

Evaluation of Biomedical and Probiotic Properties of Fermented Cocoa Pod Isolates from Idanre, Ondo State

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ABSTRACT

Cocoa (*Theobroma cacao* L.) is an economically important crop whose beans are widely used in chocolate production. Its fermentation involves diverse microorganisms that influence product quality, however, the presence of potentially pathogenic and antimicrobial-resistant organisms may pose risks to consumer safety. This study investigated the microbial profile of fermented cocoa pods from Idanre Farm Settlement, Ondo State, Nigeria, focusing on microbial diversity, antibiotic susceptibility, and growth-death kinetics. Fermentation was simulated under laboratory conditions using freshly harvested cocoa beans with mucilaginous pulp. Microorganisms were isolated and characterized using serial dilution, selective media, Gram staining, biochemical tests, and cultural and microscopic identification of fungi. Bacterial counts ranged from 1.2×10^5 to 1.5×10^6 CFU/mL, while fungal spore-forming units ranged from 0.1×10^2 to 2.9×10^3 SFU/mL. The bacterial community was dominated by *Lactobacillus* spp., with other isolates including *Staphylococcus aureus*, *Bacillus*, *Enterobacter*, *Klebsiella*, *Escherichia coli*, and *Acetobacter* spp. Fungal isolates comprised yeasts such as *Candida tropicalis* and *Pichia membranifaciens*, and filamentous fungi including *Fusarium equiseti*, *Aspergillus parasiticus*, *Aspergillus calidoustus*, and *Alternaria infestoria*. Antibiotic susceptibility testing revealed high resistance to amoxicillin and tetracycline, whereas ciprofloxacin and gentamicin showed greater effectiveness against most bacterial isolates. Killing kinetics demonstrated rapid reductions in viable bacterial populations within 6–12 hours following treatment with ciprofloxacin and gentamicin, while amoxicillin exhibited limited bactericidal activity. The coexistence of beneficial fermentative microorganisms with potential pathogens indicates the complex microbiological nature of cocoa fermentation. The findings emphasize the need for improved hygienic practices, microbial monitoring, and antimicrobial resistance surveillance to enhance product quality and protect consumer health.

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INTRODUCTION

Cocoa, scientifically known as *Theobroma cacao*, is a fascinating and highly valued plant species that holds a prominent place in both natural and cultural realms. Renowned for its delectable flavour and aroma, cocoa has captivated human societies for centuries and continues to be a cherished ingredient in various culinary delights. Beyond its culinary significance, cocoa possesses a rich biological component that contributes to its unique characteristics and remarkable versatility (Sentellas and Saurina, 2023). *Theobroma cacao* is a member of the Sterculiaceae family and is native to the tropical regions of Central and South America. The cocoa tree features large, glossy leaves, and its canopy provides shade for the delicate cocoa pods that grow directly from the trunk and branches (Essien *et al.*, 2021). The biological importance of cocoa lies primarily in its pods, which house the beans that are later transformed into the beloved cocoa products. The cocoa pods are oval or elongated in shape, with a variety of colours ranging from yellow to red to purple, depending on the cultivar. The pods contain an average of 30-50 seeds, or cocoa beans, enveloped in a sweet and tangy pulp. These beans are the essence of cocoa and serve as the raw material for various products such as

chocolate, cocoa powder, and cocoa butter (Schlüter *et al.*, 2022). Within the cocoa beans, numerous biological components contribute to their distinctive characteristics and significant economic value (Younes and Karboune, 2023). Firstly, cocoa beans are rich in various alkaloids, most notably theobromine and caffeine (Edo *et al.*, 2023). Theobromine, in particular, provides cocoa with its mildly stimulating effects and contributes to its bitter taste. (Jean-Marie *et al.*, 2021; Edo *et al.*, 2023). Caffeine, on the other hand, adds a stimulating kick, albeit in lesser quantities compared to coffee (Edo *et al.*, 2023).

In recent years, studies on microbial ecology have expanded due to the recognition of microorganisms as essential agents in sustainable agriculture, soil fertility improvement, and waste management. Fermented cocoa pod husks, often discarded as agricultural waste, have also been shown to harbour beneficial microorganisms that can be exploited for industrial and agricultural purposes such as biofertilizer production, enzyme synthesis, medicine and biocontrol of plant pathogens (Ouattara *et al.*, 2021). Understanding the microbial composition of fermented cocoa pulp/juice in a specific locality is therefore essential for optimizing cocoa fermentation processes, improving crop sustainability, and

harnessing microbial resources for biotechnological applications.



Figure 1: Image of *Theobroma cacao* (Ouattara et al., 2021).

Cocoa (*Theobroma cacao*), a revered tropical plant known for its esteemed beans that form the basis of chocolate production, thrives within a carefully tailored habitat designed by nature. Understanding the intricacies of this habitat sheds light on the conditions essential for cocoa's growth and the cultivation of its invaluable beans (Schmidt et al., 2022).

Raw cocoa beans, after pod opening, are inedible because of their bitter, astringent and unpleasant taste (Baleba et al., 2025). For this reason, fresh cocoa pulp-bean mass undergoes a natural fermentation to obtain full-flavoured cocoa and chocolate. Therefore it is without any doubt that the process of cocoa bean fermentation plays a crucial role in the entire chocolate-making process. Fermentation removes the viscous pulp around the beans, moreover contributes to colour and flavour development of beans and reduces bitterness and astringency. Cocoa fermentation is a spontaneous on-farm process that varies according to farmers, producing countries, and geographical conditions. Among the commonly used methods are the box, heap, and platform fermentations, each influencing the microbial succession, biochemical reactions, and final bean quality.

Cocoa bean fermentation requires a particular succession of well-defined microbial populations, in particular indigenous yeast, lactic acid bacteria (LAB), and acetic acid bacteria (AAB) (Campos et al., 2025). Between 24-72 hours after fermentation starts, the increasing amount of air entering the cocoa pulp-bean mass creates ideal conditions for the growth of a succession of enterobacteria, LAB and AAB (Papalexandratou et al., 2025). According to Kouamé et al. (2021), facultative Enterobacteriaceae, such as *Tatumella* species, occur frequently, but transiently; these type of microorganisms, coming from the environment (especially soil and plants) may play a role in the production of glucuronic acid from glucose, an unwanted process yielding acidity and reducing the quantity of glucose available for yeasts and LAB (Janda and Abbott, 2021).

