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RESEARCH ARTICLE

DIVERSITE DES BACTERIES LACTIQUES IMPLIQUEES DANS LA FERMENTATION DU FRUIT DU TAMARIN (*Tamarindus indica*) CÔTE D'IVOIRE

DIVERSITYOF LACTICACID BACTERIAINVOLVEDIN THE FERMENTATION OF THE TAMARIND FRUIT (*Tamarindus indica*) CÔTE D'IVOIRE

Noka Lahissa Bakayoko[1,2], Souleymane Soumahoro[3,4], Konan Bertin Tiekoura[2], Ouattara Mohamed Baguy[2], Guede Kippre Bertin[2], Coulibaly Adja Mansadja[5], Nathalie Kouadio Guessennd[2] and Marina Koussemon-Camara[1]

1. Université NanguiABROGOUA, Laboratoire de Biotechnologie et Microbiologiedes Alimentaire, 02 BP 801 Abidjan 02, Côte d'Ivoire.
2. Institutpasteur de Côte d'Ivoire, Unité des Antibiotiques, des Substances Naturelles et de la Surveillance de la Résistance aux anti-Microbiens, 01 BP 490 Abidjan 01, Côte d'Ivoire.
3. Université Pelefero GON COULIBALY, Institut de Gestion Agropastorale, Laboratoire de Biochimie, Microbiologie et de Valorisation des Agroressources, BP 1328 Korhogo, Côte d'Ivoire.
4. Unité de Recherche et de Formation des Sciences Médicales Université Felix HOUPHOUÉTBoigny Abidjan Côte d'Ivoire.
5. Laboratoired'Agrovalorisation, Université Jean LOROUGNON GUEDÉ de Daloa.BP150 Daloa.

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Abstract

In Côte d'Ivoire, tamarind fruits, from trees growing in the northern part of the country, are used for various food purposes (seasoning dishes, producing acidic beverages, etc.), typically after a fermentation process. These fermented fruits could therefore represent a source of beneficial fermentative microorganisms for the development of starters in biotechnology. A study was therefore undertaken to determine the diversity of lactic acid bacteria involved in tamarind fermentation. For this purpose, a total of 400 tamarind samples were collected from various markets in the cities of Korhogo and Abidjan. Lactic acid bacteria from these samples were screened and counted on MRS agar, and then subjected to identification. A total of 664 strains were isolated, of which 430 isolates were retained. Genus characterization was performed by 16S rRNA gene PCR. For species determination of the isolates, MALDI-TOF proteomics was applied. The results obtained showed that the lactic acid bacteria isolated from tamarind belong to the genera *Pediococcus*, *Lactobacillus*, *Lacococcus*, and *Weissella*, with a dominance of the genus *Lactobacillus* (63%) compared to the total number of identified strains, followed by the genus *Pediococcus* (16%) and finally the genera *Weissella* (12%) and *Lactococcus* (8%). The genus Lactobacillus was represented by Lactobacillus plantarum (36%), *Lactobacillus fermentum* (20.5%), *Lactobacillus acidophilus* (20%), and *Lactobacillus rhamnosus* (23.5%). The genus *Pediococcus*was represented by *Pediococcusacidilactici*, the *Lactococcus* strains

Corresponding Author:- Noka Lahissa Bakayoko
Address:- Université NanguiABROGOUA, Laboratoire de Biotechnologie et Microbiologiedes Aliments, 02 BP 801 Abidjan 02, Côte d'Ivoire.

were solely composed of *Lactococcus lactis*. As for the genus *Weissella*, only *Weissella sp*. was involved in tamarind fermentation.

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Introduction:-

A diverse array of microorganisms inhabits the surfaces of fruits, including Gram-positive and Gram-negative bacteria, yeasts, molds, and lactic acid bacteria (LAB). While LAB constitute a minor fraction of the overall microbial flora, they play a pivotal role in spontaneous fermentation (lactic fermentation) when favorable conditions (temperature, nutrients, water) arise **(Di Cagno*et al*., 2013**). These fermentations are characterized by rapid growth and acidification, effectively inhibiting the growth of Gram-negative bacteria and yeasts, thereby extending the shelf life of food products. Additionally, with the production of antimicrobial compounds, LAB also emerge as potential candidates for the biopreservation of fruits and vegetables **(Fessard., 2017**).

