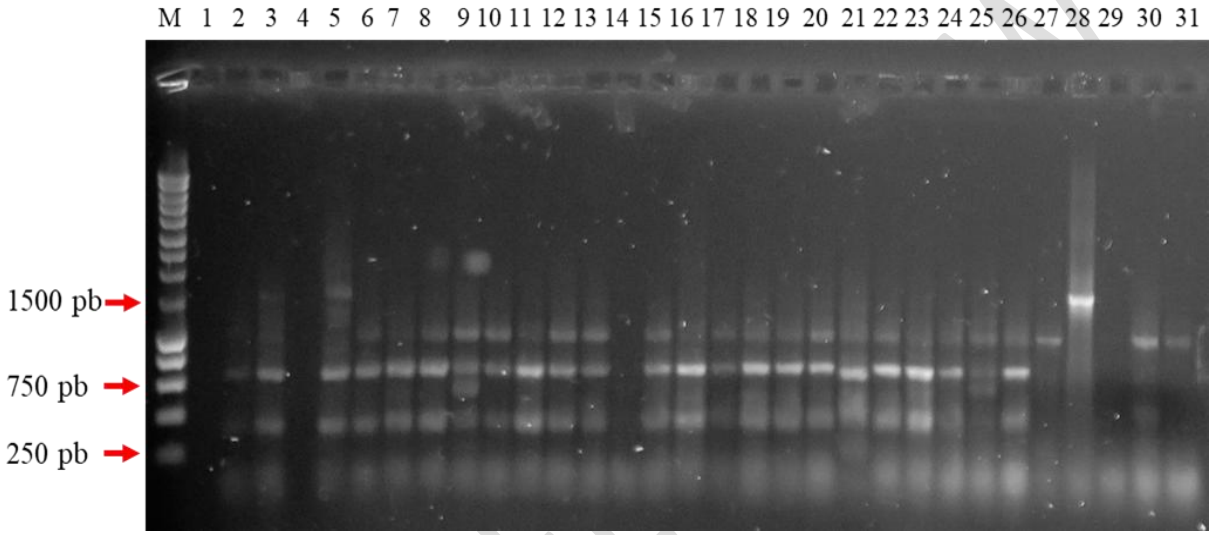

Fig 6: ARDRA patterns of cocci isolated from *nonnonkoumou* after digestion with *Hinf*I.
Lane M representmarker 250 bp Ladder.Lanes 2, 3, 6-8, 10-14 correspond to profile 1. Lanes 9, 25 correspond to profile 2. Lanes 27, 30, 31 correspond to profile 3. Lane 28 correspond to profile 4.

Based on the restriction fragment sizes as presented in **Table IV**, six groups of lactic shells were defined. Group 1 is the majority group with a prevalence of 76.67%, followed by groups 2 and 3 with a prevalence of 6.67%. The other three groups are in the minority with an identical prevalence of 3.33%.

Table IV: Fragments size and prevalence of the different groups identified

Group	Restriction fragment size (bp)		Prevalence (%)
	Hae III	*Hinf* I	
1	400 + 250	950 + 450	76,67
2	400 + 250	950 + 650	6,67
3	400 + 250	1300	6,67
4	600 + 500+ 400	1300	3,33
5	600 + 500+ 400	950 + 450	3,33
6	400 + 250	1500	3,33

4- Discussion

Nonnonkoumou is a traditional fermented milk consumed in Côte d'Ivoire. This fermented milk is a dairy product that has undergone lactic fermentation. This fermentation improves the final quality of dairy products by producing compounds of interest, including aromatic compounds.(**Ouazzani *et al.*, 2014**).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 (**Zamfir *et al.*, 2006**).So, this study has shown the presence of lactobacilli and cocci in the *nonnonkoumou*. The cocci load is between 6.7 log CFU / mL and 8.31 log CFU / mL. Similar charges have been reported by **Ren *et al.* (2015)** in traditional fermented milks from Mongolia. These authors found mean cocci loads of 8.80×10^6 to 3.47×10^8 CFU / ml after seeding on the M17 agar.The *nonnonkoumou*samples taken during this study had high lactobacilli loads with the highest load (6.12 ± 0.30 log CFU / mL) observed at Lobia belt 1 site. The abundance of these lactic acid bacteriain *nonnonkoumou* is due to their tolerance to acidic conditions and also to their ability to use the substrates present in this fermented milk for their growth. Also, it has been recognized that LAB are capable of producing inhibitory substances such as organic acid, hydrogen peroxide, bacteriocin and diacetyl (**Mishra *et al.*, 1996**).The variable maturation periods of the different products sampled, the differences in transport methods and the different sampling sites may have contributed to this variability in