Cocoa (*Theobroma cacao*), the esteemed tropical plant renowned for its delectable beans used in chocolate production, also contains a wealth of bioactive compounds that contribute to its allure and potential health benefits. Phenolics constitute a prominent group of bioactive compounds found in cocoa, including flavonoids, catechins, and procyanidins (Katz et al., 2011). Cocoa is a natural source of alkaloids, the most well-known of which are caffeine and theobromine (Edo et al., 2023). Although they are found in smaller quantities than other substances, carotenoids help to give cocoa beans their vibrant colour spectrum. Beta-carotene is the most prominent carotenoid present in cocoa (Addae, 2022). The aim of the study is to investigate the microbial diversity of fermented cocoa pods, assess the antibiotic susceptibility and growth-death kinetics of bacterial isolates,

and evaluate the implications of beneficial and pathogenic microorganisms for product quality and consumer safety.

MATERIALS AND METHODS

Study Area

The study was carried out at Idanre Farm Settlement, located in Idanre Local Government Area of Ondo State, Nigeria. Idanre Local Government Area (LGA) of Ondo State, southwestern Nigeria, lies approximately between latitudes 6°50' and 7°10' N and longitudes 5°00' and 5°40' E. The LGA covers a total land area of approximately 1,964.95 km² (Tenkap & Balogun, 2020). The area lies within the rainforest belt of southwestern Nigeria, characterized by high humidity and rainfall that favour the cultivation of cocoa. The area experiences a tropical climate characterized by distinct wet and dry seasons. The wet season generally extends from April to October and accounts for most of the annual precipitation, with rainfall varying spatially from approximately 2,000 mm in the southern parts to about 1,150 mm in the northern areas. Agriculture constitutes a major economic activity in Idanre, with farming serving as an important source of livelihood for the predominantly rural population. Women also participate actively in agricultural production, often cultivating farms independently, while engaging in complementary income-generating activities such as food processing and petty trading alongside their domestic responsibilities (Oladoyin & Aturamu, 2022). Cocoa production is particularly important to the local economy, with Idanre recognized as one of the major cocoa-producing areas in Ondo State where traditional heap fermentation of cocoa beans is commonly practised using banana leaves. The area is also culturally and environmentally notable for the Idanre Hills, a prominent landscape feature of considerable historical, cultural, and ecological significance that attracts tourists to the area. These geographical, climatic, agricultural, and socioeconomic characteristics make Idanre LGA an important setting for studies involving agricultural production, food processing, environmental health, and community-based research.

Sample Collection and Preparation of Fresh Cocoa Pods

Fresh cocoa pods were obtained from the Idanre Farm Settlement, Ondo State, Nigeria. The pods were surface-sterilized by wiping with 70% ethanol and were subsequently broken open aseptically. The beans with adhering pulp were collected into sterile containers and transported immediately to the Microbiology Laboratory for fermentation studies. Laboratory fermentation was set up by weighing approximately 500 g of fresh cocoa beans with mucilaginous pulp into sterile glass fermentation jars using shimadzu digital weighing balance. The jars were loosely covered with sterile muslin cloth to allow aeration and to mimic natural fermentation conditions. Fermentation was allowed to

proceed at room temperature ($28 \pm 2^\circ\text{C}$) for seven (7) consecutive days. Samples of the fermenting juice were aseptically collected daily using sterile pipettes and transferred into sterile bottles for microbiological and physicochemical analyses (Banahene *et al.*, 2025).

Serial Dilution and Plating

Microbial counts were determined by preparing serial dilutions of each cocoa juice sample. One millilitre of the fermented juice was aseptically transferred into nine millilitres of sterile distilled water to obtain the first dilution (10^{-1}). This process was repeated until dilutions up to 10^{-6} were obtained. Aliquots of one millilitre from appropriate dilutions (10^{-3} , 10^{-5} , and 10^{-6}) were plated using the pour plate method according to the modified method of Adegbehingbe *et al.*, 2019. The molten agar (Nutrient agar and PDA) cooled to 45°C , was aseptically poured into the Petri dishes containing the inoculum and gently swirled to ensure uniform distribution. Plates were incubated at 37°C for 24 hours for bacterial isolates and at 28°C for 72 hours for

fungi and yeasts. Distinct colonies were picked for purification (Esan *et al.*, 2025).

Microbial Plate Count

Following incubation, colonies that developed on the culture plates were enumerated using a digital colony counter. Plates with 30–300 colonies were considered countable, while plates with fewer or more colonies were recorded as too low to count or too numerous to count, respectively (Esan *et al.*, 2025).

Purification and Preservation of Isolates from Fermented Cocoa

Colonies exhibiting distinct morphology were picked and streaked onto freshly prepared agar plates to obtain pure cultures. Pure isolates were maintained on agar slants in McCartney bottles, which had been sterilized and allowed to solidify in a slanted position. The cultures were incubated at 37°C for 24 hours for bacteria and at 28°C for 72 hours for fungi. After growth, the agar slants were stored in a refrigerator at 4°C for subsequent characterization (Esan *et al.*, 2025)

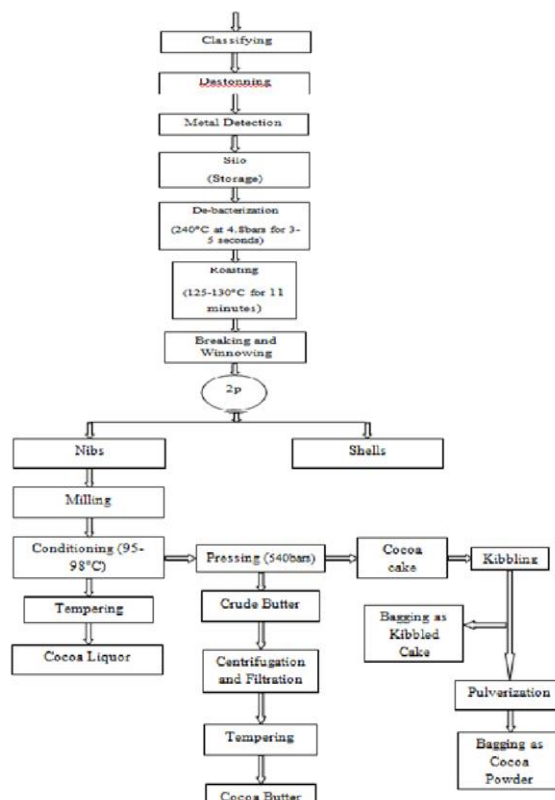


Figure 2: Fermentation process of cocoa pod
(Source: Esan *et al.*, 2025)

Identification of Bacterial Isolates from Fermented Cocoa Pod

The bacterial isolates were characterized using Gram staining and a series of biochemical tests.

Gram Staining Technique

Gram staining was performed to differentiate the bacterial isolates. Smears of each culture were prepared on clean glass slides, air-dried, and heat fixed. The smears were first stained with crystal violet for one minute, rinsed with distilled water, and treated with Gram's iodine for another minute. After rinsing, the slides were decolorized with 95% ethanol for about 20 seconds and immediately washed off to prevent over-decolorization. Safranin was then applied for one minute

as a counterstain, followed by rinsing and air-drying. The slides were examined under oil immersion ($\times 100$). Gram-positive bacteria retained the crystal violet-iodine complex and appeared purple, while Gram-negative bacteria took up the safranin counterstain and appeared pink (Escobar *et al.*, 2021).