In Côte d'Ivoire, the tamarind fruit thrives in the northern region of the country. It is consumed either raw or fermented by the local population. Post-harvest, the fruits undergo rapid decomposition due to the action of naturally occurring microorganisms and enzymes. With a limited shelf life, the fruits are often processed into various forms, such as juices, jams, cut pieces, and preparations. In most cases, stabilization treatments like blanching, pasteurization, fermentation, or drying are employed. Lactic acid fermentation, a food preservation and transformation technique employed for millennia **(Brandt *et al*., 2014)**, plays a significant role in the nutritional properties of foods, particularly fruits and vegetables.

Characterization of LAB species present in fruits represents a crucial step, both for the development of starters in fruit fermentation and for the identification of cultures for biopreservation of plant-based foods. Selected LAB should meet various technological, organoleptic, sanitary, and nutritional criteria **(Fessard., 2017**). Among the utilized LAB, Lb. plantarum and Lb. pentosus are the primary species commercialized as starters for fruit fermentation **(Tamang *et al*., 2016**). Fruits thus hold immense interest in the isolation of LAB with probiotic effects, also bearing industrial significance in vegetable fermentation.

This study aims to establish a collection of LAB strains involved in tamarind fermentation. These strains will be characterized using molecular and proteomic methods to determine their technological and antimicrobial properties, paving the way for their potential application as starter culture components in agro-industrial food production.

Materials and Methods:-

Tamarind Fruit Fermentation.

Tamarind undergoes a traditional fermentation process as depicted in Figure 1. The fermented product is then marketed in various forms, including paste, balls, alcoholic and non-alcoholic beverages, and others.

Sampling

The collection of 400 tamarind samples took place in two distinct regions of Côte d'Ivoire, namely the city of Korhogo in the northern part of the country and Abidjan in the south. Samples were obtained at different stages of fermentation, specifically at 24 hours, 48 hours, and 72 hours. Subsequently, the samples were transported to the laboratory for microbiological analyses.

Isolation and Purification of Bacteria

Lactic acid bacteria strains were isolated from tamarind. Isolation was carried out on MRS agar at 30°C for 48 hours (Institut Pasteur, Côte d'Ivoire). Purification was subsequently performed through successive subculturing on MRS agar and broth, with an incubation at 30°C for 24 hours, until colonies of uniform size, shape, and color were obtained, indicating the purity of the strains. Purified isolates were differentiated through macroscopic and microscopic examinations (Gram staining), and catalase testing. Gram-positive, catalase-negative strains were selected. Preservation of the strains was conducted in a medium containing 70% skim milk (enriched with 0.05% yeast extract and 0.05% glucose) and 30% glycerol, stored at a temperature of -20°C **(Saidi*et al*., 2002**).

