Vol. 15(6), pp. 278-285, June, 2021
DOI: 10.5897/AJMR2021.9502
Article Number: 2A7C82D66931
ISSN: 1996-0808

http://www.academicjournals.org/AJMR

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African Journal of Microbiology Research

*Full Length Research Paper*

# Monitoring of physicochemical and microbiological parameters during *nonnonkoumou* (artisanal curdled milk) production in Daloa, Côte d'Ivoire

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Received 15 February, 2021; Accepted 31 March, 2021

**This study aimed to study physicochemical and microbiological parameters during *nonnonkoumou* production. A total of 15 samples were analyzed during this study at a rate of 3 samples per fermentation time ($T_{0\ h}$, $T_{6\ h}$, $T_{12\ h}$, $T_{18\ h}$ and $T_{24\ h}$). The physicochemical analyses showed a drop in pH (from 7.03 ± 0.028 to 4.59 ± 0.021) during the 24 h of fermentation. The titratable acidity increased from 0.30 ± 0.014% at the start of fermentation to reach the maximum value of 0.88 ± 0.056% at 24 h of fermentation. The sugar level of fermenting milk samples decreased from 10.05 ± 0.071% at the start of fermentation, to 5.15 ± 0.071% at the end of fermentation. The density results showed an addition of water to the milk used for *nonnonkoumou* production. Microbiological analyses showed a similar growth of lactic acid bacteria and yeasts and molds, but yeasts and molds were absent at the start of fermentation. Aerobic mesophile flora count reached maximum value (7.56 ± 0.81 Log CFU/ml) at 12 h of fermentation. The coliform count increased up to 12 h of fermentation before decreasing and disappearing at the end of fermentation.**

**Key words:** Milk, *nonnonkoumou,* fermentation, contamination, physicochemical and microbiological analyses.

## INTRODUCTION

Milk is an edible biological liquid, usually whitish in color, produced by the mammary glands of female mammals. Rich in lactose, it is the main source of nutrients for young mammals before they can digest other types of food (FAO, 2011). Like dairy products, milk is a balanced food because it provides the human body with around 15 essential elements for the maintenance of good health (Das et al., 2015). Milk provides relatively quick incomes

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for small producers and is an important source of income (FAO, 2011). Among farmed products (skins for the preparation of leather products, meat), milk occupies an important place in African economies. In addition, milk represents a biological medium that is highly alterable by microbial means because of its high water content, its pH close to its neutrality and its richness in biodegradable components (lactose, proteins and lipids). When taken under the right conditions (Aseptic conditions), raw milk contains few bacteria ($10^3$ germs/ml). These are saprophytic bacteria and among them are the lactic streptococci (*Lactococcus*) and lactobacilli. During milking and storage, the milk can become contaminated with a variety of microflora consisting of essentially of lactic acid bacteria belonging to the following genera*: Streptococcus, Lactococcus, Enterococcus, Leuconostocs* and *Lactobacillus* (Bekhouche, 2006). Milk can also be contaminated by contaminating microorganisms (total coliforms, thermotolerant coliforms), and even pathogenic bacteria such as *Salmonella* (Assohoun et al., 2020). The high temperatures in some African countries are responsible of the quick degradation of cow milk; therefore, techniques are needed for processing milk to extend shelf life including the preparation of cheeses. Cow milk was once entirely self-consumed but is now transformed into the artisanal curdled products such as *Rayeb* and *Jben* in North Africa, *Bouhazza* in some rural areas in eastern Algeria, *Iben* in Moroccan (Kèkè et al., 2009), and also *nonnonkoumou* in Côte d'Ivoire. *Nonnonkoumou* is an artisanal curdled milk, consumed in Côte d'Ivoire and particularly in Daloa city. Its production is done in a traditional way without the use of starter cultures. This is a spontaneous fermentation initiated by natural microorganisms that are found in the milk, on the processing utensils/equipment, on the hands of producers and from the local atmosphere as natural starters (Assohoun et al., 2012). The result is a heterogeneous end product, with varying characteristics from one producer to another. The pH of the milk which is around the neutrality is very low in the *nonnonkoumou* (pH around 4) (Assohoun-Djeni et al., 2020). This variation in pH is associated with other parameters (titratable acidity rate, density, etc.) during milk fermentation for *nonnonkoumou* production and is associated with the growth of certain microorganisms such as lactic acid bacteria which produce lactic acid, hydrogen peroxide and bacteriocins. These metabolites influence the growth of certain microorganisms present in fermenting milk. To our knowledge, very few studies have been carried out on the fermentation process of milk used for *nonnonkoumou* production. In order to enhance the *nonnonkoumou* by mastering the manufacturing process, it would be interesting to deepen the understanding on the fermentation of milk in order to guarantee a stable and quality product at the end of production.

