

CHARACTERIZATION OF LACTOBACILLUS ISOLATED FROM THE MICROFLORA OF DIFFERENT FERMENTATION PROCESSES AND DODERLEIN FLORA.

Abstract

Lactobacillus contribute to the organoleptic quality of foods during the fermentation process in which they are involved. They also have the ability to inhibit the growth of undesirable germs by producing metabolites such as bacteriocins. The aim of this study was to isolate and identify the different *Lactobacillus* species from various fermentation processes and from the Doderlein flora.

Lactobacillus were isolated from raw cow's milk, curdled milk, cassava ferment (Magnan) and Doderlein flora on MRS agar. Strains were identified by mass spectrometry (MALTI-TOF).

213 *Lactobacillus* strains were isolated and identified from the different matrices. The main species isolated from raw cow's milk and curdled milk were *Lactobacillus plantarum* (31.58%), *Lactobacillus casei* (26.31%), *Lactococcus lactis* (21.05%), *Lactobacillus delbrueckii spp bulgaricus* (31%), *Lactobacillus helveticus* (30%) and *Lactobacillus fermentum* (25%) respectively.

In the cassava ferment, *Lactobacillus plantarum* (37.5%), *Lactobacillus rhamnosus* (25%) and *Lactobacillus reuteri* (25%) were the most isolated species. The main species of Doderlein flora were *Lactobacillus acidophilus* (50%) and *Lactobacillus plantarum* (25%).

This study showed a diversity of *Lactobacillus* present and involved in the different fermentation processes of foods and Doderlein flora. The qualitative and quantitative proportions of the different *Lactobacillus* species depend on the matrix. These *Lactobacillus* can potentially produce substances (bacteriocins) that inhibit the growth of pathogenic bacteria and multi-resistant bacteria.

Key words : *Lactobacillus*, Biodiversity, Fermented food, Doderlein flora.

1. INTRODUCTION

Lactobacillus form a heterogeneous group of bacteria, ubiquitous and widespread in the plant, animal and human environment [1 ; 2]. Bacteria of the genus *Lactobacillus* sp are Gram-positive, immobile, facultative anaerobic and homofermentative bacteria [3 ; 4]. The species are numerous and relatively difficult to characterise and identify. Rarely pathogenic to

humans, *Lactobacillus* probably also play a physiological role in humans and animals. They make a major contribution to many food preparations, particularly through lactic fermentation, in the production of cheeses, yoghurts and many other milk derivatives [2]. They are ubiquitous in many biotopes, such as fermented foods [5]. *Lactobacillus* are widely used in the food industry because of their fermenting power. They also contribute to the organoleptic quality of foodstuffs during the fermentation process, where they are involved [6]. Several studies have demonstrated the inhibitory power of *Lactobacillus* through the production of metabolites such as bacteriocins [7 ; 8] These bacteriocins have the ability to inhibit the growth of undesirable germs. In Ivory Coast, *Lactobacillus* are involved in several fermentation processes, such as the preparation of cassava ferment (mangna), the preparation of curdled milk and the fermentation of cocoa beans. *Lactobacillus* play a dual role in these fermentation processes. Firstly, they contribute to the final organoleptic quality of the food and, secondly, they inhibit the growth of pathogenic bacteria [9]. *Lactobacillus* are naturally present in the Döderlein flora (vaginal flora) where they play a protective role in the vaginal cavity against the proliferation of pathogenic germs [10 ; 11 ; 12]. The determination of the biodiversity of *Lactobacillus* in the various fermentation processes and in the Döderlein flora is more than necessary for studying their inhibiting power on multi-resistant bacteria. The aim of this study is to identify the different *Lactobacillus* species involved in the main fermentation processes and the Döderlein flora, for the study of their inhibitory power.

2. MATERIALS AND METHODS

2.1. Sample collection

A total of 180 samples belonging to 3 food matrices (raw cow's milk, curdled milk and manioc ferment) and to the Döderlein flora were taken in different localities in the city of Abidjan in Ivory Coast (Table I). These localities were chosen because of their high production of these food matrices. Samples (vaginal swabs) of Döderlein flora were provided by the Genital Tract Agents Unit (GT-AU) of the Institut Pasteur de Côte d'Ivoire (IPCI).

