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Microbial Diversity and Molecular Identification of *Bacillus* Species and Yeasts Isolated from Traditional Honey Beer from Nangakaha (Korhogo, Côte D'ivoire)

YORO Thierry Dezay[1,*], ATTCHELOUWA Kouadio Constant[1], OUATTARA Gnénépari Odjo Mahoua[1], KARAMOKO Detto[1], Andrée Emmanuelle SIKA[2], KOUASSI koffi II Nazaire[3]

[1]Department of Biochemistry and Genetics, Laboratory of Biotechnology and Agro-Food Valorisation (Labo BiVA), Peleforo GON COULIBALY University, BP 1328 Korhogo, Côte d'Ivoire
[2]Laboratory of Biotechnology and Food Microbiology, Department of Food Science and Technology, Nangui Abrogoua University, 02 BP 801, Abidjan, Côte d'Ivoire
[3]The Central and West African Virus Epidemiology (WAVE) for Food Security Program, Science and Innovation Division, University Felix Houphouët-Boigny (UFHB), Abidjan 22 BP 582, Côte d'Ivoire
*Corresponding author: dezay@upgc.edu.ci

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Abstract Traditional honey beer is an artisanal fermented beverage widely consumed in several regions of Africa, and its organoleptic characteristics result from the activity of a complex microbial community. The aim of this study was to evaluate the microbial diversity and to molecularly identify the *Bacillus* species and yeasts associated with the fermentation of traditional honey beer produced in Nangakaha, in the Korhogo Department, Côte d'Ivoire. Fifteen samples were collected and analyzed at different stages of fermentation (0, 24, 48, and 72 h). Microbial loads were determined by plate counting on selective culture media, while the phenotypic identification of the isolates was based on their morphological and biochemical characteristics. Molecular identification was performed by amplification of the 16S rRNA gene for bacteria and the internal transcribed spacer (ITS) region for yeasts, followed by amplicon sequencing. The results showed a progressive increase in microbial populations up to 48 h of fermentation, followed by a decline at 72 h. The highest microbial loads reached 6.06 ± 0.17 log CFU/mL for *Bacillus* sp. and 7.25 ± 0.22 log CFU/mL for yeasts. Molecular analysis identified *Bacillus cereus* and *Bacillus* sp. among the bacterial isolates, and *Candida tropicalis* and *Pichia kudriavzevii* among the yeasts. The predominance of yeasts throughout the fermentation process highlights their major role in the biotransformation of fermentable sugars. This study contributes to the understanding of the microbial biodiversity of traditional fermented beverages in Côte d'Ivoire and provides valuable information that could be used to improve fermentation processes and to select suitable starter cultures.

Keywords: *Traditional honey beer, traditional fermentation, Bacillus cereus, Candida tropicalis, Pichia kudriavzevii, microbial diversity, molecular identification*

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1. Introduction

Traditional fermented foods and beverages play a significant role in human nutrition because of their nutritional value, desirable organoleptic properties, and contribution to food security. Fermentation is an ancient biotechnological process driven by the activity of microorganisms naturally present in raw materials and the production environment. According to [1], the microbial diversity involved in traditional fermentations is essential for the development of the physicochemical and sensory characteristics of fermented products. Interactions between bacteria and yeasts strongly influence the microbiological quality, shelf life, and consumer acceptability of fermented foods [1,2].

In sub-Saharan Africa, numerous artisanal fermented beverages are produced from cereals, fruits, palm sap, or honey. These products represent an important component of local diets and constitute a significant source of income for rural communities. Their production generally relies on empirical and poorly standardized processes, promoting the development of a diverse microbial community whose composition depends on the raw materials, environmental conditions, and processing

practices [3,4].

Traditional honey beer, also known as traditional mead, is produced through the natural fermentation of a mixture of honey and water. Honey harbors a diverse microbiota, including spore-forming bacteria belonging to the genus *Bacillus* and various osmotolerant yeasts capable of growing in sugar-rich environments [5,6]. These microorganisms contribute to the conversion of fermentable sugars into ethanol, organic acids, and numerous volatile compounds responsible for the beverage's sensory characteristics [7].

