

Research Article

Characterization of Fermentative Strains Isolated from Spontaneously Fermentation of Cashew Apples (*Anacardium occidentale* L.)

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Abstract

The cashew apple is a juicy and sweet fruit that loses its nutritional quality during the first week after falling from the cashew tree. This degradation is strongly linked to microorganisms that may present beneficial biotechnological potential. Therefore, the objective of this study is to characterize lactic acid bacteria and fermentative yeasts from cashew apples. Cashew apples collected from three localities (Tioro, Morovine, and Waraniene) in the Korhogo department (Northern Côte d'Ivoire) were washed in the laboratory, crushed, and subjected to fermentation. Samples were taken every two days over one week. Morphological studies focused on macroscopic and microscopic analyses of the strains. Physiological and biochemical tests of lactic acid bacteria and yeast isolates were all carried out in tubes. Molecular typing of these isolates was performed using the restriction enzymes DdeI or HinfI, and HaeIII. Characterization of lactic isolates revealed that all lactic acid bacteria isolated were bacillary in shape with catalase activity. They are homofermentative, mesophilic, acidophilic, and do not tolerate salinity beyond 4%. Moreover, they fermented lactose, glucose, maltose, fructose, starch, sucrose, and cellulose. They also produced exopolysaccharides. At the molecular level, all bands were observed at 1500 base pairs. Furthermore, the biochemical profile and enzymatic digestion performed with restriction enzymes identified the genus *Lactobacillus*, specifically *Lactobacillus plantarum* as the presumptive species. Characterization revealed yeasts of oval, elongated shape with lateral budding. Sediments were observed in their growth broths. They degraded urea and produced acetoin. They also fermented various sugars, except cellulose, citrate, and mannitol. All bands were observed at 500 base pairs. Biochemical tests and PCR confirmed that all isolates belonged to the genus *Saccharomyces* sp. Digestion with restriction enzymes (HaeIII and HinfI) divided them into two groups of *Saccharomyces*, of which 93.75% were *Saccharomyces cerevisiae* and 6.25% were *Saccharomyces uvarum*. The use of these presumptive species could serve in the production of cashew apple wine.

Keywords

Cashew Apple, Fermentation, Lactic Acid Bacteria, Yeast, Enzymatic Digestion

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1. Introduction

Since the Neolithic era, fermentation has been used by humans to preserve food and improve its organoleptic and nutritional qualities [1, 2]. The Sumerians, in Mesopotamia, were already practicing fermentation to produce alcoholic beverages 8000 years before Christ [2]. It was not until the 18th century that the microorganisms involved in fermentation were scientifically identified [3]. Since then, fermentation has been recognized as a major biotechnological tool. From a biochemical perspective, fermentation is the transformation of a substrate under the action of microorganisms or their enzymes [4]. It can occur either spontaneously and naturally, or in a controlled manner that meets industrial requirements [5]. Thus, fermentation is no longer perceived merely as a preservation method, but as a true platform for the production of high value-added biomolecules. From an industrial standpoint, fermentation refers to technological processes that enable microbial development and metabolite production, under aerobic or anaerobic conditions [6, 7]. The cashew tree is cultivated in northern Côte d'Ivoire mainly for its fruit, especially for its nut. Once detached from the tree, the cashew apple deteriorates very quickly. Its nutritional quality declines during the first week after detachment, which limits its use. The degradation of this fruit is strongly linked to the activity of a diversity of microorganisms that may present beneficial biotechnological potential. The microorganisms of greatest biotechnological interest used in fermentation processes, particularly in the agri-food sector, are lactic acid bacteria, acetic acid bacteria, and yeasts [5], corresponding to lactic, propionic, acetic, and alcoholic fermentations. Natural fermentation of cashew apples may contain a wide range of lactic acid bacteria and yeasts whose identities are not well known. These microorganisms could have particular biotechnological interest, potentially promoting the valorization of cashew apples produced in Côte d'Ivoire. For this reason, the biochemical and molecular characterization of lactic acid bacteria and yeasts involved in cashew apple fermentation was the focus of our study.

2. Materials and Methods

2.1. Plant Material

The biological material consisted of red and yellow cashew apples collected from three localities (Tioro, Morovine, and Waraniene) in the Korhogo department, Côte d'Ivoire. These cashew apples were harvested in March 2024 from four orchards in these localities.