Table I : Distribution of the samples according to the differents localities in Abidjan

	Raw cow's milk		Curdled milk		Cassava ferment		Döderlein flora
Location	Anyama	Adiopodoumé	Abobo	Adjamé	Anyama	Cocody	IPCI
Number	20	30	15	25	30	30	30
Total	50		40		60		30

2.2. Enumeration of *Lactobacillus* sp in food matrices

For each matrix, 10 g (solid feed) or 10 ml (liquid feed) of the sample was added to 90 ml of buffered peptone water. The mixture was homogenised in a stomacher bag. This solution constituted the stock solution. From this stock solution, 1 mL was taken and mixed with 9 mL of buffered peptone water in a test tube. This gives the 10^{-1} dilution. Then 1 ml of the previous dilution is added to 9 ml of buffered peptone water to obtain the next dilution. The other decimal dilutions were made up to 10^{-8} . Petri dishes (2 dishes per dilution considered) were inoculated by spreading with a rake on the surface of MRS agar. The Petri dishes were then incubated at 37°C in a jar under anaerobic conditions and at 44°C from 24 to 48 hours. For the various counts, all Petri dishes containing at least 30 colonies and at most 300 colonies were selected. The results were expressed in Colony Forming Units (CFU) using the formula (NF ISO 7218/A1) [13].

$$N = \frac{\sum C_i}{((N_1 + 0,1N_2)d.V)}$$

Where, $\sum C$: is the sum of the colonies counted on all the plates retained from two successive dilutions

V: the volume of inoculum applied to each dish

N1: the number of plates retained for the first dilution considered

N2: the number of boxes retained for the second dilution considered

D: first dilution considered

2.3 Morphological identification of *Lactobacillus* sp

On the Petri dishes (10^{-4} to 10^{-7}) used for counting, five colonies chosen at random on the basis of their macroscopic appearance on MRS agar were identified. Gram-positive bacilli or cocci colonies, catalase and oxidase negative [14]. were selected for further work. Presumptive colonies of *Lactobacillus* sp were stored at -20°C in Eppendorf tubes containing Brain Heart Broth (BCC) plus glycerol [3].

2.4. Identification by mass spectrometry (MALDI-TOF)

To carry out this test, the colony of *Lactobacillus* sp in subculture for 24 hours is removed using a 10 μl calibrated loop and then placed on the spot on the plate. Each plate can be used to identify 24 strains. A matrix (α -Cyano-4-hydroxycinnamic acid) is used to cover each spot. The operation is carried out using a reference strain of *Escherichia coli* DH5 alpha, which was used as a quality control for the MALDI-TOF identification manipulation. The plate was then

inserted into the mass spectrometer. The various results were read on the computer workstation (laboratory computer system) associated with the spectrometer [15].

3. Results

3.1. Enumeration of *Lactobacillus* sp

The enumeration results observed according to the different temperatures ranged from $0.549 \cdot 10^7$ CFU/ml to $11 \cdot 10^7$ CFU/ml. They are reported in Table II. Enumeration shows that the average number of *Lactobacillus* strains varies from $5 \cdot 10^6$ CFU/ml to $1.67 \cdot 10^7$ CFU/ml in the Anyama commune and $2.98 \cdot 10^7$ CFU/ml in Yopougon (Adiopodoumé); in samples of raw cow's milk; in cassava ferments for the two communes the average number of *Lactobacillus* strains varies between $3 \cdot 10^6$ CFU/ml and $1.5 \cdot 10^7$ CFU/ml; in curdled milk samples (Adjamé and Abobo), the number varies between $1.9 \cdot 10^7$ CFU/ml and $11 \cdot 10^7$ CFU/ml (Table II).

Table II : Average number of colonies in CFU/ml observed by matrix for each commune

	Raw cow's milk		Cassava ferments		Curdled milk	
Location	Anyama	Adiopodoumé	Cocody	Ebimpé	Adjamé	Abobo
37 °C	$1,67 \cdot 10^7$	$2,98 \cdot 10^7$	$1,5 \cdot 10^7$	$0,875 \cdot 10^7$	$1,9 \cdot 10^7$	$9 \cdot 10^7$
44 °C	$5 \cdot 10^6$	$5 \cdot 10^6$	$3 \cdot 10^6$	$3 \cdot 10^6$	$7,123 \cdot 10^7$	$11 \cdot 10^7$

3.2 Isolation of *Lactobacillus* sp.

A total of 213 strains of *Lactobacillus* sp were isolated from different matrices

- 60 strains of raw cow's milk,
- 57 strains of curdled milk,
- 63 strains of manioc ferments
- 33 strains of human Doderlein flora. All were selected for identification by MALDI-TOF.



