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Identification of *Lactobacillus* and *Bifidobacterium* 16S rRNA Gene in Breast Milk of Some Healthy Women in Kinshasa (DR Congo)

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Abstract

Breast milk is important for infant health. Some of its benefits are due to the presence of a specific population of bacteria in the microflora. However, the microbiome of breast milk is influenced by many parameters such as maternal diet, breastfeeding and geographic location. Culture and non-culture methods have been used in studies of this bacterial population worldwide. But in the DR Congo, there was no study reporting the use of culture-independent techniques to characterize the bacterial diversity of human milk. The aim of this study was to identify the bacterial 16S rRNA gene from two genera *Lactobacillus* and *Bifidobacterium*. The 16S rRNA gene was also identified from four species: *Lactobacillus reuteri*, *Lactobacillus rhamnosus*, *Bifidobacterium longum* and *Bifidobacterium lactis*. This analytical cross-sectional study was conducted in Kinshasa. Breast milk from some healthy women was collected from February 2 to 28, 2018. A culture-independent protocol using the classical polymerase chain reaction (PCR) was used to identify the bacterial 16S rRNA gene. The 68 samples of breast milk were collected in a sterile condition. The bacteria-specific ribosomal gene 16S rRNA was detected in 91.18% of *Lactobacillus* and 32.35% of *Bifidobacterium* at genus level. At of species level, only *Lactobacillus reuteri* 16S rRNA gene was identified in 89.71%. The 16S rRNA gene from the other species could not be amplified. There was also an association between educational level and the presence of *Bifidobacterium*

and *Lactobacillus* 16S rRNA genes in the breast milk ($p = 0.008^*$, $p < 0.001^*$). **Conclusion:** This study demonstrates the presence of the bacterial 16S rRNA gene from *Lactobacillus* and *Bifidobacterium* in breast milk at the genus and *Lactobacillus reuteri* at species level. A further study on the diet, use of antibiotics during pregnancy and lactation practice will provide a better understanding of the microflora of breast milk.

Keywords

16S rRNA, PCR, *Lactobacillus*, *Bifidobacterium*, Breast Milk

1. Introduction

Breast milk is important for optimal growth and development of the infant. In fact, it contains nutrients, hormones, growth factors, immunoglobulins, cytokines, and enzymes, which contribute towards child well-being, but also a significant number of microorganisms [1]. The human milk microbiota drives the colonization of the gastrointestinal tract, also contributing to the maturation of the immune system [2].

This functional food protects the newborn through compounds passed from mother to child.

Diarrhea is one of the biggest threats to children in the Democratic Republic of the Congo. The acute form of diarrhea has a prevalence of 18% nationally and 14% in the city of Kinshasa [3]. Rotavirus and Escherichia coli are the two most common etiological agents of diarrhea in infants. Rotavirus infection accounted for almost 53.8% of acute diarrhea [4]. Another study shows that rotavirus is responsible for 80% of diarrhea in children under 12 months in Kinshasa and Lubumbashi [5]. This situation poses a risk for the child. In this situation, breast milk acts to allow for a speedy recovery and protects against the risk of chronic diarrhea [4]. This benefit of breast milk comes from its nutritional composition and microflora. Therefore, the complete characterization of the bacterial population remains important. Breast milk has long been considered sterile. However, studies show that it contains a large number of bacteria [6] [7]. In total, there are more than 700 different types of bacteria in breast milk [8]. The flora of breast milk appears to consist of a bacterial group that is present in all women. The number of species in an individual varies from 2 to 18 species [9].

Studies have shown that *Bifidobacterium* and *Lactobacillus* are among the recurring bacteria [10] [11]. The presence of *Bifidobacterium* and *Lactobacillus* is stable over time among individuals but varies widely within populations and between countries [12]. Some of the benefits of breast milk stem from the presence of *Lactobacillus* and *Bifidobacterium* [13]. In recent years, the study of these two groups of bacteria has accelerated. The scientific community agrees on the safe use of some strains as probiotics [14] [15] [16] [17]. Species such as *Lactobacillus rhamnosus*, *Lactobacillus reuteri*, *Bifidobacterium lactis* and *Bifidobac-*

terium longum have long been part of nutrition foods [17] [18]. In general, the factors that affect the bacterial composition of breast milk are related to the mother and her environment. These factors include the bacterial composition of the human skin, vagina, mouth, and gastrointestinal tract. The environmental factors are socio-economic status, cultural and dietary habits and mode of delivery. The use of antibiotics before and after birth is also important. These elements lead to differences at both individual and population levels [16] [19]-[25].