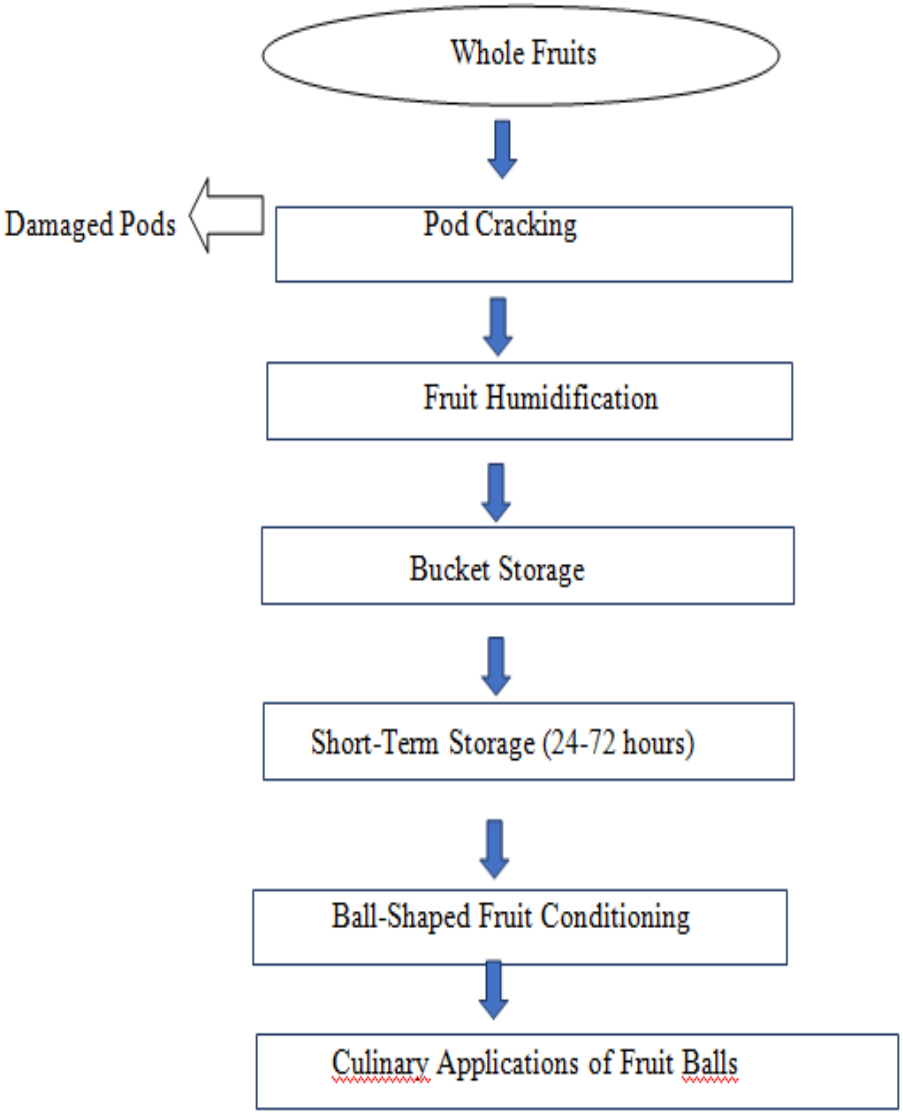


Figure 1:- Fermentation diagram of tamarind fruit.

Identification of Isolated Lactic Acid Bacteria Strains

Genus Determination

The genus identification of lactic acid bacteria isolates was conducted using the genomic method of the 16S rRNA gene.

DNA Extraction

The genomic DNA of the isolated lactic acid bacteria was extracted and purified using a Qiagen kit (Qiagen QIAamp DNA Kit). To do this, the lactic acid bacteria strains were each cultured on MRS agar and incubated at 37°C for 48 hours in an incubator. Subsequently, strain purification was performed on white agar. A sample of colonies from each bacterial culture was then mixed with 500 µL of sterile distilled water to create a bacterial suspension. 200 µL of the bacterial suspension was added to each tube containing 560 µL of lysis buffer (AL + Proteinase K). The mixture was vortexed for 15 seconds and then incubated at room temperature for 10 minutes. 560 µL of 95-100% ethanol was added to each tube, and the tubes were vortexed for 15 seconds before briefly centrifuging in a microcentrifuge to collect droplets on the tube lid. Next, for each sample, a Mini spin column was labeled and placed in a 2 mL collection tube (both provided in the kit). 630µL of the mixture was added to the column and centrifuged for 1 minute at 8000 rpm. The collection tube was emptied, and this step was repeated once more. 500 µL of AW1 buffer was added and centrifuged at 8000 rpm for 1 minute. The column from the collection

tube was placed in a new clean collection tube. Then, 500 µL of AW2 buffer was added and centrifuged at 14,000 rpm for 3 minutes. The columns from the collection tubes were placed in a sterile 1.5 mL Eppendorf tube labelled with the corresponding strain number. 50 µL of Buffer AE elution solution was introduced into the center of the column, and the mixture was incubated for 1 minute at room temperature before centrifuging at 8000 rpm for 1 minute. The column was removed and discarded; the 1.5 mL tube containing the DNA was placed on ice for immediate use or stored in the freezer at -20°C for future use.