(A)

(B)

Figure 1 : Enumeration of *Lactobacillus* strains on MRS medium (Figure A) and subculture of *Lactobacillus* strains on MRS medium for identification (Figure B).

3.3 Morphological characterisation (GRAM staining)

Microscopic observation after Gram staining identified two forms: shells and rods. The rod-shaped forms represent 90% of the total number of bacteria and are represented by *Lactobacillus* sp strains (all Gram-positive, immobile, catalase-negative bacilli). The shells observed make up the remaining 10%. **Figure 2**.

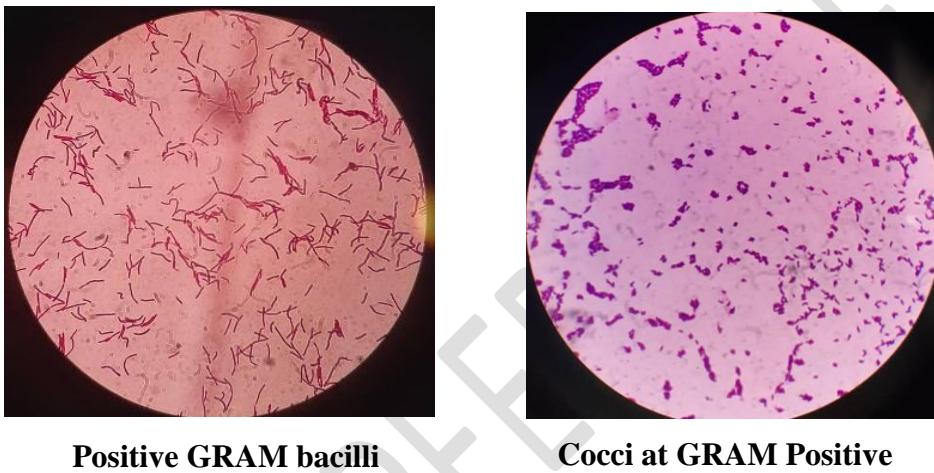


Figure 2 : different morphologies of *Lactobacillus* sp strains after Gram staining

3.4. Identification by mass spectrometry (MALDI-TOF)

Identification by MADI-TOF identified a total of 10 *Lactobacillus* species : *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus delbrueckii spp bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus fermentum*, *Lactobacillus rhamnosus* and *Lactobacillus reuteri*, *Lactobacillus paracasei*, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus* isolated from four matrices (raw cow's milk, curdled milk, cassava ferment and human Döderlein flora).

Seven (7) *Lactobacillus* species were identified from raw cow's milk. Among these, *Lactobacillus plantarum* was the most dominant species with a rate of 37.25%, followed by *Lactobacillus casei* with a rate of 31.5%. On the other hand, the species *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus reuteri* and *lactococcus lactis* were isolated very little, with proportions between 6.25 and 2.5%. (Table III).

Table III: Identification of *Lactobacillus* species isolated from raw cow's milk

<i>Lactobacillus</i>	La.	Lb.	Lb.	Lb.	Lb.	Lb.	Lb.
----------------------	-----	-----	-----	-----	-----	-----	-----

<i>species</i>	<i>lactis</i>	<i>acidophilus</i>	<i>rhamnosus</i>	<i>reuteri</i>	<i>plantarum</i>	<i>caséi</i>	<i>paracaséi</i>
<i>Number of strains</i>	2	5	5	5	30	25	8
<i>Percentage</i>	2,5 %	6,25 %	6,25 %	6,25 %	37,25 %	31,25 %	10 %

La. : *Lactococcus*

Lb. : *Lactobacillus*

The identification results revealed five (5) *Lactobacillus* species in the curdled milk (Table IV). Among the different species identified, *Lactobacillus fermentum* and *Lactobacillus bulgaricus* were the most isolated with proportions of 45.45 and 24.2% respectively. Very few *Lactobacillus plantarum* species were isolated (6.1%) (Table IV).