the number of viable counts of cocci and lactobacilli detected.This variation may also depend on the initial milk composition of lactic acid bacteria and especially the storage conditions of this food **(Ndiaye, 2002).** 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)**.

During this study, the cocci / bacilli ratio was determined. So, the average loads of cocci are higher than those of bacilli as evidenced by the cocci/bacilli ratio which vary between 1.22 and 4.These results are in agreement with the results obtained by **Erkuş (2007)** in his work which showed an average cocci load of the order of 10^8 CFU / mL in the yogurt collected in "Toros" a region of Turkey. This author determined the cocci / bacilli ratio in 12 samples and found ratios greater than 1 in 11 of the 12 samples analyzed.According to **Bozoudi*et al.* (2017),** the monitoring of the fermentation process during a 5-day period revealed that cocci (*Lactococcus lactis* and*Lactococcus spp*.) were isolated in high frequency (35% of the isolates), reaching higher numbers at day 3. These bacteria are naturally occurring in raw milk (**Berthier *et al.*, 2001**) and they multiply when they find optimal conditions.Also, **Sansanwal*et al.* (2017)** showed that the cocci / bacilli ratio would vary according to the fermentation temperature. Indeed, the work carried out on *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in milk has shown that these two microorganisms can grow at a temperature of 42-43ºC, and the optimum temperature for symbiotic growth is 42ºC. Since the optimum growth temperature for *Streptococcus thermophilus* is 37ºC and 45ºC for *Lactobacillus bulgaricus,* increasing the temperature above from 42ºC, the growth of lactobacilli will be favored while the temperatures below 42ºC results in increased growth of streptococci. Either case is resulted in a deviation in the ratio of cocci to bacilli, for the optimum yoghurt the ratio should be 1:1(**Shah, 2003**).Some authors such as **Amoroso *et al.* (1988)** indicated that the optimal ratio between bacilli and cocci present in yogurts should be between 1: 1 and 1: 2. These results therefore suggest that *nonnonkoumou,* which has a cocci / bacillus ratio of between 1.9 and 4 during this study, is not an optimal quality curd.

PCR followed by Amplified Ribosomal DNA Restriction Analysis (ARDRA) were used to identify lactic acid bacteria isolated from *nonnonkoumou*.Indeed,identification of microbial species by use of phenotypic methods may sometimes be uncertain, complicated, and time-consuming. The use of molecular methods has revolutionized identification, improving its quality and efficacy.Some molecular methods target mainly the rDNA genes or their spacer regions**(Blaiotta*et al.*, 2008).**ARDRA has been used to compare bacterial isolates within a