Biochemical Characterization of Bacterial Isolates from Fermented Cocoa Pod

Biochemical tests were carried out to identify microbial isolates in accordance with standard microbiological protocols.

Identification of Fungal Isolates from Fermented Cocoa Pod from Idanre Plantation Farm Settlement

Fungal isolates were identified based on their cultural and morphological characteristics following standard taxonomic keys (Herrera-Rocha *et al.*, 2021). Pure fungal cultures were obtained by subculturing on Potato Dextrose Agar (PDA) plates and incubating at 28 ± 2 °C for 3 days. The colonies were observed daily for macroscopic features including growth rate, pigmentation, colony diameter, elevation, texture (velvety, cottony, powdery), margin characteristics, and sporulation pattern.

Microscopic identification was carried out using the lactophenol cotton blue (LPCB) staining method to the method of Kortei *et al.*, 2022. A small portion of mycelium was aseptically removed with a sterile needle and placed on a clean glass slide. A drop of LPCB stain was added, and the specimen was covered with a sterile cover slip, avoiding air bubbles. The preparation was examined under the light microscope at 10× and 40× objectives for diagnostic features such as hyphal type (septate or aseptate), presence and arrangement of conidiophores, sporangia, vesicles, phialides, chlamydospores, and spore morphology. Photomicrographs were taken for record purposes.

Identification of the fungal species was made by comparing observed characteristics with standard descriptions in fungal identification manuals and published taxonomic reports of cocoa-fermenting fungi and environmental fungi (Barnett, & Hunter, 1998; Ruiz *et al.*, 2026). Reference strains of known *Aspergillus*, *Penicillium*, and *Mucor* species (Sánchez-Hervás *et al.*, 2008; Ao *et al.*, 2026) were used for comparative purposes to increase the accuracy of identification.

Antibacterial Susceptibility Testing of Antibiotics against Isolates from Fermented Cocoa Pod from Idanre Plantation Farm Settlement

The bacteria isolate cultures on slants in test tubes were reconstituted to 0.5 McFarland standards using sterilized Nutrient Agar broth and incubating at 37°C for 15 hours. A sterile swab stick was dipped in 1 mL of a 10^{-4} sterile water dilution of the 24-hour-old broth culture in the test tube and pressed firmly against the wall to remove excess inoculum before streaking the entire dried surface of sterile Mueller-Hinton agar plates that were well prepared with the respective isolates. Wells were bored in the agar with a 6 mm corn borer. A volume of 5.0 ml of each antibiotic concentration

(Levofloxacin (30 µg), Erythromycin (10 µg), Ciprofloxacin (50 µg)) was introduced into the wells, and the plates were incubated at 37°C for 24 hours. The diameters of the zones of inhibition were measured and recorded in millimeters, and the results were interpreted using Clinical Laboratory Standard Institute (CLSI) guidelines. The broad-range antibiotic erythromycin (0.125 mg/ml) was employed as a control (Zhou *et al.*, 2024).

Measurement of Growth Dynamics and Death Rate of Isolates using Ultraviolet Spectrophotometer

The measurement of growth dynamics involves assessing the rate at which microbial cells proliferate over time. These isolated organisms were designed to evaluate the growth rate of the isolates and determine their kill time. A colony was selected from the stocked culture slant and inoculated into nutrient broth, followed by incubation at 37°C for 24 hours. After this period, a loopful of the cultured organisms was transferred into three separate flasks of fresh nutrient broth, labeled A, B, and C. Prior to measurement, the ultraviolet (UV) spectrophotometer was calibrated to a wavelength of 480 nm and allowed to warm up for 15 minutes. The initial optical density (OD) was recorded at the zero-hour mark for all three sets, serving as the baseline control. Subsequent readings were taken every 12 hours for a total of eight readings. At the fifth reading, corresponding to the 48-hour mark in set B, Amoxicillin was introduced to evaluate the kill rate of the isolates. In set C, to assess the killing time, the antibiotics Levofloxacin, Erythromycin, and Ciprofloxacin were applied at the same 48-hour mark. The absorbance values recorded during these experiments provided insights into the growth dynamics and the effectiveness of the antimicrobial treatments applied to the isolates (Osuntokun *et al.*, 2022).

RESULTS AND DISCUSSION

Microbial Count of Fermented Cocoa Pod

Table 1 presents the spore-forming unit (SFU/mL) and corresponding bacterial count (CFU/mL) obtained from fermented cocoa pod. The results revealed considerable variation across the plates, with SFU/mL values ranging from 0.1×10^2 to 2.9×10^3 and CFU/mL counts ranging between 1.2×10^5 and 1.5×10^6 . The highest bacterial count was observed in Plate 12 (1.5×10^6 CFU/mL), while the lowest was in Plate 1 (1.2×10^5 CFU/mL).

Table 1: Microbial Count of Fermented Cocoa Pod

Plate	SFU/mL	CFU/mL
Plate 1	0.1×10^3	1.2×10^5
Plate 2	1.1×10^2	3.0×10^5
Plate 3	0.1×10^2	1.2×10^6
Plate 4	2.4×10^2	4.8×10^5
Plate 5	3.2×10^2	6.5×10^5
Plate 6	1.8×10^2	8.4×10^5
Plate 7	0.9×10^2	1.1×10^6
Plate 8	2.1×10^3	2.7×10^5
Plate 9	1.7×10^3	3.9×10^5
Plate 10	2.5×10^2	7.6×10^5
Plate 11	3.3×10^2	9.2×10^5
Plate 12	0.8×10^2	1.5×10^6
Plate 13	1.6×10^3	2.4×10^5
Plate 14	2.9×10^3	5.2×10^5
Plate 15	1.2×10^2	6.8×10^5

Cultural Characteristics of Bacteria Isolates from Fermented Cocoa

Table 2 shows colony morphology for the isolates and a broad spectrum of colony colours, elevations and opacities. Colonies ranged from golden yellow, pink and white/cream

to creamy-white, with elevations from flat to convex and forms mostly circular but with occasional irregular colonies having undulate or lobate edges. Opaque and translucent opacities were both observed.