PCR (Polymerase Chain Reaction)

Preparation of Reaction Mixtures

The polymerase chain reaction (PCR) involves amplifying the sequence of the 16S rRNA gene using specific primers (Fd1 and RD1) (Table 1) flanking the gene sequence, thus generating a 1500bp amplicon.

Table 1:- Primerusedforcarrying out the PCR.

Primer	Sequence 5'3'
Fd_1	5'-AGAGTTTGATCTGGCTCAG-3'
Rd1	5'-TAAGGAGGTGATCCAGCC-3'

PCR reactions are performed in a total volume of 25 µL, comprising 5 µL of master mix (Solis Biodyne), 13 µL of nuclease-free water (Ambion), 1 µL of each primer, and 5 µL of DNA sample. In the negative control, 5 µL of DNA is replaced with 5 µL of sterile H_2O (Table 2).

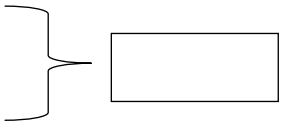
Table 2:- PCR Reaction Mixture.

Reagents (Buffer and Taq Platinum from Invitrogen)	Quantities in (µL) per tube
Masrer Mix (Solis Biodyne)	5
DNA Extract	5
Primer Fd1 (10**µ**M)	1
Primer RP1 (10**µ**M)	1
Nuclease-free Water (Ambion)	13

The microtubes are then placed in a thermal cycler (GeneAMP PCR System 9700, Applied Biosystems). Amplifications are performed according to the program outlined below.

Amplification Program.

Initial Denaturation: 94°C for 10 min
Denaturation: 94°C for 30 s
Annealing: 58°C for 1 min
Initial Extension: 72°C for 1 min
Final Extension : 72°C for 8 min



Revelation of PCR amplification products.

The revelation of the amplification products will be carried out through agarose gel electrophoresis prepared at a concentration of 2%. Within the supercooled agarose, a solution of SyBr® Safe DNA gel (Invitrogen) (8 µL per 100 mL) has been incorporated. The mixture will be thoroughly homogenized and the gel cast into a support. Migration will be conducted at 100 Volts for 25 minutes, and subsequently, the gel will be placed in an automated system equipped with Gel Doc EZ Imager (BioRad) for visualization. The presence of bands corresponding to the amplified fragment will be compared against the molecular marker's size as well as that of the positive control samples. The absence of bands will be construed as a negative result.

Species identification by MALDI-TOF

Bacterial isolates underwent MALDI-TOF MS whole-cell analysis. This method discriminates bacteria based on screening of observed peaks as protein biomarkers for bacterial identification (**Holland *et al.*, 1996**). This strategy is enhanced by utilizing one or more reference strains for each species to be included in the database (**Williams *et al.*, 2003; Chen *et al.*, 2008**).

Results:-

Isolation of lactic acid bacterial strains.

From the 400 tamarind samples, a total of 664 were obtained, from which 430 isolates were selected. Indeed, out of the 664 bacterial strains isolated through culture on MRS medium, 430 strains exhibited distinctive characteristics of lactic acid bacteria. Cultures grown on Petri dishes were visually inspected to characterize colony shape, size, appearance, and color. These colonies appeared small, circular or lenticular in shape, with a whitish color and either regular or irregular margins. The figure displays the macroscopic appearance of colonies, showing rounded, raised, white colonies of lactic acid bacteria cultivated on MRS agar, with a diameter ranging from 1 to 2 mm.

Gram staining of the isolates revealed bacilli or cocci with Gram-positive staining. The strains were also facultative anaerobes, catalase-negative, and all Gram-positive. Subsequently, these 430 strains were identified.

Genus Determination.

The genus of presumptive lactic acid bacterial isolates was confirmed through PCR targeting the 16S rRNA gene. Amplification of this gene yielded a single band of approximately 1500 base pairs, characteristic of the size of the 16S rRNA gene in lactic acid bacteria (Figure 2).