Studying the bacterial population of breast milk requires the use of precise methods. Culture-dependent bacterial techniques do not always provide an accurate representation of all species. They have also clearly demonstrated their limitations in distinguishing *Lactobacillus* and *Bifidobacterium* bacteria [26] [27]. Molecular biology techniques offer an alternative way to identify bacteria by targeting a specific gene. The PCR techniques are widely used to identify 16S rRNA genes of breast milk bacteria [8] [10] [28] [29] [30]. The presence of 16S rRNA specific gene can be established as an indicator of the quality of the breast milk microflora. A large amount of information is available on the microflora of breast milk in Europe, America and Asia. But Africa does not have the same scientific wealth. And there are few studies on this microflora in DR Congo due to the lack of accurate methods to determine the bacterial diversity of breast milk.

The aim of this study was to identify 16S rRNA genes from two genera of bacteria: *Lactobacillus* and *Bifidobacterium*. The 16S rRNA genes were also amplified to identify two *Lactobacillus* species (*Lactobacillus reuteri*, *Lactobacillus rhamnosus*) and two *Bifidobacterium* species (*Bifidobacterium lactis*, *Bifidobacterium longum*) in the breast milk of healthy Congolese women using a culture-independent technique. Some demographic factors have also been analyzed in association with the presence of 16S rRNA genes in breast milk.

2. Material and Methods

In a local laboratory, we set up a protocol to identify *Lactobacillus* and *Bifidobacterium* bacteria by direct simplex PCR. The specific 16S rRNA gene was amplified by the classic polymerase chain reaction technique. In this study, healthy breastfeeding women were recruited from February 2-28, 2018. The babies' ages were from 0 to 6 months. All of the women lived in the urban area of NGABA in Kinshasa, DR Congo. Recruitment was carried out by nutritionists from the Nutrition Department of the Hospital center of NGABA (Kinshasa, DRC). All women were volunteers and gave written informed consent to the protocol. This protocol was approved by the hospital ethics committee. The nipples and areola were cleaned with soap and sterile water and soaked in chlorhexidine. Milk samples were collected manually using sterile gloves. The first drops were discarded and 10 ml was collected in a sterile tube. The samples were then delivered to the Molecular Biology Laboratory of the National Institute of Biomedical Research (Kinshasa/DRC).

All women completed a questionnaire that included information on sociode-

mographic characteristics such as age, lactation (exclusive or mixed), level of education, employment, marital status, and aspects related to motherhood (number of pregnancies, type of delivery, number of children).

DNA Extraction

The breast milk samples were treated to remove fat and 1 mL of the samples was centrifuged at 7150 g for 20 minutes [10]. Then total DNA was isolated from the pellets using GENEJET GENOMIC DNA PURIFICATION KIT® (Thermo Scientific). DNA was eluted in 20 µL of BufferAE and the purified DNA extracts were stored at −20˚C. The bacterial DNA was quantified by UV spectrophotometry (260 - 280 nm) using the NANODROP® (Thermo Scientific). DNA was also extracted from reference strains and used as a positive control in each DNA amplification. The choice of primers was based on their proven specificity and efficiency in previous studies (Table 1). These primers were synthesized in South Africa by INQABA®. A two-step classic PCR was performed to identify the breast milk bacteria. The first amplifications were performed to identify the 16S rRNA gene at the genus level and the second amplifications were at the species level. Each 25 µL reaction mixture contained 12.5 µL of Taq, 0.5 µL of each of the specific primers, 100 ng of template DNA and nuclease-free water. The PCR conditions were as follows: 40 cycles, 95˚C for 5 minutes, annealing temperature for 30 seconds, 72˚C for 60 seconds and a final extension at 72˚C for 5 minutes as shown in Table 1. Amplicons were analyzed by horizontal electrophoresis (135 volts) on a 1.3% agarose gel in Tris-Acetate-EDTA buffer X 0.5 (VWR®) and gel loading dye purple (BIOLABS®). The DNA on the agarose gel was stained

Table 1. List of primers for 16S rRNA genes.