Table 2: Cultural Characteristics of Bacteria Isolates from Fermented Cocoa

Isolate Code	Color	Elevation	Form	Edges	Opacity
Co 10 ⁻³	Golden yellow	Convex	Circular	Entire (smooth)	Opaque
Co 10 ⁻⁶	Pink mucoid	Raised	Circular	Entire	Opaque
Co 10 ⁻³	White to cream	Low convex	Circular	Entire	Translucent to opaque
Co 10 ⁻⁶	Pink	Raised/convex	Circular	Entire	Opaque
Co 10 ⁻³	White to cream	Low convex	Circular	Entire	Translucent/opaque
Co 10 ⁻⁶	Whitish	Flat to slightly raised	Irregular	Undulate to lobate	Opaque
Co 10 ⁻³	Pink	Raised	Circular	Entire	Opaque
Co 10 ⁻⁶	White to cream	Low convex	Circular	Entire	Translucent
Co 10 ⁻³	Pink	Convex	Circular	Entire	Opaque
Co 10 ⁻⁶	Pink	Raised	Circular	Entire	Opaque
Co 10 ⁻³	White/cream	Low convex	Circular	Entire	Translucent
Co 10 ⁻⁶	Creamy-white	Flat to raised	Irregular	Undulate	Translucent
Co 10 ⁻³	White to cream	Low convex	Circular	Entire	Translucent
Co 10 ⁻⁶	Pink	Raised	Circular	Entire	Opaque
Co 10 ⁻³	White to cream	Low convex	Circular	Entire	Translucent

Biochemical Test Characteristics of Bacteria Isolates from Fermented Cocoa

Table 3 presents the biochemical test characteristics of bacteria isolated from fermented cocoa. The results confirm the presence of both Gram-positive and Gram-negative bacteria. The Gram-positive organisms identified include *Staphylococcus aureus*, *Bacillus* spp., and several *Lactobacillus* spp., while the Gram-negative isolates consist of *Enterobacter* spp., *Klebsiella* spp., *Escherichia coli*, and

Acetobacter sp. The isolates showed varied biochemical reactions, with *Lactobacillus* appearing repeatedly across samples, indicating its dominance. In contrast, *Enterobacter* and *Klebsiella* were also frequently detected, alongside occasional recovery of *E. coli*, *Bacillus*, and *Staphylococcus aureus*. This confirms that fermented cocoa harbors a diverse microbial community comprising both beneficial fermenters and potential contaminants.

Table 3: Biochemical Test Characteristics of Bacteria Isolates from Fermented Cocoa

Isolate code	Gram Stain	Sucrose	Lactose	Glucose	Dextrose	Galactose	Citrate	Urea	Oxidase	Motility	Indole	M/R	V/P	Catalase	Coagulase
Co 10 ⁻³	+	Rod	+	-	+	-	+	-	+	-	-	-	+	+	+
Co 10 ⁻⁶	-	Rod	+	+	+	+	+	+	+	+	+	-	-	+	-
Co 10 ⁻³	+	Rod	+	-	+	-	+	-	+	-	-	-	+	-	-
Co 10 ⁻⁶	-	Rod	+	+	+	+	+	+	+	+	+	+	-	+	+
Co 10 ⁻³	+	Rod	+	-	+	-	+	-	+	-	-	-	+	-	-
Co 10 ⁻⁶	+	Rod	+	+	+	+	+	+	+	+	+	-	+	+	-
Co 10 ⁻³	-	Rod	-	-	+	+	+	+	+	+	+	-	-	+	-
Co 10 ⁻⁶	+	Rod	+	-	+	-	+	-	+	-	-	-	+	-	-
Co 10 ⁻³	-	Rod	+	+	+	+	+	+	+	+	+	+	-	+	+
Co 10 ⁻⁶	+	Rod	+	-	+	-	+	-	+	-	-	-	+	-	-
Co 10 ⁻³	-	Rod	+	+	+	+	+	+	+	+	+	-	-	+	+
Co 10 ⁻⁶	+	Rod	+	-	+	-	+	-	+	-	-	-	+	-	-
Co 10 ⁻³	-	Rod	+	-	+	-	+	-	+	-	-	-	-	+	-
Co 10 ⁻⁶	+	Rod	+	-	+	-	+	-	+	-	-	-	+	-	-

Co 10 ⁻⁶	-	R od s	+	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	+	+	-
Co 10 ⁻³	+	R od s	+	-	+	-	+	-	+	-	+	-	-	-	-	-	-	+	-	-	-

Key: + = Positive, - = Negative

Cultural Characteristics of Fungal Isolates from Fermented Cocoa

Table 4 shows the cultural characteristics of fungal isolates from fermented cocoa. The colonies displayed diverse morphological features, ranging from cottony, fluffy, and spreading forms to smooth, creamy, and yeast-like

appearances. Isolates included white to pinkish colonies with powdery texture, yellow-green to olive colonies with velvety growth, and dark gray to black colonies with concentric rings showing woolly to cottony texture. Yeast-like colonies, appearing cream to beige or white, were also observed.

Table 4: Cultural Characteristics of Fungal Isolates from Fermented Cocoa

Isolate code	Colony Color	Elevation	Form	Edges	Opacity/Texture
Co 1	White to pinkish	Flat to slightly raised	Cottony/spreading	Irregular	Fluffy to powdery, opaque
Co 2	Yellow-green to olive	Low convex	Circular	Entire	Velvety, opaque
Co 3	Cream to beige, smooth	Convex	Circular	Entire	Opaque, yeast-like creamy
Co 4	Olive-green to gray; Reverse	Flat to low convex	Circular	Entire	Powdery to granular, opaque
Co 5	Cream to white, dull colonies	Raised	Circular	Entire	Opaque, yeast-like
Co 6	Dark gray to black with concentric rings	Flat to slightly raised	Irregular	Lobate/undulate	Woolly to cottony, opaque

Microscopic Characteristics of Fungal Isolates from Fermented Cocoa

Table 5 presents the microscopic characteristics of fungal isolates from fermented cocoa. The findings revealed both filamentous fungi and yeasts. Filamentous species identified included *Fusarium equiseti*, *Aspergillus parasiticus*, *Aspergillus calidoustus*, and *Alternaria infectoria*, all

characterized by septate hyphae with distinct conidial structures. In contrast, yeast species such as *Candida tropicalis* and *Pichia membranifaciens* displayed pseudohyphae, true hyphae, and budding cells typical of yeast morphology. This mixture of filamentous fungi and yeasts confirms a heterogeneous fungal community in the fermentation process