2.2. Preparation of Cashew Apple Samples

Once harvested, the apples were carefully separated from

the nuts, then transported to the laboratory where they were washed with distilled water. They were subsequently crushed using a blender (SMART model No.: STPE1110). One (1) kilogram of the crushed material of each color was collected and transferred into Erlenmeyer flasks, sealed with carded cotton and covered with aluminum foil (Figure 1). Fermentation was carried out at ambient temperature for six (6) days, with samples taken every two days during the fermentation process [8].



Figure 1. Crushed red and yellow cashew apples.

TiJ: yellow apple from Tioro

TiR: red apple from Tioro

MoJ: yellow apple from Morovine,

MoR: red apple from Morovine

2.3. Preparation of Culture Media

Isolation was carried out by surface streaking. For the isolation of lactic acid bacteria, 1% nystatin was added to the MRS medium after cooling, using a five (5) mL syringe. For the isolation of yeasts, 0.5% vancomycin and 0.5% gentamicin were added to the OGA medium (Oxytetracycline-Glucose-Yeast Extract Agar) after cooling [9]. Once the media poured into Petri dishes had solidified, streak inoculation was performed directly using a platinum loop [9]. The inoculated plates were then incubated at 30°C for 24 hours for yeasts and 48 hours for lactic acid bacteria [10].

2.4. Purification of Cashew Apple Isolates

The purification of strains was carried out through successive subculturing: on MRS agar for lactic acid bacteria and on PCA agar for yeasts. This consisted of re-streaking colonies onto Petri dishes containing the appropriate solid media, followed by incubation under temperature and duration conditions specific to each strain. This operation was repeated until pure colonies were obtained [10].

2.5. Identification of Strains

After obtaining homogeneous cultures, morphological,

physiological, biochemical, and molecular tests were performed [10, 11].

2.5.1. Morphological Study

The morphological study was based on macroscopic and microscopic observations [7]. It was carried out on pure colonies.

Macroscopic observation highlighted the size, color, and shape of the colonies [7, 12].

Microscopic observation allowed visualization of the shape and arrangement of cells as well as Gram type (for lactic acid bacteria). To achieve this, a smear was prepared on a slide from a young bacterial suspension. The slide was successively treated with gentian violet, Lugol's iodine, alcohol, and fuchsin for 30 seconds each, with washing, rinsing, and drying between the different staining steps.

For yeasts, a sample from each pure strain was taken to determine the shape of the constitutive cells (spherical or oval), cell size, mode of reproduction, and the position of the bud(s) on the mother cell [13]. The study consisted of examination under a light microscope at $\times 100$ magnification, after Gram staining for bacteria and simple staining for yeasts.

2.5.2. Study of Physiological Tests of Lactic Acid Bacteria and Yeasts

The catalase test was performed by bringing a bacterial colony into contact with hydrogen peroxide. Gas release indicated positive catalase activity.

Growth at different temperatures was tested at 10°C, 37°C, and 45°C in tubes containing MRS broth. The tubes were examined after 24 hours, and growth was assessed by the appearance of turbidity [14].

Growth at different pH values was tested in MRS broths adjusted to pH 9.6, 5, 3.8, and 3 using NaOH and HCl [14]. Growth was indicated by turbidity in the broth tubes.

The thermoresistance test of the strains was performed at 60°C for 30 minutes in MRS broth in a water bath, using a young and pure bacterial culture [15].

Growth in saline medium was tested in MRS broth containing 4% and 6.5% NaCl. The tubes were incubated at 37°C for 24 hours. *Lactic streptococci* are unable to survive in this medium [15].

2.5.3. Biochemical Study

Growth of lactic isolates in Sherman's milk was tested on skimmed milk containing 0.1% and 0.3% methylene blue [7].

The search for arginine dihydrolase activity was carried out by adding a few drops of arginine to 1 ml of young culture. After incubation at 37°C for 24 hours, arginine-positive strains prevented the color change to yellow [7].

Acetoin production was tested using the Voges-Proskauer (VP) reaction, applied as follows: in a test tube containing 1 ml of the culture to be tested, 5 drops of reagent VPI (16% NaOH solution in distilled water) and 5 drops of reagent VP II

(6% α -naphthol in 95% ethanol) were added. After resting for 5 to 10 minutes at room temperature, a positive test was indicated by the appearance of a pink ring on the surface of the medium [7].

The fermentative type of lactic isolates was determined by introducing Durham tubes into broth tubes. Gas release by heterofermentative bacteria accumulated in the Durham tube after incubation at 37°C for 24 to 48 hours [16].

Dextran production from sucrose was demonstrated on liquid and solid Mayeau, Sandine, and Elliker (MSE) medium [17]. Dextran-producing strains were characterized by the formation of large, viscous, and slimy colonies on Petri dishes.