wide range of microbial communities. The advantages of ARDRA are that it is rapid, reproducible, relates to microbial diversity, and will be invaluable in analyzing a greater number of samples together with experimental objectives such as dietary interventions **(Stackebrandt*et al.*, 1994).**In the present work, ARDRA allowed us to differentiate the LAB isolates. This differentiation was observed by the restriction fragment analysis using 2 endonucleases enzymes which are *Hae* III and *Hinf* I.During this study, each amplicon of the 16S region of the rDNA corresponding to the 64 positive isolates generated after PCR is digested with the enzymes *Hae* III and *Hinf*I. Amplified Ribosomal DNA Restriction Analysis (ARDRA) have revealed 4 *Hinf*I profiles and 3 *Hae* III for lactobacilli and 2 *Hae* III profiles and 4 *Hinf*I profiles for cocci. This technique has revealed 4 species of lactobacilli isolated from *nonnonkoumou* which are *Lactobacillus reuteri*, *Lactobacillus paracasei*, *Lactobacillus plantarum* and *Fructobacillusdurionis* and 6 groups of cocci corresponding to 6 species. So, the group 1 was found to be the dominant group in the majority of *nonnonkoumou* samples.**Ren *et al.* (2015)** also identified six species of lactic shell in fermented milk from Mangolia. These are *Enterococcus faecalis*, *E. durans*, *Lactococcus lactis* subsp. *lactis*, *Leuconostocmesenteroides* subsp. *mesenteroides*, *Leuc. lactis*, *S. thermophilus* with *S. thermophiles*as the majority species in all samples of fermented milk.A diversity of cocci has been observed in *nonnonkoumou*. However, no diversity was observed at 2 sites. According to **Ren *et al.* (2015)**, the diversity of the species present seems to vary according to the sites and the fermented dairy product. This diversity is not surprising since many factors can influence the presence or absence of a particular species. These include the different production methods, recipes and raw materials. In addition, the variability of environmental conditions at each location could also contribute to this variation, as observed by **Yu *et al.* (2012).**

The ARDRA results obtained during this study are in agreement with those of **Afaf (2012)** which used ARDRA to differentiate lactobacilli isolates. Similar results were found by **Maria & Manuel**(**2007)** which showed the presence of these different lactobacilli species in fermented milk. Also, it has been shown that *Lactobacillus plantarum* had been identified as the predominant microorganism at the end of many lactic fermentations **(Kunene *et al.*, 2000),** probably for its acidity tolerance (**Mishra *et al.*, 1996**) and its ability to use the substrates of the fermentation medium.Furthermore, it is important to mention that lactic acid bacteria play a very important role in fermented milk, through the metabolites production such as lactic acid. This is because lactic acid helps to destabilize the casein micelles, which leads to gel formation. It also gives fermented milk its distinct and characteristic tangy taste,

as it contributes to the flavor and aroma of fermented milk(**Tamine & Robinson, 1999; Singh *et al.*, 2006).**Lactic acid also acts as an inhibitor against undesirable microorganisms **(Léory*et al.*, 2002).** Lactic acid bacteria, by their acidifying activity, have a beneficial effect from the hygienic quality point of view of the product. In parallel, they generate secondary products which contribute to the organoleptic quality of the curd. From a nutritional point of view, the fermentation activity of these lactic acid bacteria promotes solubilization of the various constituents, thereby improving their bioavailability (**Courtin *et al.*, 2002; Ngounou *et al.*, 2003**). Also, certain species of Streptococci present in fermented milk such as *Streptococcus thermophilus* has a beneficial effect on constipation because its ingestion stimulates intestinal motility in the elderly (**Seki *et al.*, 1978).**Interest in the role of probiotics for human health goes back at least as far as 1908 when Metchnikoff suggested that man should consume milk fermented with lactobacilli to prolong life (**Hughes & Hoover, 1991; O'Sullivan *et al.*, 1992**). Many functional characteristics of lactic acid bacteria (LAB) are responsible for their historical and modern use in food production. Several health benefits have been claimed to be associated with the consumption of fermented milk products (**Yamamoto *et al.*, 1994**).It should be also noted that the health benefits of milk and dairy products are known to humanity and may be attributed to the biologically active compounds those lactic acid bacteria that are existing in milk.The functional role of fermented dairy products is either directly through interaction with consumed microorganisms or, indirectly, as a result of action of microbial metabolites like nutrients, generated during the fermentation process. The health promoting mechanisms of probiotic action are mostly based on the positive effect they exert on the immunity response**(Bhat & Bhat 2011)**.