The objective of this work was to study the evolution of some physicochemical and microbiological parameters during the milk fermentation for *nonnonkoumou* production in Daloa.

## MATERIALS AND METHODS

### Sampling

The samples analyzed during this study consisted of fermenting milk collected taken at different times ($T_0$, $T_{6H}$, $T_{12H}$, $T_{18H}$ and $T_{24H}$) during fermentation of milk. Indeed, 1 L of milk is taken from 3 resellers and boiled for about 10 min. 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 h, a bottle containing the fermenting milk is removed and the contents analyzed. Fermentation is thus carried out for 24 h. 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*.

### Physico-chemical analysis

#### *pH determination*

The pH of fermenting milk was determined using a pH meter (Microprocessor pH meter, pH 211, HANNA Instruments) according to the AOAC (1995) method. The instrument was calibrated using two buffer solutions at pH 7.0 and 4.0 and this was systematically done before pH measuring. The measurement was made by immersing the electrode in 20 mL of sample and the reading is repeated three times.

#### *Total titratable acidity (TA) determination*

Total titratable acidity (TA) was measured by titrating 10 mL of fermenting milk against 0.1N sodium hydroxide (NaOH) solution using phenolphthalein as indicator (Kimaryo et al., 2000) and the reading is repeated three times. The lactic acid level (corresponding to the level of titratable acidity) was determined by the following expression:

$$\text{Acid level (\%)} = \frac{\text{Vol (NaOH)} \times \text{N (NaOH)} \times 0.09}{\text{Quantity taken for test}} \times 100$$

where Vol (NaOH) = NaOH volume (in ml) used for the determination, N (NaOH) = normality of NaOH solution, quantity taken for test = 10 mL, and 0.09 = factor (in mill equivalent) for lactic acid.

### Density determination

To deduce the fraudulent practices of sellers by adding external elements, the density of the samples of fermentation milk was determined according to formula used by Pointurier (2003):

$d = ml / me$

where d = density of milk at 20°C, ml = mass of 10 ml of milk, and me = mass of 10 ml of tap water.

When milk temperature at the measurement time is above 20°C, the calculated density is increased by 0.0002 per degree above 20°C. When the temperature of the milk at the time of measurement is below 20°C, the calculated density is decreased by 0.0002 per degree below 20°C. Normal milk has a specific density at 20°C between 1.028 and 1.032.

#### Brix degree or refractometric dry extract (E.S.R) determination

The Brix degree or refractometric dry extract (E.S.R) which is the weight (in grams) of soluble dry matter contained in 100 g of product was measured using a refractometer (El Bouichou 2015). So, a drop of fermenting milk was placed on the movable jaw then this jaw was folded back. The reading is carried out in the presence of a light source and the number corresponding to the line of separation of the clear zone and of the blue zone gives the degree Brix or the refractometric dry extract (expressed as a percentage).