Yeasts are the principal biological agents responsible for alcoholic fermentation. Although *Saccharomyces cerevisiae* is considered the reference species, several non-*Saccharomyces* yeasts belonging to the genera *Candida*, *Pichia*, *Kluyveromyces*, and *Torulaspora* have been reported in various African fermented beverages [1,2,4]. Among these, *Pichia kudriavzevii* and *Candida tropicalis* exhibit distinctive metabolic properties that enable them to contribute to aroma formation and to improve the sensory characteristics of fermented products [8,9].

Members of the genus *Bacillus* are also frequently encountered in traditional fermented foods. They are recognized for their ability to produce a wide range of hydrolytic enzymes involved in the degradation of complex substrates. While several *Bacillus* species have considerable biotechnological potential, others, particularly *Bacillus cereus*, are capable of producing toxins responsible for foodborne illnesses [10,11].

Accurate identification of the microorganisms involved in spontaneous fermentations is essential for understanding the biochemical mechanisms underlying the quality of fermented products. Although phenotypic methods remain useful for preliminary identification, molecular approaches based on sequencing of the 16S rRNA gene for bacteria and the internal transcribed spacer (ITS) region for yeasts provide higher taxonomic resolution and enable more reliable identification of microbial species [12,13].

Despite the socio-economic and cultural importance of traditional honey beer produced in northern Côte d'Ivoire, information regarding its microbial biodiversity remains scarce. To date, few studies have specifically focused on the molecular identification of spore-forming *Bacillus* species and yeasts involved in its fermentation.

Therefore, the present study aimed to characterize the microbial diversity and to molecularly identify the *Bacillus* species and yeasts associated with the fermentation of traditional honey beer produced in Nangakaha, Korhogo Department, Côte d'Ivoire. The findings are expected to improve our understanding of the microbial communities involved in this fermentation process and provide a scientific basis for their potential exploitation in improving the quality, safety, and standardization of this traditional fermented beverage.

2. Materials and Methods

2.1. Study Area and Sampling

The study was conducted in the Korhogo Department, specifically in the locality of Nangakaha, considered one of the main traditional honey beer production sites located in northern Côte d'Ivoire. Traditional honey beer was collected from several artisanal producers encountered in the Nangakaha locality. In total, fifteen (15) honey beer samples were aseptically collected in sterile 500 mL bottles. The samples were transported in a cooler at approximately 4°C and delivered to the laboratory for microbiological analyses within a maximum of 24 hours.

To assess the evolution of microbial diversity during fermentation, samples were taken at different fermentation stages (0 h, 24 h, 48 h, and 72 h).

2.2. Preparation of Solution and Various Dilutions

For the preparation of the stock solution, 10 mL of honey beer was added to 90 mL of buffered peptone water (BPW, COMBOURG, France) in order to obtain a suspension corresponding to the 10^{-1} dilution. From this initial dilution, serial dilutions were performed under aseptic conditions.

2.2.1. Isolation and Enumeration of *Bacillus* isolates

Ten millilitres of each sample were homogenized, and serial decimal dilutions (10^{-2} to 10^{-6}) were prepared in buffered peptone water (BPW). A volume of 0.1 mL from each dilution was spread-plated onto Plate Count Agar (PCA) and Nutrient Agar. To select spore-forming bacteria of the genus *Bacillus*, the suspensions were heat-treated at 80°C for 10 minutes prior to inoculation. The plates were incubated at 37°C for 24 to 48 hours. Colonies exhibiting distinct morphological characteristics were repeatedly subcultured until pure cultures were obtained.