Table 5: Microscopic Characteristics of Fungal Isolates from Fermented Cocoa

Type of Hyphae	Microscopic Characteristics	Isolate Name
Co 1 Septate, hyaline	Produces sickle-shaped (falcate) macroconidia with several septa; microconidia in false heads; chlamydospores sometimes present.	<i>Fusarium equiseti</i>
Co 2 Septate, hyaline	Conidiophores rough-walled ending in globose vesicles; biserial phialides covering the entire vesicle; conidia round and rough-walled.	<i>Aspergillus parasiticus</i>
Co 3 Pseudohyphae + true hyphae	Oval budding yeast cells; blastoconidia abundant; pseudohyphae and true hyphae formed; occasional chlamydospore production.	<i>Candida tropicalis</i>
Co 4 Septate, hyaline	Short, smooth conidiophores; biserial phialides; globose vesicles; conidia smooth-walled, globose.	<i>Aspergillus calidoustus</i>
Co 5 Pseudohyphae, true hyphae sometimes	Oval to elongated yeast cells; multilateral budding; may produce ascospores in asci.	<i>Pichia membranifaciens</i>
Co 6 Septate, darkly pigmented (dematiaceous)	Conidiophores branched, bearing chains of large muriform (multi-septate) conidia with transverse and longitudinal septa.	<i>Alternaria infectoria</i>

Bacteria Isolates from Fermented Cocoa

Table 6 shows the microscopic characteristics of bacteria isolates from fermented cocoa. The findings revealed bacteria such as *Enterobacter sp.*, *Staphylococcus aureus*, *Klebsiella*

sp. This mixture of different bacteria confirms a heterogeneous bacteria community in the fermentation process

Table 6: Bacteria isolates from fermented Cocoa

Isolate Code	Probable isolates
Co 10 ⁻³	<i>Staphylococcus aureus</i>
Co 10 ⁻⁶	<i>Enterobacter sp.</i>
Co 10 ⁻³	<i>Lactobacillus sp.</i>
Co 10 ⁻⁶	<i>Klebsiella sp.</i>
Co 10 ⁻³	<i>Lactobacillus sp.</i>
Co 10 ⁻⁶	<i>Bacillus sp.</i>
Co 10 ⁻³	<i>Escherichia coli</i>
Co 10 ⁻⁶	<i>Lactobacillus</i>
Co 10 ⁻³	<i>Klebsiella sp.</i>
Co 10 ⁻⁶	<i>Enterobacter sp.</i>
Co 10 ⁻³	<i>Lactobacillus</i>
Co 10 ⁻⁶	<i>Acetobacter sp.</i>
Co 10 ⁻³	<i>Lactobacillus sp.</i>
Co 10 ⁻⁶	<i>Enterobacter</i>

Isolate Code	Probable isolates
Co 10 ⁻³	<i>Lactobacillus</i> sp.

Antibiotic Susceptibility Testing of Selected Drugs against Gram-negative Bacterial Isolates from Fermented Cocoa

Figure 3 presents the antibiotic susceptibility patterns of Gram-negative bacterial isolates recovered from fermented cocoa. The results reveal a wide variation in inhibition zone diameters across isolates and antibiotics tested. *Escherichia coli* demonstrated the highest susceptibility overall, with broad inhibition zones against penicillin (25 mm), ciprofloxacin (21 mm), ofloxacin (22 mm), levofloxacin (21 mm), and streptomycin (18 mm). This indicates a strong sensitivity profile compared to other isolates. *Klebsiella* spp. displayed mixed responses. One strain showed high susceptibility to ciprofloxacin (22 mm), levofloxacin (26 mm), tetracycline (17 mm), and gentamicin (15 mm).

However, another strain exhibited much lower inhibition, with zones not exceeding 17 mm and some as low as 8 mm against penicillin, gentamicin, and ceftriaxone, suggesting intra-species variability. *Enterobacter* spp. generally showed reduced sensitivity, with inhibition zones frequently below 15 mm. A few isolates responded moderately to ampicillin (16–19 mm), ciprofloxacin (11–18 mm), and ofloxacin (10–21 mm). However, resistance was indicated by consistently low or absent zones against chloramphenicol (0 mm). *Acetobacter* sp. demonstrated selective susceptibility. The organism exhibited high inhibition with gentamicin (21 mm) and ceftriaxone (22 mm), while showing only moderate zones (9–15 mm) against most other antibiotics.

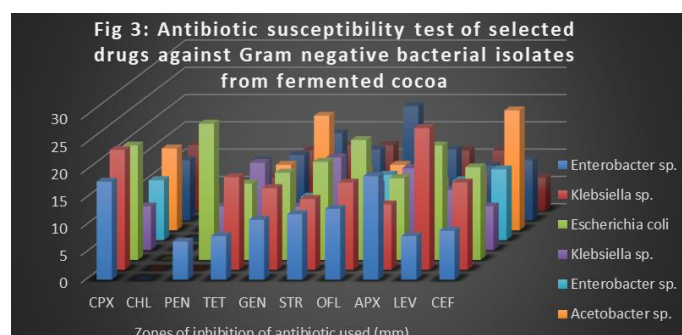


Figure 3: Antibiotic susceptibility test of selected drugs against Gram-negative bacterial isolates from fermented cocoa

Key: CPX = Ciprofloxacin, CHL = Chloramphenicol, PEN = Penicillin, TET = Tetracycline, GEN = Gentamicin, STR = Streptomycin, OFL = Ofloxacin, APX = Ampicillin, LEV = Levofloxacin, and CEF = Ceftriaxone mm = milliliter

Antibiotic Susceptibility Testing of Selected Drugs against Gram Positive Bacterial Isolates from Fermented Cocoa

Figure 4: Presents the antibiotic susceptibility profiles of Gram-positive bacterial isolates obtained from fermented cocoa. The inhibition zones reveal differential sensitivity across species and antibiotics. *Bacillus* sp. exhibited the broadest susceptibility profile among the Gram-positive isolates. Strong inhibition was recorded with levofloxacin (20 mm), ciprofloxacin (17 mm), ofloxacin (17 mm), and pefloxacin (18 mm). Moderate sensitivity was noted with erythromycin (10 mm), while no inhibition was observed with oxytetracycline and ceftriaxone, suggesting intrinsic resistance to these agents. *Staphylococcus aureus* showed

appreciable inhibition with ofloxacin (20 mm), ciprofloxacin (18 mm), azithromycin (17 mm), and levofloxacin (16 mm). However, weak activity was observed with tetracycline (8 mm) and ceftriaxone (7 mm), while oxytetracycline and moxifloxacin exhibited no inhibitory effect. *Lactobacillus* spp. demonstrated variable susceptibility depending on the strain. Most isolates displayed relatively high inhibition against levofloxacin (16–18 mm), ciprofloxacin (11–18 mm), ofloxacin (16–20 mm), and azithromycin (16–19 mm). A few strains also responded moderately to pefloxacin (13–14 mm) and tetracycline (6–14 mm). In contrast, oxytetracycline and moxifloxacin consistently produced no inhibition, while ceftriaxone showed weak effects (≤ 6 mm) in some strains.