The sugars used were: hexoses (D-glucose, D-fructose), disaccharides (lactose, maltose, sucrose), and polysaccharides (starch, cellulose). The sugars used as carbon sources were prepared at 2%. After 24 to 48 hours of incubation at 37°C, a color change to yellow indicated sugar utilization by bacteria or yeasts [7].

Urease activity was detected on Christensen medium. A positive reaction was indicated by the appearance of a bright pink to red coloration in the culture medium after 3 days of incubation at 30°C.

Simmons citrate was used as a carbon source, with central streak inoculation performed along the slope of the tubes, from the bottom to the surface of the medium. Color change was observed after 24 hours of incubation at 30°C.

On mannitol-motility medium, inoculation was performed by central stabbing, stopping 1 cm from the bottom of the tube. After 24 hours of incubation at 30°C, color change and turbidity of the medium were noted.

For the coagulation test, lactic isolates were inoculated into flasks containing sterile skimmed milk reconstituted at 10%. Observation was carried out after 24 hours of incubation at 37°C [18].

2.5.4. Molecular Study of Lactic Acid Bacteria and Yeasts

DNA extraction from the strains was carried out following the protocol described by El Sheikha *et al.* [19]. Strains were collected from Petri dishes under aseptic conditions and introduced into 2 mL Eppendorf tubes (in duplicate) using sterile distilled water with 0.1% Tween 80. Glass beads (0.3 g) with a diameter of 425–600 μm (Sigma, France) were added to the tube contents. The mixture was vigorously shaken for 30 minutes in a Vortex mixer and then centrifuged at 8000 g for 15 minutes to remove the supernatant. The cell pellet was resuspended in a solution composed of 300 μL hydrolysis buffer, 100 μL TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), 100 μL lysozyme solution, and 100 μL proteinase K solution. The mixture was incubated at 42°C for 20 minutes. Then, 50 μL of 20% SDS were added to each tube, followed by incubation at 42°C for 10 minutes. In addition, 400 μL of MATAB were added to each tube and incubated at 65°C for 10 minutes after vigorous shaking for 5 minutes. Furthermore, 700 μL of phenol/chloroform/isoamyl alcohol (25/24/1, v/v/v) were added

twice to the tube contents, shaken in a vortex for 5 minutes, and centrifuged again at 8000 g for 15 minutes. The aqueous phase was transferred to an Eppendorf tube, and residual phenol was removed using 600 μ L chloroform/isoamyl alcohol (24/1) after centrifugation at 8000 g for 15 minutes. The aqueous phase was collected, and DNA was stabilized in 30 μ L sodium acetate (3 M, pH 5), followed by precipitation with an equal volume of chilled isopropanol. DNA in isopropanol was stored at -20°C for 12 hours (overnight). After centrifugation at 8000 g for 15 minutes, the supernatant was discarded, and the DNA pellets obtained were washed with 500 μ L of 70% ethanol. Ethanol was removed after centrifugation. Finally, DNA was resuspended in 50 μ L ultrapure water and stored at -20°C until analysis.

Composition of the PCR reaction mixture was performed in a reaction volume containing 5 μ L template DNA, 5 μ L sample DNA, 1 μ L of each primer, 1 μ L deoxynucleoside triphosphates (dNTP, 2.5 mM, brand D0056, Sangon Biotech.,

Shanghai, Co., Ltd., China), and 20 μ L DNA polymerase (DyNAzyme EXT) [20]. The universal 16S DNA primers for bacteria used were:

- 1) 7F 1540R (5-AGA GTT TGA TCC TGG CTC AGG AGG TGA TCC AGC CGC A-3)
- 2) 1492R (5-AGT TTGATCMTGGCTCAG GGT TAC CTT GTT ACG ACT T-3)

These amplify fragments of 1500 bp [20].

The 26S DNA primers for yeasts used were:

- 1) NL1 (5-GCATATCAATAAGCGGAGGAAAAG-3)
- 2) NL4 (5-GGTCCGTGTTTCAAGACGG-3)

These amplify fragments of 500 bp [20].

Amplification of lactic acid bacteria and yeast genes was carried out using a PCR thermal cycler (2720 thermal cycler, Applied Biosystems) under the conditions shown in the following Table 1. Finally, the amplicons were stored at 4°C [21].

Table 1. Gene Amplification Program.