2.2.2. Isolation and Enumeration of Yeast isolates

Yeasts were isolated from the same serial decimal dilutions described above by inoculating 0.1 mL onto Sabouraud agar supplemented with chloramphenicol in order to inhibit bacterial growth. The plates were incubated at 30°C for 48 to 72 hours. Distinct yeast colonies, differentiated by their color, shape, elevation, and texture, were selected and subcultured onto fresh medium to obtain pure strains

2.3. Phenotypic Characterization of the Isolates

2.3.1. Phenotypic Identification of *Bacillus* Isolates

The bacterial isolates were subjected to morphological and biochemical characterization. Gram staining was performed to determine the cellular morphology of the isolates by light microscopy. Catalase and oxidase activities were evaluated by observing bubble formation following the addition of hydrogen peroxide (H_2O_2) and by detecting the color change produced with the oxidase reagent, respectively, to support the phenotypic identification of the isolates according to standard microbiological methods.

The presence of endospores in *Bacillus* isolates was assessed using the Schaeffer–Fulton endospore staining method, followed by microscopic examination to distinguish endospores from vegetative cells.

The ability of the isolates to hydrolyze starch was evaluated on starch agar. After incubation, the agar surface was flooded with Lugol's iodine solution. The appearance of a clear halo surrounding the colonies was considered a positive result, indicating amylolytic activity.

Nitrate reduction was determined by inoculating the isolates into nitrate broth, followed by the addition of Griess reagents (sulfanilamide and α-naphthylamine). The development of a red color indicated the reduction of nitrate to nitrite. In the absence of color development, zinc powder was added to confirm or rule out the nitrate-reducing ability of the isolates.

Preliminary identification of *Bacillus* species was performed based on the phenotypic characteristics described in the literature**.**

2.3.2. Phenotypic Identification of Yeast Isolates

For phenotypic identification, yeast isolates were characterized using several conventional identification techniques.

Yeast colonies were examined for their color (white to cream), surface appearance (smooth or rough), consistency (creamy or dry), elevation (flat or convex), and margin characteristics. These colony features were used for the phenotypic characterization of the isolates.

Microscopic examination of the yeast isolates was performed after methylene blue staining to assess cell morphology, budding pattern, and cell viability. Viable cells remained unstained or only faintly stained, whereas non-viable cells absorbed the dye and appeared blue.

Pseudomycelium formation was evaluated by culturing the isolates on Corn Meal Agar, followed by incubation at 30°C for 48–72 h. The cultures were subsequently examined microscopically for the presence of elongated cells and chains of blastoconidia, which are characteristic of pseudomycelium formation.

The carbohydrate assimilation and fermentation abilities of the yeast isolates were assessed using glucose, galactose, sucrose, maltose, and lactose according to standard microbiological methods. Assimilation tests were performed on media containing each carbohydrate as the sole carbon source, whereas fermentation tests were carried out in liquid media containing the respective sugars. Yeast growth was interpreted as a positive assimilation result, while gas production was considered indicative of positive fermentation.

Presumptive identification of the yeast isolates was based on the morphological and physiological characteristics described in the literature.

2.4. Genomic DNA Extraction

For genomic DNA extraction, the isolated microbial strains were cultured on appropriate agar media (Plate Count Agar (PCA) for *Bacillus* isolates and Sabouraud agar supplemented with chloramphenicol for yeast isolates) for 24–48 h. Genomic DNA was extracted and purified using the InstaGene Matrix Kit (Bio-Rad, USA) according to the manufacturer's instructions. DNA quality and concentration were assessed by 1% agarose gel electrophoresis and spectrophotometric measurement at an A260/A280 ratio.

2.5. PCR Amplification

2.5.1. Amplification of the 16S rRNA Gene of *Bacillus* Isolates

PCR amplification of the bacterial 16S rRNA gene was performed using the universal primer pair 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 519R (5′-GAATTACCGCGGCGGCTG-3′). The 20 µL PCR reaction mixture consisted of 4 µL of 5× FIREPol Master Mix (Solis BioDyne), 0.75 µL of each forward and reverse primer (10 µM), 14.5 µL of ultrapure water, and 5 µL of genomic DNA template. PCR amplification was carried out with an initial denaturation step at 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 s, primer annealing at 54°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. PCR products were analyzed by electrophoresis on a 1% agarose gel prepared in 1× TBE buffer and stained with SYBR Safe.