Table IV: Identification of *Lactobacillus* species isolated from curdled milk

<i>Species of Lactobacillus</i>	<i>Lb bulgaricus</i>	<i>Lb helveticus</i>	<i>Lb fermentum</i>	<i>Lb plantarum</i>	<i>Lb caséi</i>
<i>Number of strains</i>	8	3	15	2	5
<i>Percentage</i>	24.2%	9.1%	45.45%	6.1%	15.15%

Lb. : *Lactobacillus*

Four (4) *Lactobacillus* species isolated from cassava ferments were identified (Table V). It should be noted that *Lactobacillus plantarum* was the most dominant species with a rate of 50% out of 35 strains tested, followed by *Lactobacillus* species (*Lactobacillus rhamnosus* and *Lactobacillus reuteri* (21.43%) and finally the least dominant species was *Lactobacillus casei* with a rate of (7.15%) (Table V).

Table V: Identification of *Lactobacillus* species isolated from cassava ferment.

<i>Species of Lactobacillus</i>	<i>Lb rhamnosus</i>	<i>Lb reuteri</i>	<i>Lb caséi</i>	<i>Lb plantarum</i>
---------------------------------	---------------------	-------------------	-----------------	---------------------

<i>Number of strains</i>	15	15	5	35
<i>Percentage</i>	21.43 %	21.43 %	7.15 %	50 %

Lb. : Lactobacillus

Of the five (5) *Lactobacillus* species identified in the Döderlein flora, *Lactobacillus acidophilus* was the most dominant, with a percentage of 46.7%. The *Lactobacillus bulgaricus* species was the least dominant, with a low rate of 6.7%. It should also be noted that the *Lactobacillus plantarum* species was isolated to a lesser extent with a rate of 23.3% (Table VI).

Tableau VI : Identification of *Lactobacillus* species isolated from the Döderlein flora

<i>Species of Lactobacillus</i>	<i>Lb acidophilus</i>	<i>Lb plantarum</i>	<i>Lb bulgaricus</i>	<i>Lb paracaséi</i>	<i>Lb fermentum</i>
<i>Number of strains</i>	14	7	2	3	4
<i>Percentage</i>	46,7 %	23.3 %	6,7 %	10 %	13 %

Lb. : Lactobacillus

1. DISCUSSION

The results of identification by mass spectrometry (MALDI-TOF) showed a diversity of *Lactobacillus* species isolated from raw cow's milk, curdled milk, Döderlein flora and cassava ferments.

Seven (7) species of *Lactobacillus* (*Lactococcus lactis*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus reuteri*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus paracasei*) isolated from raw cow's milk were identified using the MALDITOF technique. Our results are similar to those reported by [16 ; 17] who identified 5 of our *Lactobacillus* species namely *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus plantarum* *Lactobacillus rhamnosus* and *Lactococcus lactis*. These species have been identified in raw cow's milk and camel's milk. The wide diversity of *Lactobacillus* species isolated from our samples could be explained by the different methods used to identify the different species. According to the results obtained in our experiment, we also agree with those reported by [18] who identified by MALDITOF and PCR the same *Lactobacillus* species (*Lactobacillus bulgaricus*, *Lactobacillus fermentum*, *Lactobacillus*

plantarum, *Lactobacillus rhamnosus*, *Lactobacillus acidophilus* and *Lactobacillus casei*) isolated from sheep's milk; cheese; fermented milk products; other dairy and meat products.

Our results are in line with those reported by [19] who identified a strain of the *Lactobacillus plantarum* species isolated from samples of raw cow's milk [20 ; 21 ; 22 ;24]. These authors identified two species of *Lactobacillus* (*Lb. plantarum* and *Lb. rhamnosus*) in raw cow's milk, whereas in our samples of raw cow's milk this species was largely dominant and represented 30 strains of *Lactobacillus*. We can deduce that this large difference could be due to the diversity of samples used in our work to identify this species.

Our results are virtually identical to those reported by [24 ; 25] who in their study identified two *Lactobacillus* strains isolated from raw cow's milk and a lactic ferment and belonging to the following species namely *Lactobacillus acidophilus* (6 strains), *Lactobacillus helveticus* (1 strain), *Lactobacillus lactis* (1 strain) and *Lactobacillus bulgaricus* (1 strain).

Lactobacillus species such as *Lactobacillus bulgaricus*, *Lactobacillus helveticus*, *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus casei* were identified and isolated from curdled milk in our study. Two species from our study were also highlighted by [26 ; 27] who in their study identified *Lactobacillus fermentum* and *Lactobacillus plantarum* species isolated from fermented milk. The diversity of *Lactobacillus* species identified in our raw cow's milk samples could be explained by the large number of isolates isolated from raw cow's milk, unlike those isolated in their study.