Amplification program	Lactic acid bacteria	Yeasts
Initial denaturation	95°C / 4 min	95°C/3 min
Denaturation	95°C / 60 s	95°C / 30 s
Annealing	57°C / 60 s	55°C / 60 s
Extension	72°C / 1 min	72°C / 1 min
Final extension	72°C / 5 min	72°C / 10 min

Gel electrophoresis migration, agarose (1.2% w/v) was dissolved by heating in 1X TAE buffer. An intercalating DNA agent, ethidium bromide (EtBr), was then added at a final concentration of 0.2 $\mu\text{g/mL}$. After polymerization, the gel was placed in an electrophoresis tank containing 1X TAE buffer. Subsequently, 4 μ L of each prepared DNA sample was mixed with 1 μ L of 10% (v/v) loading dye and loaded into the wells. Electrophoresis was performed at 90 V for 45 min for lactic acid bacteria and at 150 V for 20 min for yeasts. DNA bands were visualized under UV light using an Infinity Capt imaging system (Fisher Scientific, Bioblock). The presence of bands corresponding to the amplified fragments was compared with the molecular size marker [21].

Composition of the reaction mixture for gene digestion, PCR-amplified DNA fragments were purified using the EZ-10 Spin Column PCR DNA Extraction Kit. The reaction mixture for restriction digestion of bacterial and yeast genes consisted of 12.3 μ L of double-distilled water, 2 μ L of 10X RE buffer, 0.2 μ L of acetylated SAB (10 $\mu\text{g}/\mu\text{L}$), 0.5 μ L of each restriction enzyme (DdeI, HaeIII, and HinfI), and 5 μ L of DNA from each sample. All digestion reactions were incubated at 37°C for 4 h. Enzymatic reactions were terminated by

incubation at 65°C for 20 min to inactivate the enzymes [21].

3. Results

Inoculation allowed the isolation of pure colonies of lactic acid bacteria and yeasts. Consequently, three (03) lactic acid bacterial isolates and three (03) yeast isolates were selected during sampling. From 24 samplings obtained from six (06) samples, a total of 144 isolates were recovered, including 72 lactic acid bacteria and 72 yeasts (36 isolates from red cashew apples and 36 from yellow cashew apples) during the fermentation of cashew apple pulp. For subsequent analyses, 16 lactic acid bacterial isolates and 16 yeast isolates were retained after morphological and physiological tests.

3.1. Characteristics of Fermentative Lactic Acid Bacterial Isolates from Cashew Apples

Macroscopic analysis of the cultivated bacterial colonies revealed circular, rounded, slightly convex, white colonies with a creamy and shiny appearance and a homogeneous aspect on

MRS agar (Figure 2a). Gram staining showed that all isolates were Gram-positive. They were sometimes arranged in chains,

as diplobacilli, or as single cells (Figure 2b).

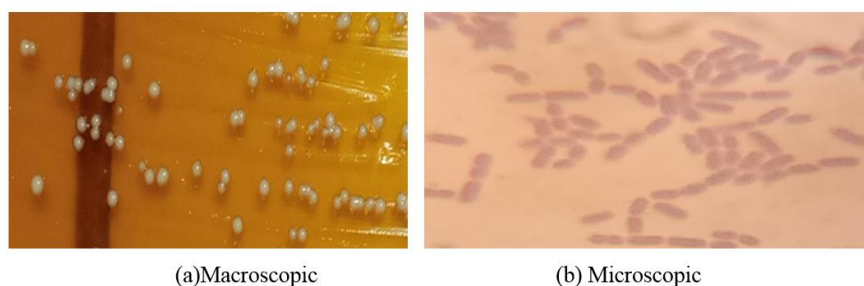


Figure 2. Appearance of Lactic Acid Bacteria.

All isolates were catalase-negative (–) and urease-negative (–). They did not grow at pH 3 or at pH 9.6. The isolates survived at 60°C for 30 minutes but did not grow at 10°C or 45°C. All isolates grew at 37°C. This characterization revealed that all isolates were acidophilic, thermoresistant, mesophilic, and tolerant only to low salinity (4%).

The biochemical study indicated that all lactic acid bacterial isolates were homofermentative, with positive results for the fermentation of skimmed milk, glucose, sucrose, fructose, lactose, maltose, and starch. Citrate, methylene blue (Sherman milk), ADH, and mannitol were not degraded. The isolates produced exopolysaccharides but were negative for acetoin production.