#### Enumeration of microorganisms

Preparation of stock solutions, inoculation of agar plates, and cultivation and quantification of microorganisms were carried out according to Coulin et al. (2006). For all determinations, 10 ml of the 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 coliforms was carried out using violet crystal and neutral red bile lactose (VRBL) plates containing agar (VRBL agar, Oxoid Ltd., Basingstore, UK), incubated for 24 h at 30°C for total coliforms and 44°C for fecal coliforms. Yeasts and molds were enumerated on Sabouraud chloramphenicol agar (Fluka, Biochemica 89579, Sigma–Aldrich Chimie GmbH, India) incubated at 30°C for 4 days. Aerobic mesophiles were enumerated on Plate Count Agar (PCA Oxoid.) and incubated at 30°C for 2 days. Enumeration of LAB was carried out using Man, Rogosa and Sharpe (MRS) agar (Merck, Darmstadt, Germany), which were incubated under anaerobic conditions (Anaerocult A, Merck) at 37°C for 72 h.

#### Colony enumeration

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

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

where 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).

#### 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.

## RESULTS

### Change of pH, titratable acidity, refractometric dry extract and density

The pH dropped significantly during the *nonnonkoumou* production from 7.03 ± 0.028 at 0 h of fermentation to 4.59 ± 0.021 at 24 h of fermentation contrarily to the total titratable acidity which amount increased at the same time from 0.30 ± 0.014 to 0.88 ± 0.056%. Regarding density, the values are not significantly different at the 5% threshold. With a value of 1.01 ± 0.028 at the start of fermentation (T0 h), the density decreased very slowly and reached the value of 0.98 ± 0.021 at 18 h of fermentation. A slight increase (1.01 ± 0.007) was observed at the end of fermentation (T24 h). As for the refractometric dry extract (E.S.R.), its value is 10.05 ± 0.071% at the start of fermentation. This value decreased during fermentation until it reached its minimum value of 5.15 ± 0.071% at 24 h of fermentation (Table 1).

### Enumeration of the different bacterial groups

Enumeration of microorganisms in this study showed that LAB and yeasts and molds had similar growth throughout the fermentation, but their counts were significantly different ($P < 0.05$) at the different fermentation times (Figure 1). Indeed, LAB mean load was 1.27 ± 0.12 Log CFU/ml at the start of fermentation but yeasts and molds were absent. Yeasts and molds appear after 6 h of fermentation with a load of 3.95 ± 0.32 Log CFU/ml. The LAB and yeasts and molds counts increased during the process and reached highest values (8.56 ± 0.18 Log CFU/ml for LAB and 6.31 ± 0.54 log CFU/g for yeasts and molds) at 18 h of fermentation, before decreasing until the end of fermentation (Figure 1). However, the LAB count stayed significantly higher than that of yeasts and molds. Contrarily to the fermenting microorganisms (LAB and yeasts and molds), the total and fecal coliform populations, with respective initial loads of 4.22 ± 0.52 Log CFU/ml and 3.73 ± 0.45 Log CFU/ml increased slightly to reach their maximum at 12 h of fermentation. After this 12 h of fermentation, these microorganisms decreased rapidly and disappeared after 18 h of fermentation. However, the aerobic mesophiles count was very high (6.22 ± 0.56 Log CFU/ml) at the start of fermentation. This count increased further during the process to reach the value of 7.56 ± 0.81 Log CFU/ml at 12 h of fermentation before experiencing a slight decrease at the end of fermentation (Figure 2).