Genus and Species	Primers			
	Name	Sequences 5'-3'	Annealing temp. (˚C)	References
Bifidobacterium spp.	BIF 164	GGGTGGTAATGCCGGATG	66	Kok, 1996 [37]
	BIF 662	CCACCGTTACACCGGGAA		
B. lactis	LW420C	GGATGCTCCGCTCCATCG	66	Kok, 1996 [38]
	LW420D	GGGAAACCGTGTCTCCAC		
B. longum	BILO-1	TTCCAGTTGATCGCATGGTC	65	Markiewicz, 2005 [38]
	BILO-2	GGGAAGCCGTATCTCTACGA		
Lactobacillus spp.	LAC-1	TGGAAACAGGTGCTAATACCG	60	Orist, 2002 [39]
	LAC-2	CCATTGTGGAAGATTCCC		
L. rhamnosus	RHAM-1	GTCGAACGAGTTCTGATTATTG	63	Sul, 2007 [40]
	RHAM-R	GAACCATGCGGTTCTTGGAT		
L. reuteri	LREU-1	AGAGTTTGATCCTGGCTC	54	Egervärn, 2007 [16]
	LREU-2	CGGGAACGTATTCACCG		

with 2% ethidium bromide. The sequences were visualized with a BIODOCK Trans-Illuminator. The sizes of the amplicons are presented in Table 2.

Statistical analysis

The parameters that influence the presence of bacterial 16S rRNA gene in breast milk were collected through a survey. All data were analyzed by IBM SPSS Statistics 23®. The ANOVA test (=0.05) was used to investigate the association between the presence of 16S rRNA in milk and the mean maternal age or number of pregnancies. The FISCHER test (=0.05) was used to investigate the association between the presence of 16S rRNA in milk and some sociodemographic factors.

3. Results

Figures 1-3 showed the results of PCR using 16S rRNA specific primers with amplified fragments isolated from breast milk for *Bifidobacterium*, *Lactobacillus* and *Lactobacillus reuteri*.

Breast milk was collected from 68 healthy women. The average age of the women questioned (n = 68) was 27.7 ± 5.3 and the number of children per woman was 2.03 ± 1.29. In this study, 75.36% of the women were married. Of these, 18.84% reached primary school, 57.97% secondary school and 23.19% university studies. The most common occupation was housekeeping at 68.12%. In 55.07% of cases, women exclusively breastfeed their children.

The results in Figure 4 show that the bacterial 16S rRNA gene was identified at the genus level, with 91.18% (n = 62/68) for *Lactobacillus* and 32.35% (n = 22/68) for the *Bifidobacterium*. Only 8.82% (n = 6/68) of the samples contained

Table 2. PCR products of 16S rRNA genes.

Genus and Species	Primers	PCR product (bp)
Bifidobacterium	Bif 164 - Bif 662	523 bp
B. lactis	Lw 420C - Lw 420D	845 bp
B. longum	Bilo 1 - Bilo 2	831 bp
Lactobacillus	Lac 1 - Lac 2	247 bp
L. reuteri	Lreu 1 - Lreu 2	1411 bp
L. longum	Rham 1 - Rham R	158 bp

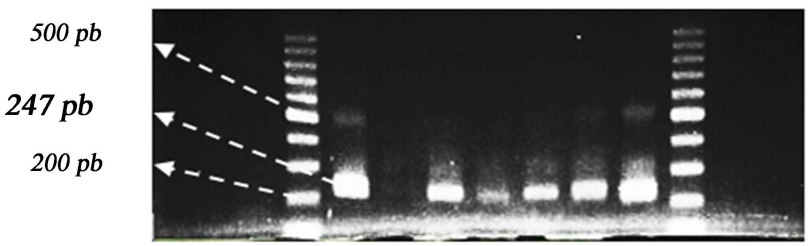

Figure 1. *Lactobacillus* 16S rRNA gene detection.