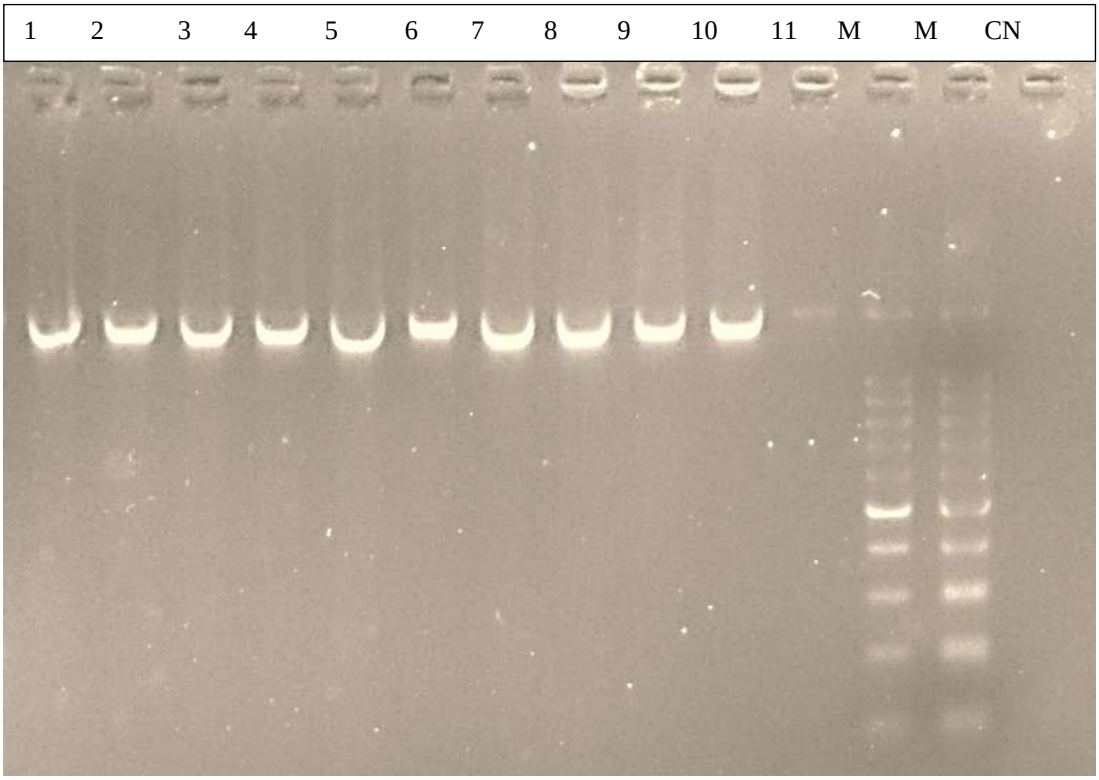


Figure 2:- PCR Profile Obtained after amplification of the 16S rRNA gene of Lactic acid bacterial isolates (Electrophoretic profile of the 16S rDNA gene of lactic acid bacterial isolates).

M: 1500 bp size marker
CP: Positive control
Lane 1 to 10: PCR profile of the gene encoding for the 16S rRNA of lactic acid bacterial isolates obtained from tamarind.
bp: base pairs

Diversity of lactic acid bacterial genera in tamarind.

The results of identifying the 430 lactic acid bacterial isolates using MALDI-TOF (**Figure 3)** showed the presence of four lactic acid bacterial genera in the analyzed tamarind samples. These genera are*Lactobacillus, Pediococcus, Weissella,* and *Lactococcus,* noted with frequencies of 64%, 16%, 12%, and 8%, respectively.