Genotype analysis showed that all lactic acid bacterial isolates aligned at 1500 base pairs on the electrophoresis gel (Figure 3a). The letter M represents the molecular weight marker. Digestion with the restriction enzymes DDeI and HaeIII revealed identical fragment patterns for all lactic acid bacterial isolates on the electrophoresis gel (Figure 3b and 3c). Furthermore, the sum of the fragment sizes generated by DDeI and HaeIII digestion was identical both to each other (Figure 3b and 3c) and to the 16S rDNA gene size (Figure 3a). This resulted in the formation of a single group of lactic acid bacteria after enzymatic digestion. This lactic acid bacterial group is therefore likely the dominant lactic species involved in cashew apple fermentation.

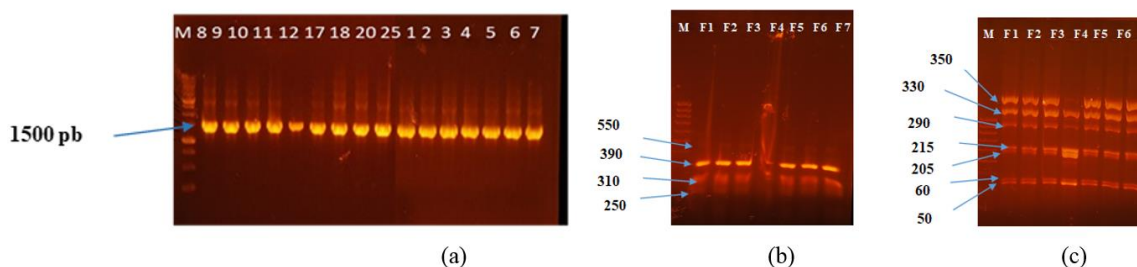


Figure 3. Electrophoretic profile of the 16S gene and restriction digestion profiles of lactic acid bacterial genes using DDeI and HaeIII.

(a) Electrophoretic profile of the 16S gene of lactic acid bacteria

(b) Digestion profile using DDeI

(c) Digestion profile using HaeIII

Lactic acid bacterial isolates: F1, F2, F3, F4, F5, F6, F7, F8, F9, F11, F12, F17, F18, F20 and F25

3.2. Characteristics of Yeast Isolates Obtained During Cashew Apple Fermentation

All isolated yeasts exhibited a white color with a regular circular margin, smooth surface, and convex elevation, and

were oval in shape (Figure 4a). Growth of the yeasts in YPD broth showed the absence of a surface film and the presence of a white sediment (pellet) at the bottom of the tubes after 24 h of incubation at 30°C, with the exception of yeast Z.

Microscopic examination using simple staining on slides at 100× magnification revealed yeasts of varying sizes (elongated and short oval forms) with lateral budding (Figure 4b).

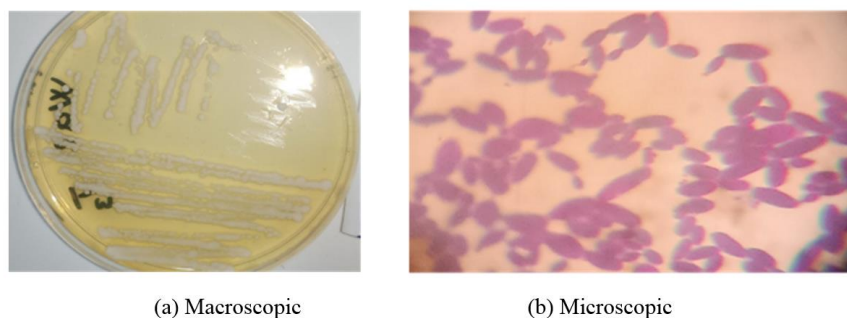


Figure 4. Appearance of yeasts.

All physiological tests performed on the yeast isolates yielded positive results, except in media with pH values of 3 and 9.6. Biochemical test indicated that the yeasts showed variable fermentation of Simmons citrate medium and nitrate–mannitol medium. Furthermore, all yeasts produced acetoin and fermented glucose, sucrose, fructose, lactose, maltose, and starch, except cellulose.

The electrophoretic profile showed that all isolated yeasts exhibited a similarity of 500 base pairs on the electrophoresis gel (Figure 5a).

The letter M represents the molecular weight marker. Digestion with the restriction enzymes *HinfI* and *HaeIII* revealed identical fragment patterns for all yeasts on the electrophoresis gel, except for yeast Z (Figure 5b and 5c). The restriction enzymes allowed the grouping of the isolated yeasts into two distinct groups (Figure 5b and 5c). However, the sizes of the different genomic fragments generated by *HinfI* and *HaeIII* digestion differed between the enzymes. The molecular size difference observed between yeast Z and the other yeasts enabled their classification into two groups after enzymatic digestion.