**Table 1.** Evolution of pH, titratable acidity, refractometric dry extract (ESR) and density of *Nonnonkoumou* samples taken at different fermentation times.

| Fermentation time (h) | Parameter | | | |
|---|---|---|---|---|
| | ESR (%) | Density | Titratable acidity (%) | pH |
| 0 | 10.05±0.071$^{a}$ | 1.01±0.028$^{a}$ | 0.30±0.014$^{d}$ | 7.03±0.028$^{a}$ |
| 6 | 9.65±0.071$^{b}$ | 1.00±0.028$^{a}$ | 0.33±0.035$^{d}$ | 6.54±0.014$^{b}$ |
| 12 | 6.05±0.071$^{c}$ | 0.99±0.021$^{a}$ | 0.47±0.028$^{c}$ | 6.21±0.021$^{c}$ |
| 18 | 5.95±0.071$^{c}$ | 0.98±0.021$^{a}$ | 0.69±0.007$^{b}$ | 5.63±0.028$^{d}$ |
| 24 | 5.15±0.071$^{d}$ | 1.01±0.007$^{a}$ | 0.88±0.056$^{a}$ | 4.59±0.021$^{e}$ |

ESR: Refractometric dry extract. Values at each time point are the means of our replicates ± SD (error bars). The same letter in the same column indicated no statistical difference (P> 0.05) (Tukey, HSD).

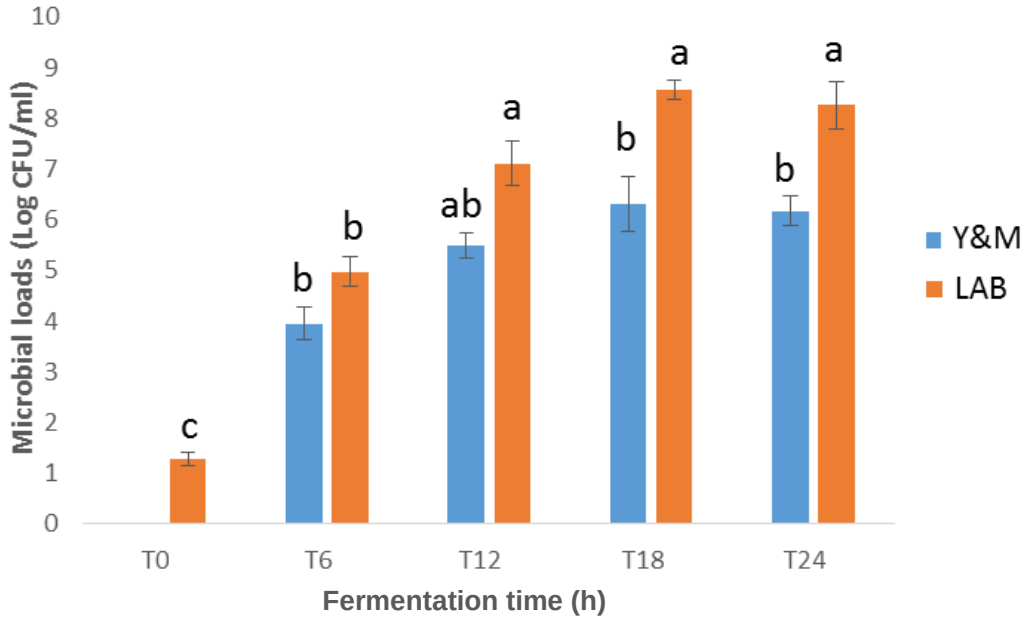


**Figure 1.** Evolution of lactic acid bacteria and yeasts and molds (fermenting microorganisms) populations in *nonnonkoumou* samples taken at different fermentation times. Values at each time point are the means of our replicates ± SD (error bars). For the same type of germ, histograms bearing the same alphabetical letter are not statistically different (P> 0.05) (Tukey, HSD). LAB: Lactic acid bacteria, Y&M: Yeast and molds.

