



# Characterization of Lactobacillus Isolated from the Microflora of Different Fermentation Processes and Doderlein Flora

**Coulibaly Adja Mansagna<sup>a,b\*</sup>, Coulibaly Ibourahema<sup>a</sup>,  
Ouattara Mohamed Baguy<sup>b,c</sup>, Tiekoura Konan Bertin<sup>b,c</sup>,  
Guédé Kipre Bertin<sup>b,c</sup>, Toty Abale Anatole<sup>b,c</sup>,  
Konan Kouadio Fernique<sup>b,c</sup>,  
Mariko Safiatou Tenin. Soumahoro<sup>b,c</sup>,  
Nathalie Kouadio Guessennd<sup>b,c,d</sup> and Le GER-BMR<sup>c</sup>**

<sup>a</sup> *Laboratoire d'Agrovalorisation, Université Jean Lorougnon Guedé de Daloa, BP 150 Daloa, Côte d'Ivoire.*

<sup>b</sup> *Département de Bactériologie et Virologie, Institut Pasteur de Côte d'Ivoire, 01 BP 490 Abidjan 01, Côte d'Ivoire.*

<sup>c</sup> *Groupe d'Etude et de Recherche Sur les Bactéries Multi-Résistantes (GER-BMR), Institut Pasteur de Côte d'Ivoire, Côte d'Ivoire.*

<sup>d</sup> *Unité de Recherche et de Formation des Sciences Médicales, Université Felix Houphouët, Boigny, Abidjan, Côte d'Ivoire.*

## **Authors' contributions**

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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\*Corresponding author: E-mail: hadjamansagna@gmail.com;

## 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 Döderlein flora. *Lactobacillus* were isolated from raw cow milk, curdled milk, cassava ferment (Magnan) and Döderlein flora on MRS agar. Strains were identified by mass spectrometry (MALDI-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 Döderlein 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 Döderlein flora (Commensal flora of the vagina). 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.

**Keywords:** *Lactobacillus*; biodiversity; fermented food; döderlein 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 lactobacilli 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 [10-12]. 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 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 1). 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 1. Distribution of the samples according to the different localities in Abidjan**

Location	Raw cow milk		Curdled milk		Cassava ferment		Döderlein flora
	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 homogenized 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 gave the  $10^{-1}$  dilution. Then 1 ml of the previous dilution was 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 of 0.1  $\mu$ L spreading with a rake on the surface of MRS agar. The Petri dishes were then incubated 37°C in a jar under anaerobic conditions and at 44°C from 24 to 48 hours. For the bacterial 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 were selected for further work [14]. 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 was removed using a 10  $\mu$ l calibrated loop and then placed on the spot on the plate. Each plate can be used to identify 24 strains. A matrix ( $\alpha$ -Cyano-4-hydroxycinnamic acid) was used to cover each spot. The operation was carried out using the 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. Results were analyzed 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.  $10^7$  CFU/ml to 11.  $10^7$  CFU/ml. They are reported in Table 2. Enumeration shows that the average number of *Lactobacillus* strains varies from 5.110<sup>6</sup> CFU/ml to 1.67.110<sup>7</sup> CFU/ml in the Anyama commune and 2.98.  $10^7$ CFU/ml in Yopougou (Adiopodoumé); in samples of raw cow's milk; in cassava ferments for the two communes the average number of *Lactobacillus* strains varies between 3.10<sup>6</sup> CFU/ml and 1.5.  $10^7$  CFU/ml; in curdled milk samples (Adjamé and Abobo), the number varies between 1.9.  $10^7$  CFU/ml and 11.  $10^7$  CFU/ml (Table 2).

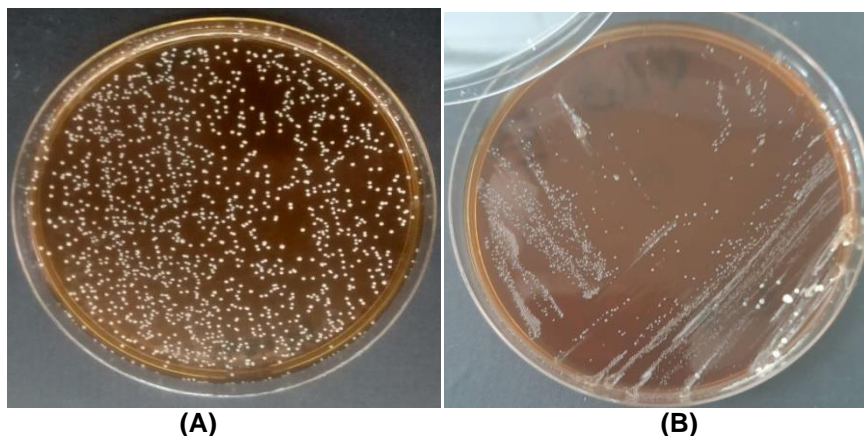
### 3.2 Isolation of *Lactobacillus* sp.