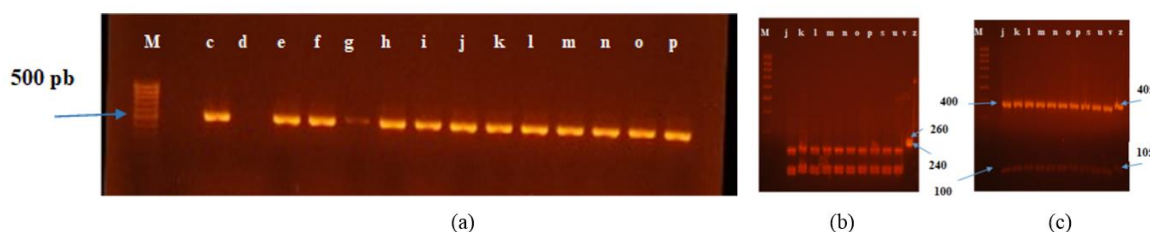


Figure 5. Electrophoretic profile of the 26S gene (a) and restriction digestion profiles of yeast genes using the enzymes *HinfI* (b) and *HaeIII* (c).

(a) Electrophoretic profile of the 26S gene of yeasts

(b) Digestion profile using *HinfI*

(c) Digestion profile using *HaeIII*

Yeast isolates: c, e, c, f, h, i, j, k, l, m, n, o, p, s, u, v, and z

4. Discussion

In this section, authors are advised to provide a thorough analysis of the results and make comparisons with relevant literature, not a short summary or conclusion. Any future research directions could also be stated in the discussion. Morphological analyses revealed that the dominant microflora during cashew apple fermentation consisted of rod-shaped lactic acid bacteria. The observed characteristics correspond to the classical descriptions of fermentative genera most frequently encountered in tropical fruits and fruit juices. The homogeneity of colony morphology highlights a relatively stable

fermentative environment largely dominated by lactic acid bacteria. This predominance can be explained by the natural richness of cashew apples in fermentable sugars (glucose and fructose), which favor their development [22]. The complete absence of cocci suggests that fermentation was mainly carried out by species of the genus *Lactobacillus*. This microflora, typical of spontaneous fermentations of tropical fruits, plays an essential role in stabilization, acidification, and nutritional valorization of the product [23]. These observations are consistent with literature data on tropical fruit fermentations (pineapple, mango, cashew apple), where *Lactobacillus plantarum* and *Lactobacillus fermentum* are generally the dominant species [24]. Physiological tests are essential criteria for the classification of lactic acid bacteria (LAB). The

growth of lactic isolates between pH 3.8 and 6.5 allows effective fermentation of cashew apple juice or pulp, thereby reducing the pH and making the environment unfavorable for the development of pathogenic microorganisms [25]. Moderate osmotic resistance at approximately 4% NaCl enables their survival in environments rich in fermentable sugars, typical of tropical fruits. Thermoresistance and growth at 37°C demonstrated optimal adaptation of these isolates to fermentation conditions in tropical regions, while remaining sensitive to extreme temperatures and harsh storage conditions. These characteristics are consistent with reports on lactic acid bacteria involved in the fermentation of tropical fruits such as cashew apple, pineapple, and mango [26]. These studies highlight the dominance of this lactic genus during spontaneous fermentation and its key role in stabilization and acidification of the final product. The uniformity of morphological and physiological test results indicates a homogeneous dominant microflora, likely composed of species well adapted to pulp fermentation. These findings corroborate those of Bekhouche [27] and Mechai [28], who confirmed that most *Lactobacillus* species thrive in acidic environments and do not tolerate salinity above 5%. Kamar [29] reported that among the collected isolates, only *Lactobacillus plantarum* was unable to grow at 45°C, which is consistent with the results obtained in the present study on lactic acid bacteria from cashew apple fermentation. The biochemical profile highlighted the functional role of lactic acid bacteria during the fermentation process. The absence of CO₂ production in Durham tubes and the lack of acetoin production suggest that the isolates are homofermentative, producing mainly lactic acid as the final product of sugar metabolism, which is ideal for product acidification [30]. The ability of the isolates to ferment various sugars indicates their adaptation to fermentable sugar-rich substrates, enabling efficient fermentation of cashew pulp and contributing to the formation of organic acids responsible for flavor and preservation [31]. The degradation of starch and cellulose demonstrates their role in converting polysaccharides into simple sugars, thereby facilitating the release of fermentable sugars and accelerating spontaneous fermentation [32]. The production of exopolysaccharides (EPS) improves texture, viscosity, and microbial stability of fermented juices or pulps while providing potential protective effects against environmental stress [33]. None of the isolates produced catalase or urease, nor degraded ADH or citrate. Meryem [34] reported that citrate lyase enzymes are present in strains belonging to the genera *Streptococcus*, *Lactococcus*, and *Enterococcus*. These findings are supported by Houali [35], who stated that *Lactobacillus* species do not possess citrate lyase capable of degrading citrate to produce acetoin. Similarly, studies by Bourgeois *et al.* [36] and Leveau *et al.* [37] showed that citrate and acetoin tests are useful for identifying *Streptococcus*, *Lactococcus*, *Pediococcus*, and *Enterococcus*. The results are consistent with those reported by Bouchibane [38] in Algeria and Morocco during LAB characterization, where *Lactobacillus*