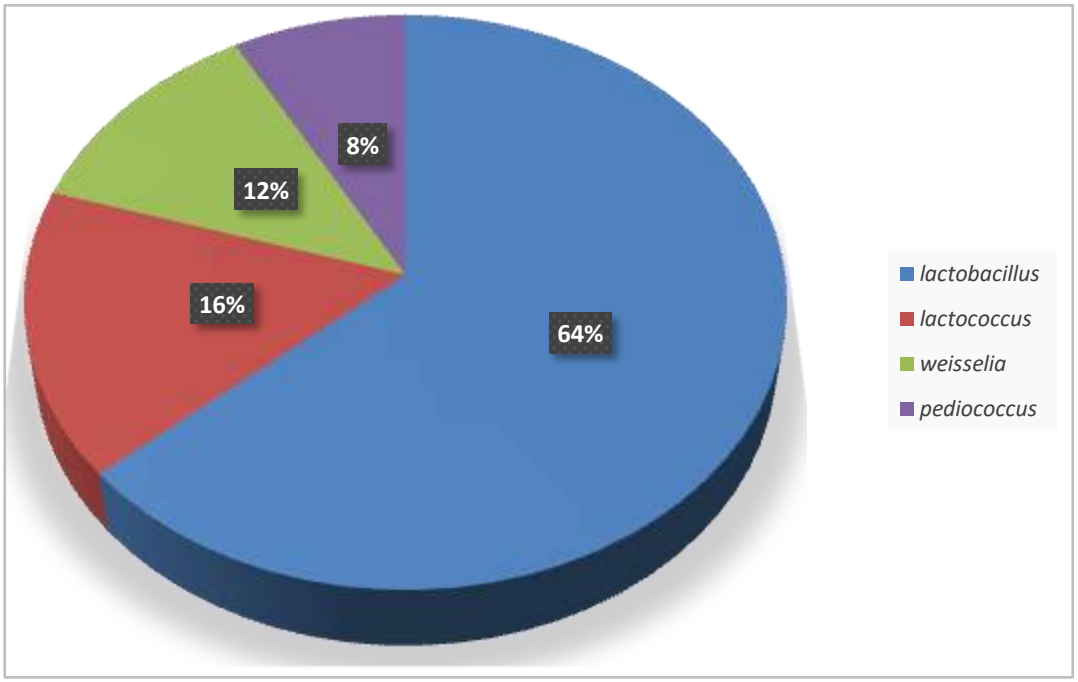


Figure 3:- Proportion of Lactic acid bacterial genera isolated from tamarind.

Different species of lactic acid bacteria involved in tamarind fermentation.

Identification via MALDI-TOF of lactic acid bacterial strains isolated from tamarind revealed species variability. Thus, the 430 isolates of lactic acid bacteria could be grouped into 6 clusters representing 6 species, namely *Lactobacillus fermentum*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, *Weissellasp*, *Pediococcusacidilactici*.

Tableau 3:-Proportionof lactic acid bacteria strainsisolatedfrom tamarindaccording to genus.

Genus	Species	Isolates	
		Number	Proportion %
Lactobacillus	*Lactobacillus fermentum*	83	36
	Lactobacillus rhamnosus	64	23,5
	Lactobacillus plantarum	57	20,5
Lactococcus	*Lactococcus lactis*	69	16
Pediococcus	*Pedicoccusacidilactici*	34	8
Weisselia	*weisseliasp*	52	12

During tamarind fermentation, a succession of lactic acid bacteria is observed. At the beginning of fermentation, four isolates were present, namely *Lactobacillus fermentum*, *Pediococcusacidilactici*, *Weissellasp*, and *Lactococcus lactis*, representing 5%, 55%, 43%, and 10%, respectively. After 24 hours of fermentation, among the identified species, *Weissellasp* becomes the predominant species, representing 47% of the isolates. *Lactobacillus plantarum* and *Lactobacillus rhamnosus* appear with low proportions of 10% and 13%, respectively. However, there is a disappearance of *Pediococcusacidilactici* and *Weissellasp* after 48 hours of fermentation, along with an increase in the number of *Lactobacillus plantarum* (60%). At the end of fermentation, *Lactobacillus fermentum* and *Lactobacillus plantarum* are the only isolated species, with a high proportion of *Lactobacillus fermentum* (85%) compared to a low proportion of *Lactobacillus plantarum* (15%).