The presence of *Lactobacillus* species such as *Lactobacillus plantarum*, *Lactobacillus reuteri*, *Lactobacillus rhamnosus* and *Lactobacillus casei* isolated from cassava ferments is thought to be due to the effect of the lactose present in cassava and the acid pH [28]. Our results are similar to those reported by these authors who identified four (4) *Lactobacillus* species, the majority of which are *Lactobacillus plantarum*.

Five (5) *Lactobacillus* species were identified from the Döderlein flora. Our results are in agreement with those reported by [10] ; who in their study identified 14 species of *Lactobacillus* all representative of the vaginal microflora, including *Lactobacillus acidophilus* and *Lactobacillus fermentum*.

Our results are practically similar to those reported by [29] who concluded that the *Lactobacillus* most frequently isolated from healthy vaginas are in approximate proportions. These *Lactobacillus* are *Lactobacillus fermentum* (1.1%), *Lactobacillus crispatus* (48.3%), *Lactobacillus jensenii* (25.3%), *Lactobacillus gasseri* (23.5%), *Lactobacillus iners* (20.5%), *Lactobacillus vaginalis* (11.6%), *Lactobacillus reuteri* (1.4%), *Lactobacillus rhamnosus* (0.9%).

1. CONCLUSION

This study enabled us to quantitatively and qualitatively identify several *Lactobacillus* species involved in the various food fermentation processes (raw cow's milk, curdled milk and cassava ferment) and present in the Döderlein flora. This diversity could be an asset, increasing the chances of finding species that produce inhibiting substances (bacteriocins). The bacteriocins produced by these species could contribute to the fight against pathogenic bacteria and multi-resistant bacteria.

7. Références

1. **Yamamoto Y., Togawa Y., Shimoska M., & Okazaki M. (2003).** Purification and characterization of novel bacteriocin produced by *Enterococcus faecalis* strain. Applied and Environmental Microbiology., 69(10) :5746–5753.
2. **Tailliez P. (2004).** Les lactobacilles : propriétés, habitats, rôle physiologique et intérêt en santé humaine. Institut National de la Recherche Agronomique, ; 6 : 35-41.
3. **Badis A., Laouabdia-Sellami N., Guetarni D., Kihal M. & Ouzrout R. (2005).** Phenotypic characterisation of lactic acid bacteria isolated from raw goat's milk from two local goat populations, Arabia and Kabyle. Science and Technologie, N°23 : 30-37.
4. **Leila I.A., (2020).** Study of the biochemical and physiological characteristics of *Lactobacillus* isolated from curdled milk and enhancement of their technological abilities. Master's thesis in Nutrition and Human Dietetics, Faculty of Natural and Life Sciences. University of Blida. P1-65.
5. **Hammi I. (2016).** Isolation and characterization of bacteriocins produced by strains of lactic acid bacteria isolated from Moroccan fermented products and different varieties of French cheeses. Doctoral thesis in Analytical Chemistry, Faculty of Pharmacy, Université strasbourg 150p.
6. **Requena T and Buist G. (2000).** Applied and Environmental Microbiologie, Aug., Pp: 3174-3179.)
7. **Mechai A., (2009).** Physiological and biochemical studies on the isolation, characterisation and purification of bacteriocins produced by indigenous lactic acid bacteria. Ph.D. Thesis in Biochemistry, University of Annaba, 99p.
8. **Dortu C., Thonart P. (2009).** Les bacteriocines des bacteries lactiques : caracteristiques et intérêt pour la bioconservation des produits alimentaires, biotechnol. Agron Soc. Environ. 13(1) 143-154.