casei subsp. casei, *Lactobacillus casei subsp. alactosus*, *Lactobacillus plantarum*, and *Lactobacillus amylophilus* degraded the same substrates. Huang *et al.* [39] demonstrated that *Lactobacillus plantarum* and *Lactobacillus fermentum* are generally the dominant species in tropical fruit fermentations (pineapple, mango, cashew apple). Moreover, *Lactobacillus fermentum* is a facultative anaerobe, unlike *Lactobacillus plantarum* [39], which supports the identification of *Lactobacillus plantarum* as the presumptive species. The morphological, physiological, and biochemical profiles of these isolates are highly similar, reinforcing the hypothesis that *Lactobacillus plantarum* is the dominant species. These biochemical characteristics confirm the fundamental functional role of *Lactobacillus plantarum* in cashew apple fermentation, in agreement with previous studies on tropical fruit fermentations. Genomic analysis revealed that all lactic isolates exhibited amplicons of approximately 1500 base pairs, confirming their identity as *Lactobacillus* species, in addition to the use of MRS selective medium and microscopic examination. Genotypic methods such as PCR and restriction fragment length polymorphism (PCR-RFLP) were used for isolate typing. All isolates showed identical 16S gene digestion patterns, indicating strong genetic similarity. Restriction enzymes (DdeI and HaeIII) generated multiple fragments with identical base pair sizes for each isolate. Each band was associated with a specific base pair size according to its migration on the electrophoresis gel. These results indicate that all isolates belong to a homogeneous restriction profile, suggesting that they represent the same species. This technique is widely used in microbiology for typing lactic acid bacteria and confirming species-level identity [40]. This confirms that the presumptive dominant species is *L. plantarum*, consistent with previous morphological, physiological, and biochemical analyses. Digestion with HaeIII revealed seven fragments of the following sizes for all isolates: 50 bp, 60 bp, 205 bp, 215 bp, 290 bp, 330 bp, and 350 bp. Aspasia *et al.* [41] obtained seven fragments for *Lactobacillus plantarum* using the MseI restriction enzyme, with fragment sizes (90 bp, 110 bp, 140 bp, 160 bp, 270 bp, 290 bp, and 480 bp) totaling approximately 1500 bp. Similar results were observed for 16S rDNA digestion in the present study. Differences in fragment sizes may be attributed to the restriction enzymes used in PCR-RFLP. Digestion with DdeI produced four fragments (250 bp, 310 bp, 390 bp, and 550 bp), totaling approximately 1500 bp for all isolates. Thus, lactic fermentation of cashew apples was dominated by *Lactobacillus plantarum*. Differences in fragment number and size are explained by the distinct restriction sites specific to each enzyme.

The characteristics of yeast colonies white color, smooth surface, convex margin, oval shape, and budding reproduction correspond to the profiles of many fermentative yeasts, particularly *Saccharomyces spp.*, *Candida spp.*, or *Pichia spp.* [42]. Lateral budding is characteristic of yeasts belonging to the family Saccharomycetaceae and constitutes a preliminary identification criterion [43]. The absence of surface film observed in all isolates indicates that these yeasts do not form