2.5.2. Amplification of the ITS Region of Yeast Isolates

PCR amplification of the internal transcribed spacer (ITS) region was performed using the primer pair ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The 20 µL PCR reaction mixture consisted of 4 µL of 5× FIREPol Master Mix (Solis BioDyne), 0.75 µL of each forward and reverse primer (10 µM), 14.5 µL of ultrapure water, and 5 µL of genomic DNA template. The amplification protocol included an initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 52°C for 30 s, and extension at 72°C for 1 min, with a final extension step at 72°C for 10 min. PCR products were analyzed by electrophoresis on a 1% agarose gel in 1× TBE buffer stained with SYBR Safe.

2.5.3. Gel Electrophoresis and DNA Sequencing

PCR amplicons were separated by electrophoresis on a 1.5% agarose gel containing an intercalating dye and visualized under ultraviolet (UV) light. Amplicons showing a single, well-defined band were purified and subjected to Sanger sequencing.

The obtained nucleotide sequences were edited, assembled, and compared with reference sequences available in the GenBank database using the Basic Local Alignment Search Tool (BLAST). Isolates exhibiting sequence similarities of ≥97% for bacterial isolates and ≥99% for yeast isolates were assigned to the corresponding species. DNA sequencing was performed by GenoScreen (Lille, France).

3. Results

3.1. Microbial Enumeration

Table 1 presents the microbial counts (log_{10} CFU/mL) of yeast and *Bacillus* isolates during the fermentation of traditional mead produced in Nangakaha, Korhogo Department, Côte d'Ivoire. The results showed a progressive increase in microbial populations from the

beginning of fermentation (0 h) to 48 h, followed by a decline at 72 h. *Bacillus* counts increased from 4.12 ± 0.21 Log CFU/mL at 0 h to 5.10 ± 0.19 Log CFU/mL after 24 h of fermentation and reached a maximum of 6.06 ± 0.17 Log CFU/mL at 48 h. At 72 h, bacterial counts decreased to 4.41 ± 0.22 Log CFU/mL.

Similarly, yeast populations increased markedly throughout the fermentation process. The initial yeast count of 3.21 ± 0.17 Log CFU/mL at 0 h increased to 5.50 ± 0.15 Log CFU/mL after 24 h and reached a maximum of 7.25 ± 0.22 Log CFU/mL at 48 h. A slight decline was subsequently observed at 72 h, with a count of 6.75 ± 0.18 Log CFU/mL, although the yeast population remained relatively high.

Overall, yeast populations exceeded those of *Bacillus* from 24 h of fermentation onward, indicating their greater adaptation to the fermentation environment and their predominant role in the conversion of fermentable sugars into ethanol. The decline in microbial populations observed at 72 h may be attributed to nutrient depletion, the accumulation of inhibitory metabolites, particularly ethanol, and the progressive acidification of the fermentation medium.

Table 1. Microbial counts in the samples

	Microbial loads	
Fermentation stage	***Bacillus* (Log CFU/mL)**	**Yeasts (Log CFU/mL)**
0 hour	4,12±0,21	3,21±0,17
24 hours	5,10±0,19	5,5± 0,15
48 hours	6,06±0,17	7,25±0,22
72 hours	4,41±0,22	6,75± 0,18

3.2. Phenotypic Characterization of *Bacillus* Isolates

Table 2 summarizes the morphological and biochemical characteristics of the three *Bacillus* isolates (S1, S2, and S3) recovered from traditional honey beer. All isolates were Gram-positive, catalase-positive, and capable of forming endospores. They also exhibited amylolytic activity, as demonstrated by starch hydrolysis, and were able to reduce nitrate.

Table 2. Morphological and Biochemical Characteristics of *Bacillus* Isolates

Isolate	Gram reaction	Catalase	Oxidase	Endospore formation	Starch Hydrolysis	Nitrate reduction
S1	+	+	+	+	+	+
S2	+	+	+	+	+	+
S3	+	+	-	+	+	+

Isolates S1 and S2 displayed identical biochemical profiles, both testing positive for oxidase activity, whereas isolate S3 differed by exhibiting a negative oxidase reaction. This difference suggests the existence of phenotypic diversity among the isolated strains.