9. **Giraffa, G., Chanishvili, N., Widyastuti, Y., (2010).** Importance of lactobacilli in food and feed biotechnology. *Research in Microbiology* 161, 480–487.
10. **Benamoura W., & Bouhelais F. (2012),** Vaginal lactobacilli: Isolation, identification and study of probiotic abilities Master's thesis, Option: Applied Microbiology Université de Jijel P1-88.
11. **Bergogne-Bérézin E. (2007).** Normal vaginal flora, bacterial vaginitis and vaginosis: diagnosis and treatment. *Antibiotique*, 9,139-144.
12. **Belgy Arcangeli.M.T. (2015).** From vaginosis to vaginitis and sometimes more. *Les entretiens de bichat*.1-4
- 13.
14. **Ghazi F., Henni DE., Benmchernene Z., & Kihal M., (2005).** Phenotypic and Whole Cell Protein Analysis by SDS-PAGE for Identification of Dominants Lactic Acid Bacteria Isolated from Algerian Raw Milk. *World Journal of Dairy & Food Sciences*, 4 (2009) 78-87.
- 15.
16. **Sadi1 F., Dilmi Bouras A., Ghomari F. N., Hallouz F. & Noui A. (2017).** Phenotypic, molecular and technological characterization Of autochthonous lactobacilli strains isolated from cow's Milkand goat of algerian populations *Fundam Appl Sci*. 9(1), 339-353
17. **Van Hoorde K., Vandamme P., Huys G., (2008).** Molecular identification and typing of lactic acid bacteria associated with the production of two artisanal raw milk cheeses. *Dairy Sci. Technol.* 88 (2008) 445–455
18. **Dušková M., Šedo O., Kšicová K., Zdráhal Z., Karpíšková R., (2012).** Identification of lactobacilli isolated from food by genotypic methods and MALDI-TOF MS. *International Journal of Food Microbiology* 159 (2012) 107–114
19. **Bekhouche F., & Boulahrouf A. (2005).** Quantitative and qualitative studies of lactic bacteria in raw milk produced by local cows belonging to six breeding stations in Constantine. Departement of Nutrition and Agri-Food Technologies (D.N.A.T.A.A) Faculty of Sciences Mentouri University Constantine Algeria. No. 23, pp. 38-45.
20. **Moulay M., Benlahcen K., Aggad H., et Kihal M., (2013).** Diversity And Technological Properties Of Predominant Lactic Acid Bacteria Isolated From Algerian Raw Goat's Milk. *Adv. Environ. Biol.*, 7(6) : 999-1007, 2013

21. **Dworkin, M., S. Falkow, E. Rosenberg, K.H. Schleifer and E. Stackebrandt, (2006).** The prokaryotes, A Handbook on the Biology of Bacteria Volume 4 : Bacteria: Firmicutes, Cyanobacteria 6. *Edition*, Spinger. Singapore., pp : 205-311.
22. **Liu, M., J.R. Bayjanov, B. Renckens, A. Nauta, R.J Siezen, (2010).** The proteolytic system of lactic acid bacteria revisited: à genomic comparison. *BMC Genomics*, 11(36) : 2-15
23. **Mami, A., J.E. Henni and M. Kihal, (2012).** Screening of autochthonous *Lactobacillus* species from Algerian raw gaot's milk for the production of bacteriocin-like compounds against *Staphylococcus aureus*. *Afr. J. Microbiol. Res.*, 6(12) : 2888-2898.
24. **Allouche F. N., Hellal A. & Laraba A. (2010).** Study of the antimicrobial activity of thermophilic lactobacillus strains used in the dairy industry. *Nature et Technologie" journal*. N° 03 :13-20.
25. **Bouzaid M., Chatoui R., Hasib A., Mennane Z., (2012).** Hygienic quality of hawker milk sampled from outlets in the city of Rabat. *Laboratory technologies - 2012*, Volume 7, N°26.
26. **Bhardwaj A., Puniya M., Sangu K. P., Kumar S., Dhewa T., (2012).** "Isolation and biochemical characterization of lactobacillus species isolated from Dahi," *Research & Reviews : A Journal of Dairy Science and Technology*, vol. 1, pp. 18–31
27. **Goa T., Beyene G., Mekonnen M., Gorems K., (2020).** Isolation and Characterization of Lactic Acid Bacteria from Fermented Milk Produced in Jimma Town, Southwest Ethiopia, and Evaluation of their Antimicrobial Activity against Selected Pathogenic Bacteria. *International Journal of Food Science* Volume 2022, Article ID 2076021, 15 pages.
28. **Assanvo J.B., Agbo G.N., Behi Y.E.N, Coulin P., Farah Z. (2002).** The microflora of cassava ferment for the production of attiéké adjoukrou in Dabou (Côte d'Ivoire) *Bioterre, Rev. Inter. Life and Earth Science*, p15.
29. **Verhelst R. et al. (2005).** Comparaison between Gram stain and culture for the characterization of vaginal microfloral : Définition of a distinct grade that resembles grade I microfloral and revised categorization of grade I microflora. *BMC Microbiol.* 5 : 61.