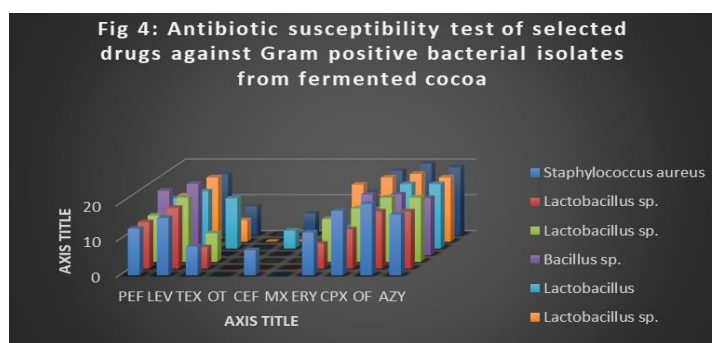


Figure 4: Antibiotic susceptibility testing of selected drugs against Gram positive bacterial isolates from fermented cocoa

Key: PEF = Pefloxacin, LEV = Levofloxacin, TEX = Teicoplanin, OT = Oxytetracycline, CEF = Ceftriaxone, MX = Moxifloxacin, ERY = Erythromycin, CPX = Ciprofloxacin, OF = Ofloxacin, and AZY = Azithromycin, mm = milliliter

Killing kinetics of bacterial Isolates

Figure 5 presents the killing kinetics of bacterial isolates treated with erythromycin over 24 hours. A general decline in optical density (OD) was observed across most isolates, indicating progressive inhibition of bacterial growth. *Staphylococcus aureus* reduced from 0.230 at 0 hr to 0.019 at 24 hr, while *Enterobacter* sp. dropped from 0.464 to 0.022.

Escherichia coli showed a significant decline from 0.517 to 0.069. *Klebsiella* sp. demonstrated a slower reduction, retaining a higher OD value of 0.041 at 24 hr. *Bacillus* sp. showed a sharp decrease from 0.168 to 0.022, while various *Lactobacillus* spp. and *Acetobacter* sp. also exhibited progressive decreases over time, reflecting the bactericidal effect of erythromycin.

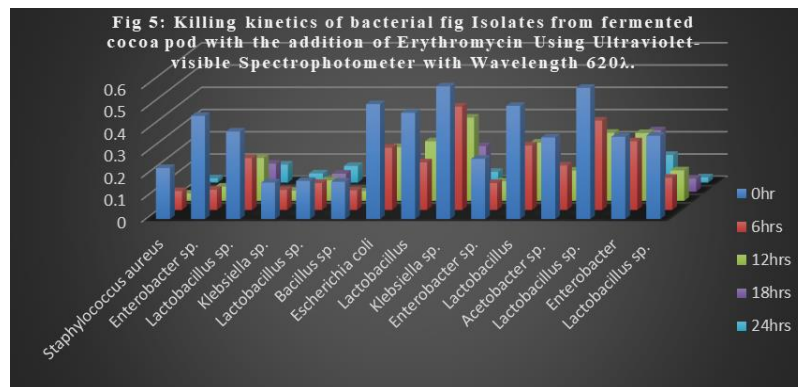


Figure 5: Killing kinetics of bacterial Isolates from fermented cocoa pod with the addition of Erythromycin Using.
Key: hr = hours

Ultraviolet-visible Spectrophotometer with Wavelength 620nm.

Figure 6 further illustrates the killing kinetics of bacterial isolates with Ciprofloxacin treatment. Many isolates reached minimal or negative OD values by 24 hr, indicating strong suppression or complete inhibition. *Staphylococcus aureus* decreased from 0.301 at 0 hr to 0.023, and *Escherichia coli*

declined from 0.391 to 0.064. *Klebsiella* sp. showed a marked reduction from 0.492 to 0.286, while *Enterobacter* sp. and *Lactobacillus* spp. also demonstrated progressive declines, reaching very low OD values. The data suggest effective bactericidal activity, with several isolates likely experiencing complete inhibition or cell death under Ciprofloxacin exposure.

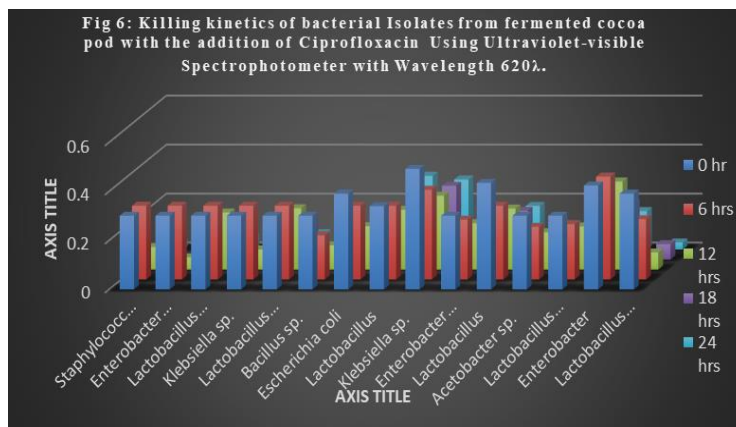


Figure 6: Killing kinetics of bacterial Isolates from fermented cocoa pod with the addition of Ciprofloxacin Using
Key: hr = hours

Ultraviolet-visible Spectrophotometer with Wavelength 620nm.

Figure 7 shows Levofloxacin effect on bacterial killing kinetics with more variable patterns. *Staphylococcus aureus* dropped from 0.214 to 0.044 at 24 hr, and *Bacillus* sp. decreased from 0.243 to 0.022. *Escherichia coli* showed overall reduction from 0.301 to 0.046 despite minor fluctuations at intermediate time points. *Klebsiella* sp. and

Enterobacter sp. exhibited mixed responses, with slight OD increases at some hours before final reductions. *Lactobacillus* spp. displayed inconsistent responses, including partial recovery at later hours. Overall, erythromycin strongly inhibited bacterial growth, though some isolates demonstrated transient tolerance or adaptive fluctuations during exposure.