Overall, the observed characteristics are consistent with those commonly reported for the genus *Bacillus*, including Gram-positive spore-forming cells, catalase production, and the ability to hydrolyze complex substrates such as starch.

3.3. Phenotypic Characterization of Yeast Isolates

Table 3 summarizes the morphological and physiological characteristics of the yeast isolates (L1, L2, L3, and L4) recovered from traditional honey beer. All isolates exhibited an oval cell morphology and were capable of forming pseudomycelium. However, differences were observed in their carbohydrate assimilation and fermentation profiles.

Isolate L1 was able to utilize glucose, galactose, sucrose, and maltose but was unable to metabolize lactose. In contrast, isolate L4 metabolized only glucose and tested negative for the fermentation of galactose, sucrose, maltose, and lactose. These results reveal physiological diversity between the two groups of isolates and suggest that they belong to different yeast species.

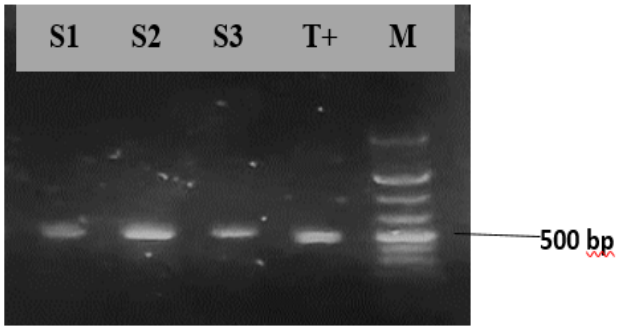
The negative lactose fermentation observed in both groups of isolates is consistent with the characteristics of many fermentative yeasts, which generally lack the β-galactosidase enzyme required for lactose hydrolysis.

Table 3. Morphological and Physiological Characteristics of Yeast Isolates

Isolates	Shape	Pseudomycelium	Glucose	Galactose	Sucrose	Maltose	Lactose
L1,L2 and L3	ovale	+	+	+	+	+	-
L4	ovale	+	+	-	-	-	-

3.4. PCR Amplification of *Bacillus* and Yeast Isolates

The electrophoretic analysis of the 16S rRNA gene PCR products from the *Bacillus* isolates (Figure 1) revealed the presence of single, well-defined DNA bands for the three isolates (S1, S2, and S3). The observed amplicons had an approximate size of 500 bp, corresponding to the expected size of the amplified bacterial 16S rRNA gene fragment. The presence of these amplicons indicates that the extracted DNA was of good quality and that the primer pair specifically amplified the target gene. In addition, the positive control (T+) produced a DNA band of the expected size, confirming the validity of the PCR assay.

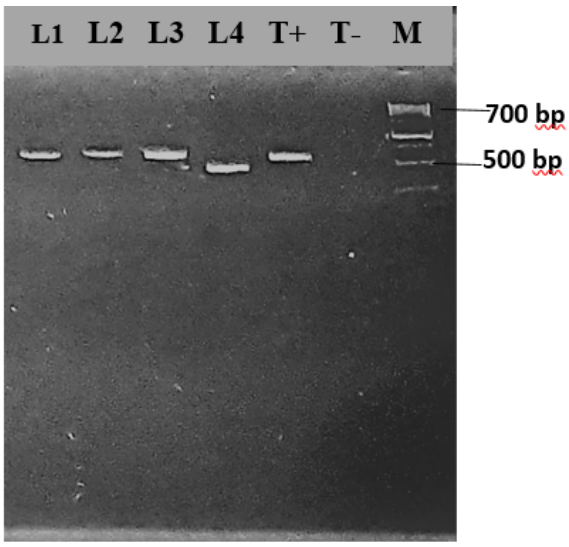


M: 100-bp DNA molecular weight marker,lanes S1–S3 : *Bacillus* isolates; T+: positive control.