A total of 213 strains of *Lactobacillus* sp were isolated on MRS (fig.1) from different matrices

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

**Table 2. Average number of colonies in CFU/ml observed by matrix for each commune**

Location	Raw cow milk		Cassava ferments		Curdled milk	
	Anyama	Adiopodoumé	Cocody	Ebimpé	Adjamé	Abobo
37 °C	1,67.10 <sup>7</sup>	2.98.10 <sup>7</sup>	1,5.10 <sup>7</sup>	0.875.10 <sup>7</sup>	1,9.10 <sup>7</sup>	9.10 <sup>7</sup>
44 °C	5.10 <sup>6</sup>	5.10 <sup>6</sup>	3.10 <sup>6</sup>	3.10 <sup>6</sup>	7,123.10 <sup>7</sup>	11.10 <sup>7</sup>



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

### 3.3 Morphological Characterization (GRAM Staining)

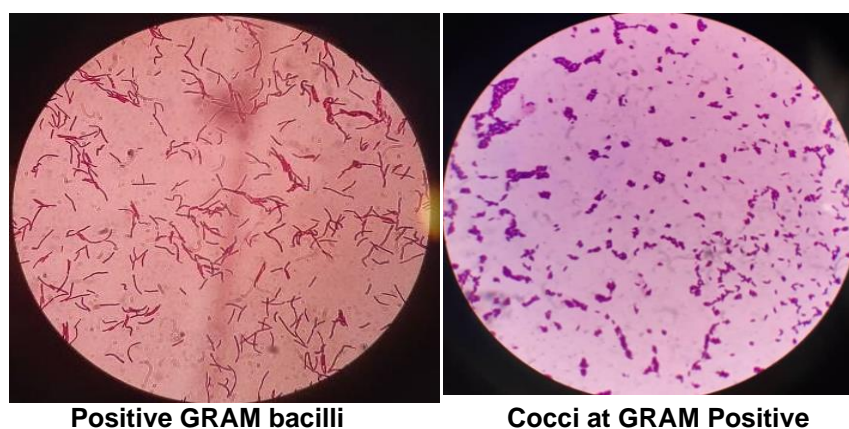
Microscopic observation after Gram staining identified two forms: shells and rods. The rod-shaped forms represent 90% of the total number of bacteria and are represented by *Lactobacillus* sp strains (all Gram-positive, immobile, catalase-negative bacilli). The shells observed make up the remaining 10%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

### 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 milk, curdled milk, cassava ferment and human Döderlein flora).

Seven *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 3).



**Fig. 2. Different morphologies of *Lactobacillus* sp strains after Gram staining**

**Table 3. Identification of *Lactobacillus* species isolated from raw cow milk**

<i>Lactobacillus</i> species	La. lactis	Lb. acidophilus	Lb. rhamnosus	Lb. reuteri	Lb. plantarum	Lb. caséi	Lb. paracaséi
Number of strains	2	5	5	5	30	25	8
Percentage	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 4). 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 4).

Four *Lactobacillus* species isolated from cassava ferments were identified (Table 5). 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 5).

Of the five *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 6).

#### 4. 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 species of *Lactobacillus* (*Lactococcus lactis*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus reuteri*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus paracasei*) isolated from raw cow milk were identified using the MALDI-TOF 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

**Table 4. Identification of *Lactobacillus* species isolated from curdled milk**

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

Lb. : *Lactobacillus*

**Table 5. Identification of *Lactobacillus* species isolated from cassava ferment**

<i>Lactobacillus</i> species	Lb rhamnosus	Lb reuteri	Lb caséi	Lb plantarum
Number of strains	15	15	5	35
Percentage	21.43 %	21.43 %	7.15 %	50 %

Lb. : *Lactobacillus*

**Table 6. Identification of *Lactobacillus* species isolated from the Döderlein flora**

<i>Lactobacillus</i> species	Lb acidophilus	Lb plantarum	Lb bulgaricus	Lb paracaséi	Lb fermentum
Number of strains	14	7	2	3	4
Percentage	46,7 %	23,3 %	6,7 %	10 %	13 %

Lb. : *Lactobacillus*

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 MALDI-TOF 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 [20-24]. 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 identical to those reported by [24,25] who in their study identified two *Lactobacillus* strains isolated from raw cow 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 *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%).

Among the *Lactobacillus* species isolated in our samples of the commensal flora of the vagina, *Lactobacillus acidophilus* is the most dominant species with a rate of 46.7%. Our results are similar to those reported by [30,31] who also found this species to be dominant in Chinese pregnant women. Our results are also in agreement with those reported by [32,33], who identified several *Lactobacillus* species in the vagina of women with normal flora, including *Lactobacillus acidophilus*.

## 5. CONCLUSION

This study enabled us to quantitatively and qualitatively identify several *Lactobacillus* species involved in the various food fermentation processes (raw cow 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.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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