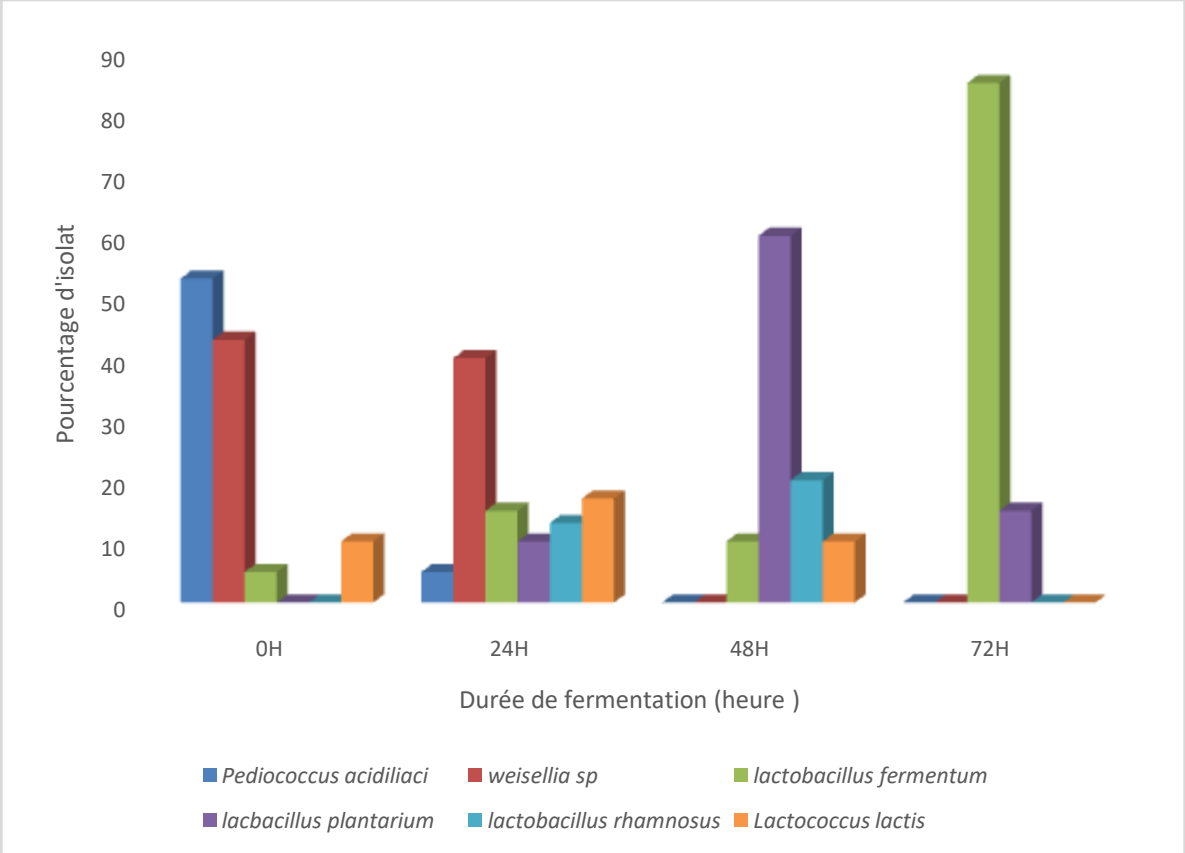


Figure 4:- Succession of lactic acid bacteria during tamarind fermentation.