## DISCUSSION

*Nonnonkoumou* is an artisanal curdled milk consumed in Côte d'Ivoire. Its production is done in an artisanal way and its fermentation is spontaneous. The present study focused on the common, fermentative and contaminating microflora of *nonnonkoumou* throughout the manufacturing process. The study also aimed to determine some physicochemical characteristics of the *nonnonkoumou* samples taken at different fermentation times during the production of this food. Acidity is an important quality indicator of fermented milk, which is closely related to the texture and flavor of the product (Li et al., 2017). This is also the case of Moroccan *Iben* which is a dairy beverage prepared by spontaneous fermentation and coagulation of whole raw milk. This dairy product has a low pH (4.2), responsible for the texture of the final product (Tantaoui-Elaraki and El Marrakchi, 1987). Appropriate acidity gives the product a unique flavor and inhibits the growth of spoilage bacteria and food-borne pathogens (Mufandaedza et al., 2006). Changes in pH and titratable acidity are shown in Table 1. Indeed, the pH of the milk at the start of fermentation is 7.03 ± 0.028. There is significant acidification resulting in

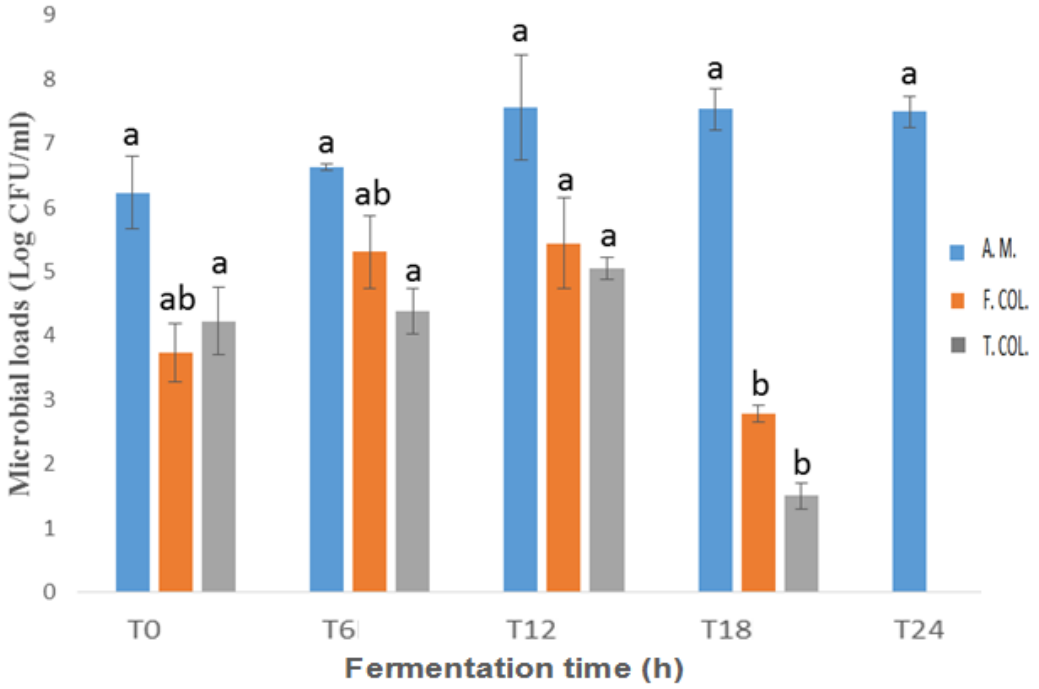


**Figure 2.** Evolution of aerobic mesophiles, total and fecal coliforms populations (non fermenting microorganisms) in *nonnonkoumou* samples taken at different fermentation times. Values at each time point are the means of our replicates ± SD (error bars). For the same type of germ, histograms bearing the same alphabetical letter are not statistically different (P> 0.05) (Tukey, HSD). A.M.: Aerobic mesophiles, T. COL.: Total coliforms, F. COL.: Fecal coliforms.