5- Conclusion

It may be concluded from this study that the *nonnonkoumou*samples have high lactobacilli and cocci loads with a preponderance of cocci in all the *nonnonkoumou* samples taken in comparison to bacilli through the cocci / bacilli ratio. PCR followed by Amplified Ribosomal DNA Restriction Analysis (ARDRA) after enzymatic digestion with *Hae* III and *Hinf* I have revealed 4 *Hinf* I profiles and 3 *Hae* III for lactobacilli and 2 Hae III profiles and 4 Hinf I profiles for cocci. This technique has revealed 4 species of lactobacilli isolated from *nonnonkoumou* which are *Lactobacillus reuteri*, *Lactobacillus paracasei*, *Lactobacillus plantarum* and *Fructobacillusdurionis* and 6 groups of cocci corresponding to 6 species.

References

Afaf IS. Molecular identification of probiotics lactobacillus Strain isolates by: Amplified Ribosomal DNA Restriction Analysis (ARDRA). Sci. J. Microbiol.2012; 6(12), 3034-3041

Assohoun-Djeni NMC, Kouassi KC, Kouassi KA, Yao KC, Djeni NT, Konate I. Physicochemical Characteristics and Microbial Contamination of Raw Milk and Artisanal Milk Curd (*Nonnonkoumou*) in Daloa, Côte D'Ivoire. Int. J. Curr. Microbiol. Appl. Sci.2020; 9(03): 2667-2683.

Attchelouwa CK, N'guessan FK, Aké FMD, Marcellin KD. Molecular identification of yeast, lactic and acetic acid bacteria species during spoilage of tchapalo, a traditional sorghum beer from Côte d'Ivoire. *W. J.Microbiol.Biotech.* 2018 ;34:173-183.

Berthier F, Beuvier E, Dasen A, Grappin R. "Origin and diversity of mesophilic lactobacilli in Comte cheese, as ´revealed by PCR with repetitive and species-specific primers," Int. Dairy J. 2001; 11(4-7): 293–305.

Bhat Z, Bhat H. Milk and dairy Products as Functional Foods: A review. Int. J. Dairy Sci.2011; 6(1):1-12.

Blaiotta G, Fusco V, Ercolini D, Aponte M, Pepe O,Villani F. Lactobacillus strain diversity based on partial hsp60 gene sequences and design of PCR- restriction fragment length polymorphism assays for species identification and diff erentiation, Appl. Environ. Microbiol.2008; 74: 208–215.

Bozoudi D., Agathokleous M., Anastasiou I., Papademas p. &Tsaltas D. Microbiological Characteristics of Trachanas, a Traditional Fermented Dairy Product from Cyprus. J. Food Qual.2017;1-6.

Coulin P, Farah ,Assanvo J, Spillman H,Puhan Z. Characterization of the microflora of attiéké, a fermented cassava product during traditional small-scale production. Int. J. Food Microbiol. 2006; 106:131-136

Courtin P, Monne M, Rul F. Cell-well proteinase PrtS and PrtB have a different role in *Streptococcus thermophilus* / *Lactobacillus bulgaricus* mixed culture in milk. *Microbiol.* 2002 ; 48 :3413-3421.

Erkuş O (2007). Isolation, phenotypic and genotypic characterization of yoghurt start bacteria. Master of science In Food Engineering, Tukey, 2007; 117 p.

Holzapfel WH. Appropriate starter culture technologies for small-scale fermentation in developing countries. Int. J. Food Microbiol. 2002; 75: 197-212.

Hughes DB, Hoover DG. Bifidobacteria: Their potential for use in American dairy products. *Food Technol*.1991; 45(4): 74–83.

Jespersen L, Halm M,Kpodo K, Jacobson M. Significance of yeasts and moulds occurring in *maise*dough fermentation for *kenkey* productionInt. J. Food Microbiol.1994; 24:239–248.

Kunene NF, Ifigenia G, Geornaras I, Von Holy A, Hastings JW. Characterization and determination of origin of lactic acid bacteria from a sorghum-based fermented food by analysis of soluble proteins and amplified fragment length polymorphism fingerprinting. Appl. Environ. Microbiol.2000; 66:1084-1092.

Leory F. Degeest B, De vuyst L. (2002). A novel area of predictive modeling: describing the functionally of beneficial micro-organisms in foods**.** Int. J. Food Microbiol.2002; 73:251-259.