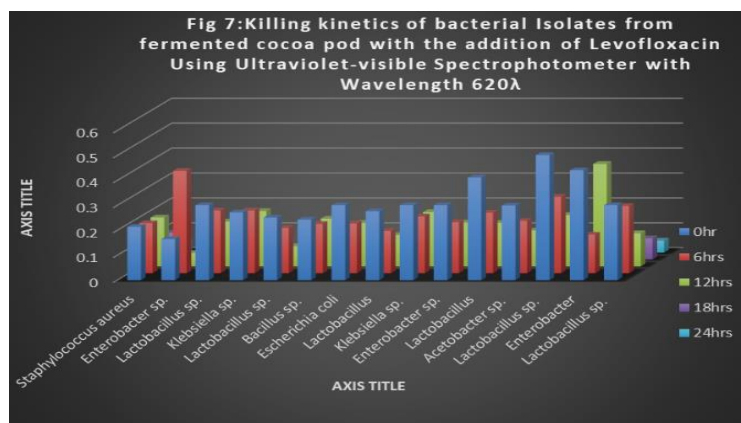


Figure 7: Killing kinetics of bacterial Isolates from fermented cocoa pod with the addition of Levofloxacin Using Ultraviolet-visible Spectrophotometer with Wavelength 620
Key: hr = hours

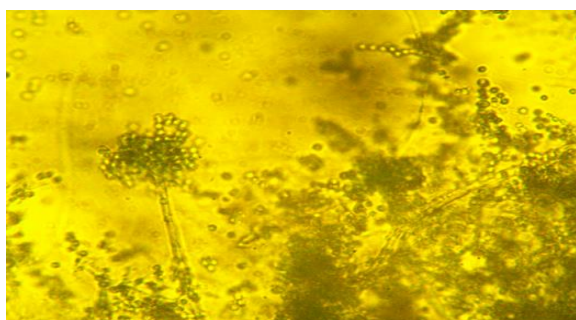


Plate 1: Micrograph of *Alternaria infectoria*

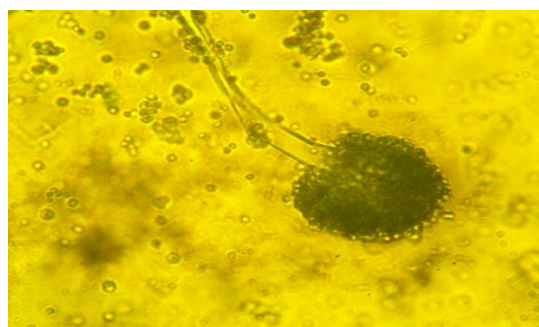


Plate 2: Micrograph of *Aspergillus calidoustus*

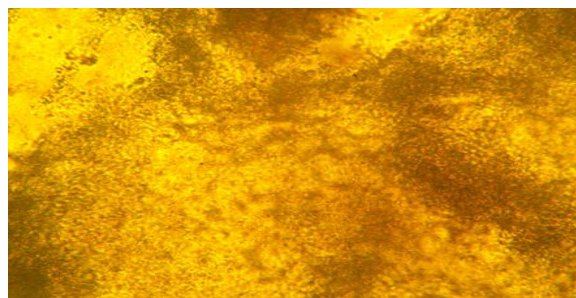


Plate 3: Micrograph of *Aspergillus parasiticus*

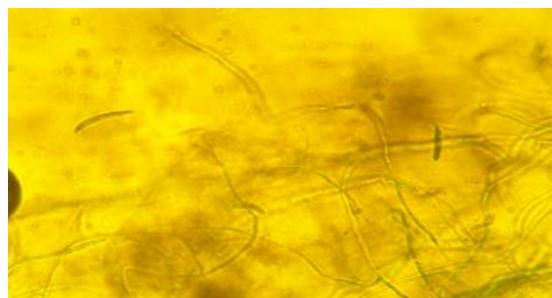


Plate 4: Micrograph of *Candida tropicalis*

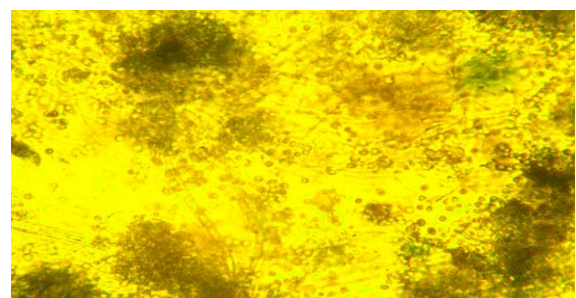


Plate 5: Micrograph of *Fusarium equiseti*

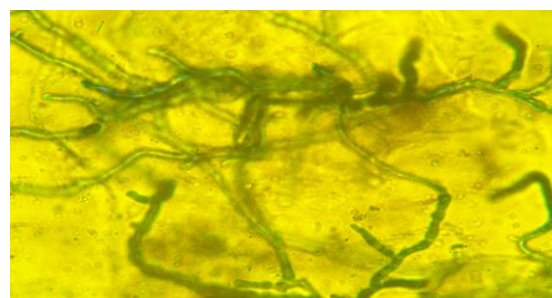


Plate 6: Micrograph of *Pichia membranifaciens*

Discussion

This study investigated the microbial community associated with fermented cocoa pod juice and examined their antibiotic susceptibility as well as the killing kinetics of selected bacterial isolates. The findings demonstrated that fermented cocoa supports a rich and diverse microbiota composed of both bacteria and fungi, which play distinct roles in fermentation and pose varying implications for food quality

and safety. The spore-forming units (SFU/mL) ranged from 0.1×10^2 to 2.9×10^3 , while the bacterial counts (CFU/mL) varied between 1.2×10^5 and 1.5×10^6 , with Plate 12 showing the highest bacterial load and Plate 1 the lowest. This variation reflects substantial microbial proliferation during fermentation, supported by the nutrient-rich environment of cocoa pulp, which contains sugars, amino acids, and organic acids that promote microbial growth. These observations are

consistent with previous reports (Campos *et al.*, 2025; Hirko *et al.*, 2023) and align with the findings of Mota-Gutierrez *et al.* (2021), who highlighted that microbial density is a key factor influencing cocoa flavour and overall quality through its effect on fermentative metabolic activity.

Biochemical and microscopic identification confirmed the presence of both Gram-positive and Gram-negative bacteria. Dominant Gram-positive isolates included *Staphylococcus aureus*, *Bacillus* spp., and *Lactobacillus* spp., while Gram-negative isolates comprised *Enterobacter* spp., *Klebsiella* spp., *Escherichia coli*, and *Acetobacter* sp. The repeated occurrence of *Lactobacillus* spp. highlights its importance as a key fermenter in pulp degradation, consistent with reports that lactic acid bacteria dominate early fermentation stages (Fan & Han, 2023; Martínez-Miranda *et al.*, 2022). The recovery of *Enterobacter* and *Klebsiella* is notable since Enterobacteriaceae are often linked to environmental contamination and spoilage risks (Janda & Abbott, 2021). Similarly, the presence of *E. coli* and *S. aureus* points to possible hygiene lapses, as these organisms are associated with faecal and skin contamination, respectively.