Figure 1. Agarose gel electrophoresis of PCR products of the bacterial 16S rRNA gene from *Bacillus* isolates

Figure 2 shows the PCR amplification results of the internal transcribed spacer (ITS) region of the yeast isolates. All four isolates produced distinct DNA bands ranging from approximately 500 to 700 bp. No amplification was observed in the negative control (T−), confirming the absence of contamination during the PCR reaction, whereas the positive control (T+) exhibited the expected amplification profile. The differences in amplicon size among the yeast isolates suggest the presence of genetic diversity within the yeast population.

Overall, the PCR results confirmed the successful amplification of the targeted molecular markers and demonstrated that both the *Bacillus* and yeast isolates were suitable for subsequent DNA sequencing and molecular identification.



M: DNA molecular weight marker,lanes 1–5 : yeasts isolates showing PCR amplicons ranging from approximately 500 to 700 bp,T−: negative control; T+: positive control.

Figure 2. Agarose gel electrophoresis of PCR products of the ITS region from yeast isolates

3.5. Molecular Identification of *Bacillus* Isolates

The sequencing results of the *Bacillus* isolates (Table 4) showed that isolate S1 was identified as *Bacillus cereus*, exhibiting 99.80% sequence similarity with the reference sequence deposited in the GenBank database under accession number PV876509.1. Isolate S2 showed 99.80% sequence similarity with a reference sequence identified as *Bacillus* sp. (Accession no. OP986994.1), confirming its affiliation with the genus *Bacillus* but not allowing reliable assignment to the species level. In contrast, isolate S3 could not be identified, as no significant sequence similarity was obtained with any reference sequence available in the consulted databases.

Table 4. Molecular Identification of *Bacillus* Isolates

Bacterial strain	Species identified	Homology %	Genbank ID
S1	*Bacillus cereus*	99,80	PV876509.1
S2	*Bacillus* sp	99,80	OP986994.1
S3	ND	-	-

3.6. Molecular Identification of Yeast Isolates

Sequencing of the ITS region of the yeast isolates (Table 5) enabled the identification of two distinct yeast species. Isolates L1, L2, and L3 were identified as *Candida tropicalis*, exhibiting 100% sequence similarity with the GenBank reference sequence (accession no. PP808742.1). In contrast, isolate L4 was identified as *Pichia kudriavzevii*, showing 99.61% sequence similarity with the reference sequence deposited in GenBank under accession number OP526919.1.

These findings demonstrate the diversity of yeast species associated with the fermentation of traditional honey beer produced in Korhogo.

Table 5. Molecular Identification of Yeasts Isolates

Yeasts strain	Species identified	Homology %	Genbank ID
L1, L2 et L3	*Candida tropicalis*	100	PP808742.1
L4	*Pichia kudriavzevii*	99,61	OP526919.1

4. Discussion

The investigation of the microbial dynamics of traditional honey beer from Nangakaha revealed a progressive increase in the populations of *Bacillus* spp. and yeasts until 48 h of fermentation, followed by a decline at 72 h. Yeast counts increased from 3.21 ± 0.17 Log CFU/mL at the beginning of fermentation to 7.25 ± 0.22 Log CFU/mL after 48 h. This pattern is characteristic of spontaneous fermentations and reflects the gradual adaptation of microorganisms to the physicochemical conditions of the fermentation medium. The increase in microbial populations observed between 0 and 48 h may be attributed to the availability of fermentable sugars and the abundance of nutrients provided by honey. Previous studies on mead fermentation have reported that yeast populations generally reach densities ranging from 10^6 to 10^8 CFU/mL (6–8 Log CFU/mL), which is consistent with the values obtained in the present study [14,15]. Likewise, *Bacillus* counts increased from 4.12 ± 0.21 Log CFU/mL at 0 h to a maximum of 6.06 ± 0.17 Log CFU/mL at 48 h before declining to 4.41 ± 0.22 Log CFU/mL at 72 h. The initial presence of these bacteria may be associated with the natural microbiota of honey, dilution water, or fermentation equipment. Honey and apicultural environments are known to harbor bacterial populations reaching approximately 5 Log CFU/g, thereby facilitating their involvement during the early stages of spontaneous fermentation. The decline observed after 72 h is likely due to substrate depletion, ethanol accumulation, the production of other inhibitory metabolites, and medium acidification, all of which restrict microbial growth. Similar microbial succession patterns have been reported in several African fermented beverages, including tchapalo, dolo, and pito, where microbial populations typically reach their maximum during the early stages of fermentation before progressively declining [1,3,16].