a considerable drop in pH of around 2.44 units (from 7.03 ± 0.028 to 4.59 ± 0.021 units at the end of fermentation) correlated with an increase in the titratable acidity rate ranging from 0.30 ± 0.014% at 0 h of fermentation to 0.88 ± 0.056% at 24 h of fermentation. The pH values decreased while titratable acidity increased during the period of fermentation of milk mostly due to the accumulation of lactic acid by the metabolism of LAB (Coulin et al., 2006; Panda et al., 2007). These results are in agreement with those of Zhihai et al. (2020) which showed a decrease in pH correlated with an increase in titratable acidity during the fermentation of goat milk in China. Furthermore, there are no significant differences between the densities of the *nonnonkoumou* samples taken at different fermentation times. The *nonnonkoumou* samples density varied from 0.832 ± 0.02 at 0 h of fermentation to 0.860 ± 0.01 at the end of fermentation. According to Pointurier (2003), normal milk has a density at 20°C between 1.028 and 1.032 (Table 1). Thus, a density of less than 1.028 indicates the addition of water in the milk (“wet” milk) while a density greater than 1.032 indicates that the milk has been heated or skimmed. It can therefore deduce that the milk used for the *nonnonkoumou* production during this study, which has a density of less than 1.028, is “wet” milk. In other words, the resellers added water to the milked milk at the pasture before the sale in order to increase their turnover. This practice, unfortunately used fraudulently by several resellers, can influence the quality of the finished curd product which is *nonnonkoumou* in the present study. The density of milk also varies according to the dry matter content and the fat content. So it decreases with the increase in fat (Mahamedi, 2015). Also, the refractometric dry extract (E.S.R.) which is the weight (in grams) of soluble dry matter contained in 100 g of product was measured during this study using the refractometer which allowed the determination of the sugar content of *nonnonkoumou*. Thus, at the start of fermentation, the refractometric dry extract has a value of 10.05 ± 0.071. This value decreases considerably during fermentation until reaching the value of 5.15 ± 0.071 at 24 h of fermentation. In fact, microorganisms, particularly lactic acid bacteria, use the sugars present in the milk for their growth during fermentation. Hence, the considerable drop in the sugar content during the production of this artisanal curdled milk commonly called n*onnonkoumou* in malinke language in Côte d'Ivoire. These results are in agreement with those of O'Mahony (1988) who showed that the lactose content of milk could vary during fermentation. The decrease in the sugar level could also