Kim M, ChunJ. "Bacterial community structure in kimchi, a Korean fermented vegetable food, as revealed by 16S rRNA gene analysis," Int. J. Food Microbiol.2005; 103(1): 91–96,

Mirah-dpp. Annuaire des statistiques des ressources animales et halieutiques. Direction de la Planification et des programmations, Ministère de Ressources Animales et Halieutiques, Abidjan, Côte d'Ivoire, 2012 ; 26 p.

Mishra C, Lambert J. Production of anti-microbial substances by probiotics. *Asia Pac J Clin Nutr*. 1996; 5(1):20–4

Morandi S, Brasca M. Lodi R."Technological, phenotypic and genotypic characterization of wild lactic acid bacteria involved in the production of Bitto PDO Italian cheese," Dairy Sci. Technol., 2011; 91(3): 341–359.

Ndiaye M. Contribution à l'étude de conformité de l'étiquetage de la qualité microbiologique des yaourts commercialisés à Dakar. Thèse de Doctorat, Université Cheikh AntaDiop de Dakar, Dakar. 2002 ; 11 p

Ngassam TC. Caractérisation de la flore lactique des laits fermentés artisanaux au Sénégal : cas de la zone de Niaye. Thèse de Doctorat, Université Cheikh Anta Diop de Dakar, Dakar, 2007 ; 109p.

Ngoumou C, Ndjouenkeu R, Nbofung F. Noubi I. Mise en évidence de la biodisponibilité de calcium et de magnésium au cours de la fermentation du lait par des bactéries lactiques isolées du lait caillé Zébu.J. Food Eng.2003 ; 57:301-307.

O'Sullivan M.G., Thornton G., O'Sullivan G.C. & Collins J.K. Probiotic bacteria: myth or reality? *Trends Food Sci. Technol*. 1992; 3: 309–314.

Ouazzani TN, Arfaoui A. Fadli M. Evaluation de la qualité microbiologique du lait cru dans la région du Gharb, Maroc. *Inter. J.Inn. and Scientific Res.*, 2014 ;9:487-493.

Prescott LM, Harley J. P. Klein D. A. Microbiologie. De Boeck. 2003; 1162 p.,.

Ren Y, Liu W, Zhang H. Identification of Coccoidal Bacteria in Traditional Fermented Milk Products from Mongolia, and the Fermentation Properties of the Predominant Species, *Streptococcus thermophilus*.Korean J Food Sci Anim Resour.2015;35:683-391.

Sansanwal R., Ahlawat U, Dhanker R, Yoghurt: A Predigested Food for Lactose-Intolerant People. Int. J. Curr. Microbiol. Appl. Sci.(2017; 6(12): 1408-1418

Seki M, Igarashi M, Fukuda Y, Simamura S, Kawashima T,Ogasa K. The effect of Biftdobacterium cultured milk on the 'regularity' among the aged group. *J Jpn Soc Food Nutrit.*1978; 31:379-387.

Shah N. The product and its manufacture. In Encylopedia of Food Science and Nutrition. Edited by Cabarello B, Trugo L, Finglas P. London: Academic Press. 2003; pp6252-6262

Stackebrandt E. & Goebel B. M.. Taxonomic note: a place for DNA- DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int. J. Syst. Bacteriol.1994; 44:846-849

Weisburg WG, Barms SM, Pelletier DA,Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. *J.Bacteriol*.1991; 173:697-703.

Yamamoto N, Akino A, Takano T.Antihypersensitive effects of different kinds of fermented milk in spontaneously hypersensitive rats. *BioSci. BiotechnolBiochem*, 1994; 58: 776–778.

Yu Y, Song C, Zhang Q, Dimaggio PA, Garcia BA, York A, Carey MF, Grunstein M. Histone H3 lysine 56 methylation regulates DNA replication through its interaction with PCNA. *Mollen Cell*.2012; 46(1):7-17

Zamfir M, Vancanneyt M, Makras L, Vaningelgem F, Lefebvre K, Pot B, Swings J. De Vuyst L. Biodiversity of lactic acid bacteria in Romanian dairy products. Syst. Appl. Microbiol.2006; 29:487–495.