pellicles, unlike *Pichia* or *Candida* species [43]. Consequently, the genus *Saccharomyces* is most likely involved. Cashew apples appear to be colonized mainly by a group of morphologically identical yeasts, possibly due to their high sugar content. The presence of white, oval, budding, and readily sedimenting yeasts is typical of yeasts used in alcoholic fermentation [43]. All yeast isolates shared very similar morphological characteristics, suggesting a uniform yeast population likely dominated by a single species. Physiological tests provided insight into the metabolic behavior, enzymatic activity, and resistance of yeast isolates from cashew apple fermentation. All yeast isolates exhibited the same enzymatic profile. Catalase activity is common among fermentative yeasts and promotes survival in acidic, sugar-rich environments. Catalase positivity indicates the ability to degrade hydrogen peroxide, allowing adaptation to oxidative environments generated during fermentation. The isolates tolerated hyperosmotic conditions, a characteristic of yeasts originating from sugary fruits. Optimal growth of fermentative yeasts generally occurs between pH 3 and 6, confirming their good adaptation to cashew apple fermentation [44]. Yeasts isolated from cashew apples exhibited a metabolic profile enabling utilization of not only simple sugars (glucose, fructose, sucrose) but also polysaccharides such as starch. The ability of all isolates to ferment glucose, fructose, maltose, and sucrose indicates a biochemical profile clearly associated with species of the genus *Saccharomyces*, particularly *Saccharomyces cerevisiae* [45]. Partial reactions with mannitol and lactose suggest poor assimilation of polyols such as mannitol [46]. These yeasts metabolize lactose poorly or not at all, a characteristic of *Saccharomyces* species [47]. Biochemical profiles indicated that 15 out of 16 isolates corresponded to *Saccharomyces cerevisiae*, the most common yeast in fruit fermentations. The ability of these yeasts to ferment multiple sugars suggests their potential application in bioethanol production. Acetoin production by these isolates may also be of interest to the food and fermented beverage industries. The 26S rDNA (NL region) of yeasts isolated from cashew apples was digested using two restriction endonucleases (HinfI and HaeIII). Two groups of isolates were subsequently defined based on cleavage profiles. Restriction products generated by HinfI and HaeIII indicated that the isolates belonged to two presumptive *Saccharomyces* species. Yeast Z alone constituted the second group, representing a distinct species due to its different migration pattern. Enzymatic digestion results were consistent with biochemical test outcomes. Antonio *et al.* [48] reported the presence of two fragments produced by HinfI digestion in *Saccharomyces cerevisiae* strains, with PCR-RFLP profiles identical to those observed in the present study. Furthermore, digestion with HaeIII generated fragments of approximately 520 bp (110 bp + 480 bp), which are generally similar to those described by Andrea *et al.* [49]. These authors obtained fragments of 480 bp (180 bp + 300 bp) with HaeIII and a single fragment of 770 bp with EcoRI for *Saccharomyces uvarum*. For *Saccharomyces cerevisiae*, they observed a 480 bp fragment with HaeIII

and two fragments of 770 bp (500 bp + 270 bp) following EcoRI digestion. Variations in fragment sizes depending on the enzymes used may be explained by the specificity of restriction sites unique to each enzyme.

5. Conclusion

This part of the doctoral research, which falls within the framework of identifying lactic acid bacteria and yeasts involved in the fermentation of cashew apples, led us to perform morphological, physiological, biochemical, and molecular characterizations. Regarding lactic acid bacteria, morphological, physiological, and biochemical identification showed that *Lactobacillus plantarum* was the only species present. Similarly, molecular identification and enzymatic digestion using the restriction enzymes DdeI and HaeIII confirmed the presence of a single group of *Lactobacillus plantarum*. With respect to yeasts, morphological, physiological, molecular, and biochemical identification indicated that the yeasts responsible for alcoholic fermentation of cashew apples belong to the genus *Saccharomyces*. Restriction analysis of isolate genes using the enzymes HinfI and HaeIII allowed the classification of *Saccharomyces* isolates into two distinct groups.

Abbreviations

TiJ	Yellow Apple from Tioro
TiR	Red Apple from Tioro
MoJ	Yellow Apple from Morovine
MoR	Red Apple from Morovine
PCR	Polymerase Chain Reaction
PCA	Plate Count Agar
OGA	Oxytetracycline-Glucose-Agar
MRS	De Man, Rogosa, and Sharpe
VP	Voges-Proskauer
dNTP	Deoxynucleoside Triphosphates
RFLP	Restriction Fragment Length Polymorphism
YPD	Yeast Peptone Dextrose
EDTA	Ethylenediaminetetraacetic Acid
SDS	Sodium Dodecyl Sulfate
DNA	Deoxyribonucleic Acid
ADH	Arginine Dihydrolase Activity
EtBr	Ethidium Bromide
bp	Base Pairs

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Data Availability Statement

The data supporting the outcome of this research work has been reported in this manuscript.

Conflicts of Interest

The authors declare no conflicts of interest.

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