The results also demonstrated that yeasts became the dominant microbial group after 48 h of fermentation,

reaching 7.25 ± 0.22 Log CFU/mL compared with 6.06 ± 0.17 Log CFU/mL for *Bacillus* spp. This predominance confirms the central role of yeasts in alcoholic fermentation. These microorganisms efficiently metabolize glucose and other fermentable sugars into ethanol, carbon dioxide, and aroma compounds responsible for the sensory characteristics of fermented beverages. According to [7], yeasts play a major role in the synthesis of higher alcohols, esters, and volatile compounds that contribute to the sensory quality of fermented products. Similar observations have been reported by [2] and [4], who showed that yeasts generally constitute the dominant microbiota in traditional African fermentations. Therefore, the present findings indicate that the first 48 h of fermentation represent the period of highest microbiological activity during the production of traditional honey beer from Korhogo. The progressive dominance of yeasts accompanied by the subsequent decline in bacterial populations reflects the typical microbial succession occurring during spontaneous alcoholic fermentation.

Phenotypic characterization of the bacterial isolates revealed that all strains were Gram-positive, catalase-positive, and capable of forming endospores. These characteristics are consistent with the classical features of the genus *Bacillus* described in *Bergey's Manual of Systematic Bacteriology* [10]. The amylolytic activity exhibited by all isolates suggests their ability to hydrolyze complex substrates, thereby releasing simple sugars required for fermentation. This enzymatic activity is a well-recognized property of *Bacillus* species, which are known producers of extracellular enzymes such as amylases, proteases, and lipases [11,17]. These enzymes play an essential role in the degradation of organic matter and contribute to improving the organoleptic properties of fermented products. Differences observed in oxidase activity among isolates S1, S2, and S3 indicate a certain degree of phenotypic diversity within the bacterial population. Similar variability has been reported in microorganisms associated with traditional fermented foods [1,18]. Nevertheless, phenotypic methods have limitations due to biochemical variability and the close genetic relationships among certain bacterial species. Consequently, molecular approaches are currently regarded as more reliable tools for microbial taxonomic identification [13].

Sequencing of the 16S rRNA gene identified isolate S1 as *Bacillus cereus* with 99.80% sequence similarity. This species is frequently detected in honey and traditional fermented products because of its ability to produce highly resistant endospores that withstand adverse environmental conditions [5,10]. The occurrence of *B. cereus* in traditional honey beer may result from contamination of raw materials, processing utensils, or the fermentation environment. Although certain *B. cereus* strains may contribute to enzymatic substrate degradation, this species is also recognized as a potential foodborne pathogen capable of producing enterotoxins responsible for food poisoning [11,19]. Its detection therefore highlights the importance of improving hygienic practices during traditional honey beer production.

Isolate S2 was identified only as *Bacillus* sp., whereas isolate S3 showed no significant similarity with sequences available in the GenBank database. This lack of identification may be attributed to poor sequence quality, partial amplification of the target gene, or the existence of a strain that is poorly represented in international sequence databases. Similar limitations have been reported by [13], who emphasized that emerging or poorly characterized bacterial species may not be accurately identified based solely on the 16S rRNA gene. Complementary approaches, including whole-genome sequencing or multilocus sequence analysis, could provide more accurate characterization of these isolates.

The carbohydrate assimilation profiles of the yeast isolates also revealed considerable physiological diversity. The ability of certain strains to utilize a wide range of carbon sources provides an adaptive advantage in carbohydrate-rich environments such as honey. Conversely, the inability of all isolates to assimilate lactose is consistent with the physiological characteristics of many fermentative yeasts, which lack the β-galactosidase enzyme required for lactose hydrolysis [20].