be due to the addition of water to raw milk before making the curd milk (El Bouichon, 2015). The microbiological parameters of milk and *nonnonkoumou* were also analyzed in this study. Indeed, the results obtained have shown the presence of LAB and yeasts and molds in artisanal curd milk (*nonnonkoumou*). As shown in Figure 1, the total LAB count in *nonnonkoumou* samples increased during fermentation and exceeded the minimum bacteria populations ($10^6$ cfu/mL), required for probiotic foods to possess health claims. Vieira-Dalode et al. (2007) indicated in their work the presence of lactic acid bacteria, yeasts and molds and their importance in fermented products. Also, Zhihai et al. (2020) showed the presence of these microorganisms during the fermentation of goat's milk in China. These fermentative microorganisms are abundant in *nonnonkoumou* samples and present a similar evolution. This is in agreement with the work of Chaves-López et al. (2014), who reported that co-culturing with yeasts was able to promote the multiplication of LAB. This phenomenon might be attributed to the release of amino acids and vitamins through the metabolism of yeasts (Zhang et al., 2017), which might provide more nutrients for the growth of LAB. Unlike contaminating microorganisms, the abundance and maintenance of yeasts, molds and 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. Yeasts would also make a useful contribution to the flavor and acceptability of fermented products according to these same authors. 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). Also, the milk collected at 0 h of fermentation was free of yeasts and molds. This absence of yeast and mold in our milk samples is probably due to the pasteurization of the milk collected from the resellers before the start of fermentation. Yeasts and molds are said to be heat-sensitive, that is, they do not tolerate high temperatures at all, hence the extermination of their population in these milk samples at 0 h of fermentation. Other microorganisms (total coliforms, fecal coliforms and aerobic mesophiles) were also present in samples analyzed in this study. Indeed, large aerobic mesophile and coliform loads were detected in the samples collected at different times ($T_0$, $T_{6\ h}$, $T_{12\ h}$, $T_{18\ h}$ and $T_{24\ h}$) of fermentation (Figure 2). These contaminating microorganisms would contaminate the milk during milking under generally unsanitary environmental conditions. The surrounding air, the utensils used to extract milk and water could be the main sources of milk contamination. Chye et al. (2004) indeed indicated that such elements (water, utensils, etc.) are major sources of contamination of milk and dairy products. Also, the non-compliance with the rules of hygiene by the sellers, the transport conditions of milk of the pastures to the sale places, the dust as well as the flies which arise on the ladle serving as a measure during the sale, could also be contamination sources (Assohoun-djeni et al., 2020). Millogo et al. (2018) have demonstrated that containers used during milking and in the manufacture of fermented milk can cause contamination of the milk as well as the final product. In addition, Ndiaye (2002) stated that the milk pH is favorable for microorganism growth, but their number decreased as the fermented milk aged. In addition, total and thermotolerant coliforms were detected in large number in milk and fermenting milk collected during this study. The incidence of coliforms in raw milk has received considerable attention, partly due to their association with contamination of fecal origin and the consequent risk of more pathogenic fecal organisms being present, partly because of the spoilage their growth in milk at ambient temperatures can produce. The presence of coliforms in dairy products is not acceptable by safe food consumption standards. Some of these microorganisms can become highly pathogenic and may cause serious diseases for human. Coliform counts regularly in excess of 2 Log cfu/mL are considered as evidence of unsatisfactory production hygiene (Directive 92/46/EEC, 1992). Sporadic high coliform counts may also be a consequence of unrecognized coliform mastitis, mostly caused by *Escherichia coli* (Torkar and Teger, 2008). Their presence in the samples of the present study is linked to poor hygienic practice during the production of *nonnonkoumou*. On the other hand, these coliforms are completely absent in the *nonnonkoumou* samples collected at the end of fermentation (after 18 h of fermentation). This phenomenon is certainly due to the acidity of this food at the end of fermentation which has a low pH (around 4) (Assohoun et al., 2020). This low pH prevents the growth of most spoilage and pathogenic organisms (Varga, 2007). Such results have already been reported by some authors (Karamoko et al., 2012) in their work on fermented foods. Also, Steinkraus (1996) specified that coliforms do not tolerate low pH. This disappearing of coliforms was certainly due to the sensitivity of these bacteria to the substances produced by LAB. It was proved that LAB exerts antimicrobial action through the production of lactic acids, bacteriocins, diacetyl and hydrogen peroxide.

## Conclusion

The general objective of this study was to study the evolution of some physicochemical and microbiological parameters during the production of *nonnonkoumou* in Daloa city. The results of the physicochemical analyses showed a considerable drop in pH during the entire

fermentation process. This drop in pH led to an acidic end product (*nonnonkoumou*) with a pH between 4 and 5. These results also showed that the fermenting milk samples analyzed during this study had a sugar level that gradually decreased during fermentation. The density results showed an addition of water to the milk used to *nonnonkoumou* production. Furthermore, the results of the microbiological analyses showed a similar growth of lactic acid bacteria and of yeasts and molds, but yeasts and molds were absent at the start of fermentation. These results of microbiological analyses also showed high loads of Aerobie mesophile microflora. As for coliforms, their load increased up to 12 h of fermentation before decreasing and disappearing after 18 h of fermentation.

## CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

## ACKNOWLEDGEMENTS

This research was made possible through funds provided by The World Academy of Sciences, Italy (N° 19-120 RG/BIO/AF/AC_I), with the important contribution of the microbiology team of Laboratory of Biotechnology and Food Microbiology, Faculty of Food Sciences and Technology, University Nangui Abrogoua, Cote d'Ivoire. The authors gratefully acknowledge Prof. Marcellin Dje Koffi, the director of the laboratory.

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