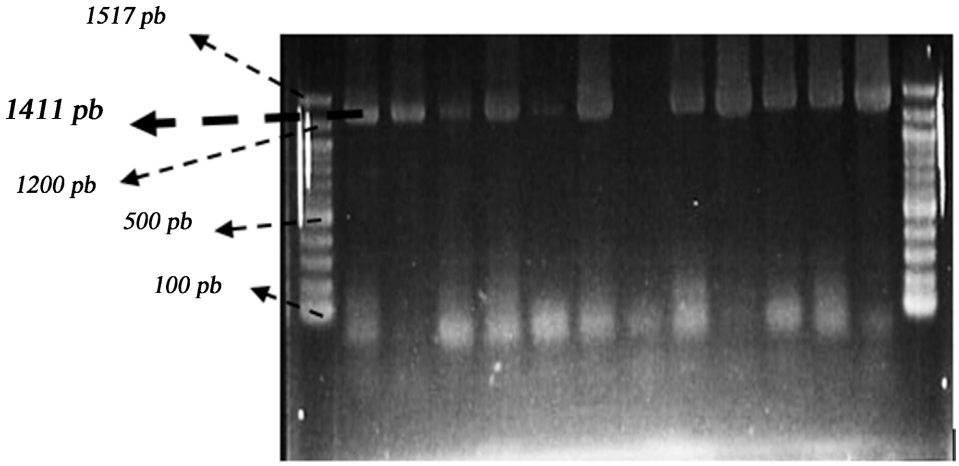

Figure 2. *Lactobacillus reuteri* 16S rRNA gene detection.

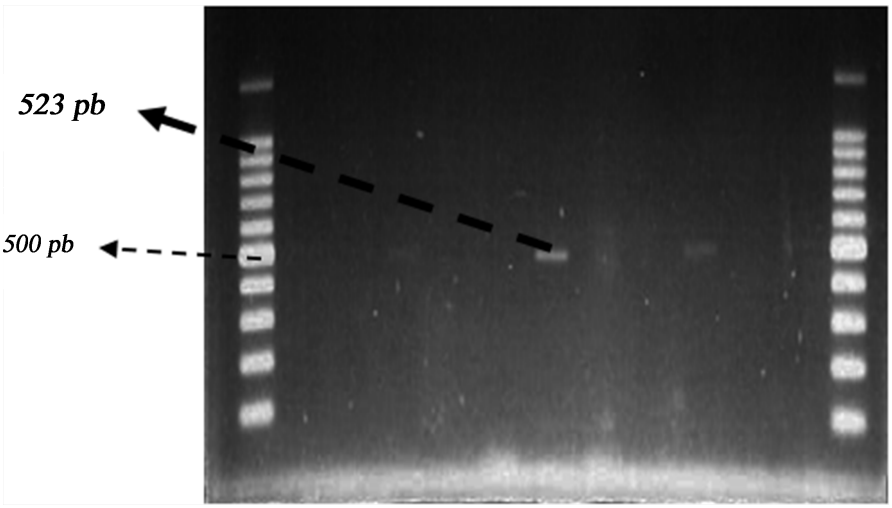

Figure 3. *Bifidobacterium* 16S rRNA gene detection.

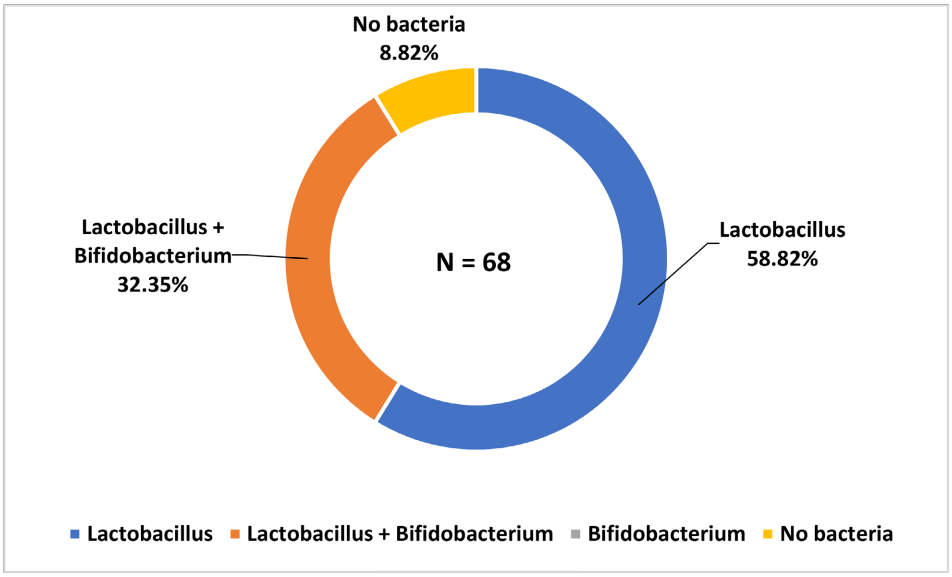

Figure 4. Frequency of *Lactobacillus* and *Bifidobacterium* 16S rRNA genes.

no *Lactobacillus* or *Bifidobacterium* 16S rRNA gene.