Discussion:-

The objectiveofthisstudywasto characterizethe lacticflorapresentduringthe fermentation of tamarind.Duringthisstudy, 430strainsof lacticacid bacteriawere isolated.Theseresultsare in agreementwith those of **Ayala-Zavala (2011)** and **Yang (2010)** who in their research showed that tropical fruit pulps contain a wide variety of interesting microorganisms, in particular lactic acid bacteria (LAB).Also, the majorityof the isolates belongedto thelactobacillus genera (64%) compared to the total number of strains identified.The predominance of lactobacilliduringtamarindfermentationcouldbe explainedby the fact that they are one of the main microorganisms responsible for fruit fermentation.This result corroborates several results already obtained which show that various bacteria isolated from the fruits were lactobacillus such as Lactobacillus rossiae from pineapple **(Di Cagno*et al.*, 2010a)**; L. plantarum from tomato, pineapple, plum, kiwi, papaya, grapes, strawberries and cherries **(Di Cagno*et al.*, 2011a; Naeem *et al.*, 2012)**; L. brevis from tomato **(Di Cagno et *al.*, *2011*a; Naeem *et al.*, 2012); Cagno*et al.*, 2008)**.Afteridentificationin the Maldives *Lactobacilusfermentum*, *Lactobacilusrhamnosus*, *Lactobacilusplantarium*were the lactobacillus speciescharacterizedwiththe respective percentages of 36 23.5 and 20.5.Accordingto **(Vitali*etal.*, 2012; Argyri*et al.*, 2013)***L.plantarum*, and *L. fermentum* are among the lactobacilli most frequently isolated in fruits and vegetables.As for *Lactobacillus rhamnossus* species, itis oneof the mostcommonstrainsfoundin variousfermentedfoodsin manycountries.They have been isolated from traditional fermented foods in Malaysia **(Hamid and Fuzi 2020)**, kombucha in China **(Pei *et al.*, 2020)**, traditional fermented milkin Cameroon (Sohanang et al.; 2021), Travnik dairy products in Serbia **(Terzic-Vidojevic*et al.*, 2014)**, traditional fermented milkan in Cameroon **(Sohanang*et al.*,2021)**, and cocoa bean fermentation in West Africa West **(Visintin*et al.* 2016)**.The diversityof the LABELS showsthatafter the lactobacillus, Lactococcusisthe more isolated genusconsistingexclusivelyof *lactococcuslacti*this result isnot surprising. Indeed *Lactococcuslactis*is involvedin the fermentation of foods, in particular cheese, yogurt, sauerkraut and other similar products, which has earned it to be recognized as safe by the Food and Drug Administration (FDA).The genusPediococcuswasalso isolatedduringthe fermentation of tamarindwiththe only species *Pediococusacidilliaci*. Theseresults are in agreement with those of **(Bhagat *et al.* 2020; Fugaban*et al.* 2022; Surachat*et al.*, 2021)** who, during their work, showed that the strains of *P. acidilactici* are in association with fermented food products of plant and animal origin.The strains of *Weissella*

wereisolatedin smallquantities(8%). The genus*Weissella*is characteristicof severalfermentedproductsbecausethey are indigenousin manyplaces. For examplethe strainsW.cibaria, W.confusaandW.koreensishavebeen detectedin fermented foods of vegetable origin **(Fusco *et al*., 2015, Lynch *et al*., 2015)**.In addition, the *W. beninensis*bacteriumwasisolatedduringthe s fermentation of cassava **(Padonou, *etal*., 2010**) andthe *W. ghanensis* and *W. fabaria* bacteriaweredetectedin pilesoffermented Ghanaiancocoabeans (**DeBruyne, *etal*., 2010**). During the fermentation of tamarind *Lactobacillusplantarum*and *Lactobacillusfermentum*werethe predominantspeciesindeedtheyare presentduringthe fermentationfrom 24 h to 72 h. *Lactobacillusplantarium*and*Lactobacillusfermentum*have been reportedto be good associatesinspontaneouslacticfermentation**(kuenne*etal*., 2000)**it has also been noted that *Lactobacillusplantarum*had been identifiedas the predominantmicroorganismat the end of numerouslacticfermentation.

Conclusion:-

The resultsof this study showedthe presenceand variation of lactic acid bacteria germs during tamarind fermentation.The loadsof lacticacid bacteriaincreaseduringthe first hours of fermentationbeforeregressingatthe end of fermentation. The identificationof the germs isolatedduringthe fermentationof the tamarindmadeitpossible to observethatthe fermentedtamarindcontainsa diversityof lacticacid bacteriarepresented bythe genera *Lactobacillus*, *Lactococcus Wesselia* and *Pediococcus*.During fermentation, differentspeciestook turnsnamely*Lactobacillusplantarium*, *Lactobacillusfermentum*, *Weissellasp*, *LactobacillusrhamnosusLactococcuslactis*and*Pedicoccusacidilactici*. The most predominant of the species were *Lactobacillusfermentum*and*Lactobacillusplantarium*whicharethe predominantbacteriaat the end of fermentation.

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