Molecular identification of the yeast isolates revealed the presence of *Candida tropicalis* and *Pichia kudriavzevii*. These species have previously been reported in several African and Asian fermented beverages [1,4,9]. *Candida tropicalis* is capable of assimilating multiple carbon sources and producing metabolites that contribute to the sensory attributes of fermented foods [8]. The ability of isolate L1 to ferment glucose, galactose, sucrose, and maltose is consistent with the physiological characteristics commonly reported for this species.

The identification of *Pichia kudriavzevii* in traditional honey beer is particularly noteworthy because of the recognized technological properties of this yeast. This species exhibits high tolerance to ethanol, low pH, and relatively high temperatures, enabling it to persist throughout the different stages of fermentation [9]. It is also capable of producing aroma compounds that contribute significantly to the organoleptic quality of fermented beverages [7]. Several studies have reported the occurrence of *P. kudriavzevii* in spontaneous fermentations of cocoa, coffee, and various traditional African beverages [1,21].

Successful amplification of the bacterial 16S rRNA gene and the yeast ITS region, demonstrated by the presence of single, well-defined bands following agarose gel electrophoresis, confirmed the quality of the extracted DNA and the efficiency of the primers used. The 16S rRNA gene and ITS region are widely recognized molecular markers for microbial taxonomic identification because of their conserved nature and high discriminatory power [12,13]. The combined use of phenotypic and molecular approaches therefore enabled a more reliable identification of the microorganisms associated with traditional honey beer.

Overall, this study highlights the rich microbial biodiversity involved in the fermentation of traditional honey beer from Nangakaha. The results demonstrate that this beverage represents a complex microbial ecosystem dominated by yeasts while also harboring spore-forming bacteria belonging to the genus *Bacillus*. A better understanding of this microbial diversity is essential for improving fermentation control, enhancing the microbiological and sensory quality of the final product,

and selecting appropriate starter cultures for a more standardized and controlled production process. Furthermore, the detection of *Bacillus cereus* emphasizes the need to strengthen hygiene and food safety practices in order to minimize potential health risks to consumers.

5. Conclusion

The present study characterized the microbial diversity associated with the fermentation of traditional honey beer produced in Nangakaha, Korhogo Department, Côte d'Ivoire, and molecularly identified selected *Bacillus* and yeast species involved in this fermentation process. The results revealed a progressive increase in microbial populations up to 48 h of fermentation, followed by a decline at 72 h, reflecting the natural succession of microorganisms during the fermentation process.

Phenotypic and molecular identification revealed the presence of *Bacillus cereus*, *Bacillus* sp., *Candida tropicalis*, and *Pichia kudriavzevii*. The predominance of yeasts throughout fermentation confirms their essential role in sugar bioconversion and in the development of the organoleptic characteristics of this traditional beverage. However, the detection of *Bacillus cereus*, a potentially pathogenic species, highlights the need to improve hygienic practices and optimize the manufacturing process to ensure the microbiological safety of the final product.

This study provides new insights into the microbial biodiversity of traditional fermented beverages from Côte d'Ivoire and establishes a scientific basis for the selection of suitable starter cultures aimed at standardizing the fermentation process and improving the microbiological quality, technological performance, and overall consistency of traditional honey beer.

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Authors' Contributions

YORO Thierry Dezay, ATTCHELOUWA Kouadio Constant, and OUATTARA Gnénépari Odjo Mahoua carried out sample collection and processing, data collection, laboratory analyses, and interpretation of the results. KARAMOKO Detto and Andrée Emmanuelle SIKA performed the data analysis and statistical analyses. The visualization of the results and the literature review were carried out by YORO Thierry Dezay and ATTCHELOUWA Kouadio Constant. KARAMOKO Detto and KOUASSI Koffi II Nazaire critically revised the manuscript and supervised the study. All authors contributed to the writing, revision, and approval of the final version of the manuscript.

Conflict of Interest

The authors declare that they have no competing interests related to the content or the findings reported in this article.

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