When all positive samples were amplified to the species level in the second round, only the *Lactobacillus reuteri* was detected in 89.71% (n = 61/68). All other species tested including *Lactobacillus rhamnosus*, *Bifidobacterium lactis* and *Bifidobacterium longum* were not detected as shown in Figure 5.

Further analysis concerned the factors linked to the presence of bacteria in breast milk. The women were divided into three groups based on the bacterial 16S rRNA gene found in their breast milk: women with *Bifidobacterium* and *Lactobacillus*, women with *Lactobacillus* alone, and women without either genus. The numbers of women in the different groups, their means age and the numbers of pregnancies are shown in Table 3. The mean age of the women did not differ between the three groups (p = 0.129). Thus, the age of the women in this study does not influence the presence of *Lactobacillus* or *Bifidobacterium* in breast milk. The mean number of pregnancies per woman did not differ between the three groups (p = 0.579). The number of maternal pregnancies did not affect the presence of *Lactobacillus* or *Bifidobacterium* in milk. Using the FISCHER test, there was an association between the presence of the *Lactobacillus* 16S rRNA gene in milk and the educational level of the women (p = 0.008*). There was also an association between educational level and the presence of *Bifidobacterium* and *Lactobacillus* 16S rRNA genes in the same samples (p < 0.001*). The presence rate of 16S rRNA genes was higher in low-educated groups of women.

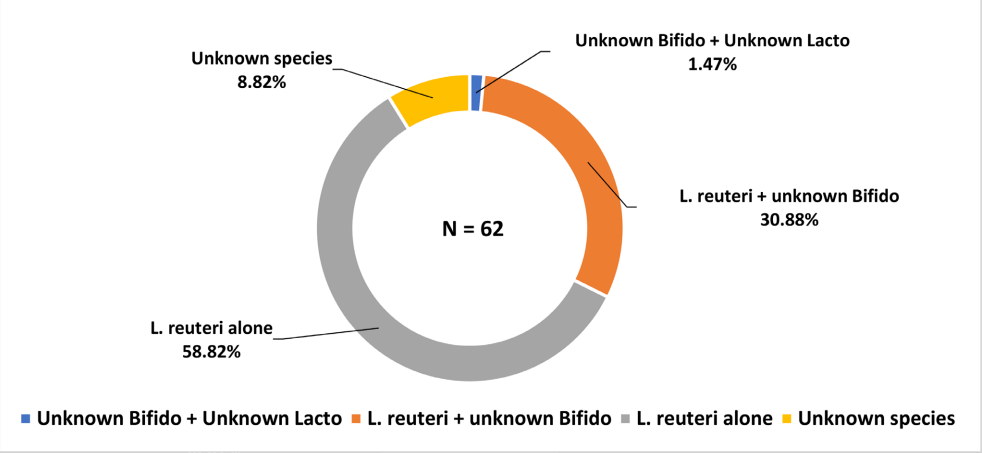

Figure 5. Frequency of *Lactobacillus* and *Bifidobacterium* species 16S rRNA genes.

Table 3. Age and number of children in different groups of women.

Bacterial species	16S rRNA gene presence in breastmilk			p-value
	Bifidobacterium + *Lactobacillus*	*Lactobacillus*	None	
N	22	40	6	
Age	26.36 ± 5.99	28.79 ± 4.93	25.5 ± 3.5	0.129
Number of children	2.31 ± 1.52	2.64 ± 1.51	2.14 ± 0.89	0.579

4. Discussion

In this study, only 55.07% of mothers practiced exclusive breastfeeding, while the MICS reported almost 53.5% in DRC in 2018 (National Institute of Statistics 2017-2018). The mean age of 27.7 ± 5.3 in the present study is close to that of another study that looked at bacteria and fatty acids in breast milk. In that study (n = 80), the average age of the women examined was 33 years. In addition, the bacterial identification result was similar to ours, with the *Lactobacillus* family (Lactobacillaceae) being the most common in human milk [29].