Fungal analysis also revealed a diverse community. Filamentous fungi such as *Fusarium equiseti*, *Aspergillus parasiticus*, *Aspergillus calidoustus*, and *Alternaria infectoria* were recovered, which is consistent with reports from cocoa fermentations in tropical regions (Hassen *et al.*, 2022; Gutiérrez-Ríos *et al.*, 2022). The detection of *Aspergillus parasiticus* is particularly concerning because of its aflatoxin-producing potential. Yeasts such as *Candida tropicalis* and *Pichia membranifaciens* were also isolated, confirming their role as initiators of fermentation through pulp sugar hydrolysis and ethanol production (Boekhout *et al.*, 2021; Del Fresno *et al.*, 2022). These results support the consensus that cocoa fermentation is a multi-microbial process requiring both yeasts and bacteria for effective flavour development (Escobar *et al.*, 2021; Farrera *et al.*, 2021).

Antibiotic susceptibility testing revealed variable inhibition patterns. Among Gram-negative isolates, *E. coli* recorded the widest inhibition zones, particularly with ciprofloxacin (21 mm), penicillin (25 mm), ofloxacin (22 mm), and levofloxacin (21 mm), suggesting broad sensitivity to fluoroquinolones and β -lactams. This observation agrees with Janda and Abbott (2021), who noted that *E. coli* remains relatively sensitive to broad-spectrum antibiotics despite growing multidrug resistance. *Klebsiella* spp. showed moderate inhibition, especially with levofloxacin (26 mm), while *Enterobacter* spp. exhibited weaker responses, often below 15 mm, implying strain-level variability. Interestingly, *Acetobacter* sp. displayed notable sensitivity to gentamicin (21 mm) and ceftriaxone (22 mm), reflecting differential drug responses.

Among Gram-positive isolates, *Bacillus* sp. showed the widest inhibition zones with levofloxacin (20 mm) and ciprofloxacin (17 mm), while *S. aureus* was strongly inhibited by ofloxacin (20 mm) and ciprofloxacin (18 mm). These results are consistent with reports that Gram-positive organisms are moderately susceptible to fluoroquinolones but show limited response to tetracyclines and β -lactams (Gupta *et al.*, 2022). *Lactobacillus* spp. demonstrated selective susceptibility, with higher inhibition zones against levofloxacin (16–18 mm), ofloxacin (16–20 mm), and azithromycin (16–19 mm), supporting earlier findings that lactic acid bacteria exhibit strain-specific tolerance to antibiotics (Milanesi *et al.*, 2025). The findings are slightly different from Okafor *et al.*, 2021 where *S. aureus* has only 9 mm with ciprofloxacin and Aboh *et al.*, 2021 with *B. subtilis*

having 14 mm and 17 mm. These differences may reflect variation in bacterial strains, geographic-strain-specific and epidemiological variations in susceptibility, antibiotic susceptibility profiles, experimental conditions, influence of antibiotic-disc quality on inhibition-zone measurements.

The killing kinetics of bacterial isolates treated with erythromycin demonstrated a progressive decline in optical density (OD) for most organisms, indicating potent bactericidal activity. *S. aureus*, *Enterobacter* sp., *E. coli*, *Bacillus* sp. was sharply reduced respectively. In contrast, *Klebsiella* sp. retained an OD of 0.041 at 24 hrs, suggesting partial tolerance. These findings align with Campos *et al.* (2025) and Fan & Han (2023), who noted that macrolides such as erythromycin are broadly effective in fermentation systems but may be less effective against certain Enterobacteriaceae that includes *Enterobacter* spp. particularly *Enterobacter cloacae* complex, *Klebsiella* spp., including *K. pneumoniae*, *Escherichia coli*, *Pantoea* spp., *Salmonella* spp.

Ciprofloxacin treatment (Figure 6) produced stronger bactericidal effects, with many isolates reaching minimal OD by 24 hr. *S. aureus* decreased from 0.301 to 0.023, *E. coli* from 0.391 to 0.064, and *Bacillus* sp. from 0.243 to 0.022. *Klebsiella* sp. showed a substantial but incomplete reduction (0.492 $\rightarrow$ 0.286), while *Enterobacter* sp. and *Lactobacillus* spp. also declined. These results reaffirm the potency of fluoroquinolones against Gram-negative bacteria, corroborating findings of selective but strong inhibition in other cocoa fermentation studies (Gupta *et al.*, 2022; Milanesi *et al.*, 2025).

Levofloxacin treatment showed variable patterns. While *S. aureus* (0.214 $\rightarrow$ 0.044) and *Bacillus* sp. (0.243 $\rightarrow$ 0.022) were strongly inhibited, *E. coli* showed fluctuations before a net reduction (0.301 $\rightarrow$ 0.046). *Klebsiella* sp. and *Enterobacter* sp. displayed intermediate declines, and some *Lactobacillus* strains partially recovered at later hours. This variability reflects strain-level adaptive mechanisms, supporting reports that lactic acid bacteria and Enterobacteriaceae can modulate metabolic activity under antibiotic stress (Fan & Han, 2023; Campos *et al.*, 2025). These findings confirm that fermented cocoa pod juice harbours a complex microbial ecosystem of beneficial fermenters and potential contaminants. While yeasts and lactic acid bacteria drive pulp degradation and flavour development, the persistence of opportunistic pathogens such as *Klebsiella*, *Enterobacter*, and *Aspergillus parasiticus* poses risks to food safety and consumer health. The antibiotic susceptibility results revealed strong activity of fluoroquinolones and erythromycin, while penicillin and tetracycline were largely ineffective. This suggests that resistance traits may be widespread among cocoa-associated microbes, possibly driven by environmental exposure and adaptive mechanisms (Gupta *et al.*, 2022; Milanesi *et al.*, 2025).

CONCLUSION

This study demonstrated that fermented cocoa pod juice contains a diverse microbial community, including bacteria (*Lactobacillus*, *Bacillus*, *Staphylococcus aureus*, *Enterobacter*, *Klebsiella*) along with different fungi *Fusarium equiseti*, *Aspergillus parasiticus*, *Aspergillus calidoustus*, and *Alternaria infectoria*. Antibiotic susceptibility testing revealed that fluoroquinolones and erythromycin were most effective, while penicillin and tetracycline showed poor activity. Killing kinetics confirmed erythromycin's strong inhibitory effect, though some strains demonstrated tolerance. These findings underscore both the functional role of

microbes in cocoa fermentation and the potential food safety concerns arising from opportunistic contaminants.

- i. Strict sanitary practices should be enforced during cocoa fermentation to minimize contamination by pathogenic bacteria and toxigenic fungi.
- ii. Controlled use of beneficial yeasts and lactic acid bacteria should be encouraged to dominate fermentation and suppress contaminants.
- iii. Regular screening for *Aspergillus* species and aflatoxins should be prioritized to ensure food safety.
- iv. Although erythromycin and fluoroquinolones demonstrated strong activity, their use in food systems should be limited to avoid resistance development.
- v. Future studies should explore molecular characterization of resistant isolates and investigate biocontrol strategies, such as bacteriocins or plant extracts, for safer cocoa fermentation.

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