The present study reports the presence of *Lactobacillus* in 91.18% of women, while *Bifidobacterium* was found in only 32.35% of them. In contrast, a study by the University of Madrid describing the bacterial diversity of 50 women in Spain showed a higher 100% presence rate for both *Lactobacillus* and *Bifidobacterium* in women's breast milk [22]. In comparison, the mean age in another study conducted by Soto in 2014 was 31.82 years, the *Lactobacillus* was the most prevalent genus at a lower rate (67.5%) than in this study (91.18%). The genus *Bifidobacterium* was also less represented with 25.6%. Different species were identified in the same study, including *Lactobacillus reuteri* (11.8%), *Lactobacillus rhamnosus* (8.1%), *Bifidobacterium longum* and *Bifidobacterium lactis* (4.3%) [28]. The present study showed only 89.71% of *Lactobacillus reuteri*. The other species have not been detected.

The association of more than two types of bacteria in the same breast milk sample was also found in another study by Soto *et al*. reported in 2014, with 1.2% *Lactobacillus reuteri*/*Lactobacillus rhamnosus* association and 1.2% *Lactobacillus reuteri*/*Bifidobacterium longum* association. In the present study, a higher percentage of the association of *Lactobacillus reuteri* and unidentified *Bifidobacterium* (30.88%) was reported in **Figure 5**. It has been suggested that the high levels of *Lactobacillus* and low levels of *Bifidobacterium* in breast milk are due to excessive weight gain in women during pregnancy [8]. In fact, a correlation has been found between body mass index and bacterial diversity in breast milk in Mexican women [30]. Unfortunately, the body mass index was not evaluated in the present study.

The results reported above show variation between these different studies; This can be explained by the peculiarities of each population due to genetic factors, diet, geographic location, and inter-individual variations [31]. Although the origin of lactic bacteria is the subject of many hypotheses, all authors agree that maternal skin flora, maternal intestinal flora, infant oral cavity flora are the most likely source of microbial colonization of breast milk [7] [9] [32] [33] [34] [35] [36].

Our results further showed that only educational level was associated with the presence of the 16S rRNA gene in breast milk (p = 0.008*). Women with beneficial bacteria in their breast milk were those with low-education. Our results are consistent with Gomez in 2016, who noted that all factors that can modulate the composition of the microflora of the mother's skin, oral cavity, vagina and gut

probably do as well modulate the composition of the microbiota in breast milk. The level of education of the woman affects the observance of hygiene rules (food, skin and vagina). Also, the level of education through its influence on the mother's lifestyle would determine the presence of bacteria in the milk. Women with high educational level adopt a modern lifestyle that has a negative impact on the effectiveness of the mother's immune system. This is the most likely route for transmission of bacteria from the digestive tract into breast milk [31].

In 2013, Leonides showed the differences between the composition of the skin microbiota and the microflora of breast milk, whereby not all bacterial species of women's skin were present in breast milk. Also, the breast milk bifidobacteria are anaerobic bacteria that make the skin or mouth an unfavorable environment for their growth. Consequently, the gut and vaginal microflora could be the most likely source of *Lactobacillus* and *Bifidobacterium* in breast milk [13].

5. Conclusions

The aim of this study was to identify the specific 16S rRNA gene of *Lactobacillus* and *Bifidobacterium* in the breast milk of women living in the NGABA area of Kinshasa. Four species of these bacteria were selected according to the beneficial effects of some of their strains on infant health. The genus *Lactobacillus* and *Bifidobacterium* were identified by amplification of the specific 16S rRNA gene. Using a conventional PCR protocol, the presence of *Lactobacillus* and *Bifidobacterium* in the breast milk of the selected women was confirmed. Most women have been found with the combination of both bacteria. *Lactobacillus reuteri* (16S rRNA gene) was the only species that could be identified in the samples containing the *Lactobacillus*. In this study, none of the *Bifidobacterium* could be identified at the species level.

These preliminary results require further studies using more appropriate techniques such as multiplex PCR and 16S rRNA gene sequencing for a better understanding of the bacterial diversity of breast milk in the DR Congo.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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