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Evaluation of The Rate of Microbial Activity in Coconut (Cocos Nucifera L.) Milk When Stored at Different Time Intervals

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ABSTRACT

Introduction: Coconut milk is a very nutritional milk or drink. The processor of coconut into milk using various forms has been of serious risk and health hazard.
Objective: This study aims to describe or evaluate the rate of microbial activity in coconut milk when stored.
Methods: The coconut milk was extracted from the coconut meat using an aseptic technique.
Results: The initial ᴘH value was 6.10, it was observed that fermentation started from the 12th hour, and at the 24th hour, the ᴘH of the coconut milk was 4.48. After 48 hours, the pH was 4.20 and at the 72nd hour time, the pH value was 3.99. It was also observed that the 24 hours stored coconut milk sample has the highest Bacterial growth rate of 4.12×10^2 and a fungal growth rate of 1.2×10^2.
Conclusions: Biochemical test carried out on the bacterial isolate showed *Bacillus spp*, *Staphylococcus aureus*, and *Bifidobacterium bifidum* were observed to be present at 24 hours and 48 hours of the coconut milk sample and at the 72nd hours of the coconut milk sample *Pseudomonas spp*, *Lactobacillus spp*, and *Aeromonas caviae* were observed to be present. A drop of pH value to 3.99 at the 72nd hour was observed due to the activity of Lactobacillus spp.

Introduction

The coconut (Cocos nucifera L.), a member of the palm family (Arecaceae), is a tropical plant typically found within latitudes 20°N to 20°S (Alfred, 2010). It produces a large, oval nut with a hard shell and a fibrous outer husk, containing thick white flesh surrounding a cavity filled with nutrient-rich liquid, known as coconut water or milk. Despite being classified as a fruit, it is often mistaken for a nut and is technically a one-seeded drupe. Coconut is widely valued for its versatility, serving as a key ingredient in coconut oil, milk, cream products, and is consumed fresh or processed into desiccated coconut, coconut water, and coconut milk. Additionally, the immature coconut has a range of uses, including applications in food production, animal feed, and the manufacture of soaps,

detergents, and cosmetics (Alyaqoubi et al., 2015).

Coconut production and processing have been a dominant economic activity in rural areas of many tropical regions, particularly in Southeast Asia, the South Pacific, and to a lesser extent, along Africa's west coast. The traditional method of producing coconut oil from "copra" (dried coconut meat) represents the largest segment of the coconut industry (Alfred, 2012). Although copra contains proteins of relatively good nutritional value, its use as food is limited due to factors such as lipid oxidation and microbial contamination, which result from high temperatures and unsanitary conditions during drying and storage (Alfred, 2010).

In many coconut-producing countries, local animal milk production is minimal, and most dairy products are manufactured using imported milk. The significant reliance on imported milk to meet consumer demand has raised concerns among governments, producers, and consumers due to its high cost and the impact on foreign exchange reserves.

Recent innovations in coconut-based products have shown potential for providing essential nutritional benefits, particularly due to their low cholesterol-inducing fat levels. The saturated fats in coconut milk are considered beneficial as they are easily metabolized, providing quick energy (Ekanayaka, 2013). Moreover, coconut possesses anti-carcinogenic, antibacterial, and antipathogenic properties and is less likely to contribute to weight gain compared to polyunsaturated oils (Ekanayaka, 2024).

Most previous studies have focused on coconut milk's preparation and stability maintenance. Asiri *et al.,* (2020) studied the antioxidant and nutritional properties of domestic and commercial coconut milk preparations. Alyaqoubi *et al.,* (2015) studied the antioxidant activity and physicochemical properties of coconut milk (pati santan) in Malaysia. Coconut possesses many health benefits due to its fibre and nutritional benefits because of its fat, and coconut oil (DebMandal and Mandal, 2011).

Coconut meat is rich in essential minerals, including magnesium, iron, fiber, copper, calcium, and phosphorus, all of which are vital for maintaining a healthy muscular and skeletal system. While the magnesium content in coconut meat is relatively modest (32 mg per 100 g), it surpasses the levels found in all animal-based foods, such as meat, fish, milk, and eggs (Pamplona-Roger, 2007).

Coconut water is made from the clear liquid inside green coconuts. It’s not to be confused with coconut milk, which is made from the water and the flesh inside of a mature coconut. Over 95 per cent of

coconut water is water. In most cases, coconut water comes from small and scarce coconut tree plantations more related to "gardens". Consequently, coconut water remains a traditional and under-used resource that could thus be considered an exotic beverage by most people living far from the coconut production area (Mat, 2022).

Coconut milk is a key ingredient in many Asian cuisines and is gaining popularity in the functional food market due to its health benefits, particularly its lauric acid content, which boosts immunity against bacterial diseases. This growing market has attracted health-conscious consumers, especially from Europe and the USA, who seek functional foods that promote health and well-being (Sethi,2016).

However, coconut milk is highly susceptible to contamination and supports the growth of spoilage microorganisms, which can be introduced through contaminated shells, utensils, processing equipment, or handlers (Mabesa and DelRosario, 1979; Hafidh et al., 2010). During natural fermentation, extracted coconut milk undergoes microbial fermentation, often facilitated by lactic acid bacteria (LAB) (Omobayonle, 2020).

LAB, widely used as starter cultures in fermented foods and beverages, enhance nutritional value, sensory properties, and shelf life. They are known to produce compounds like ethanol, bacteriocins, aroma components, exopolysaccharides, and enzymes, contributing to the desired sensory characteristics and microbiological safety of fermented products (De Vuyst and Leroy, 2004). LAB in coconut milk offers health benefits by improving gastrointestinal function and acting as antimicrobial agents through the production of organic acids, hydrogen peroxide, and bacteriocins (Hafidh et al., 2010).

Methods

Sample collection and identification

Three (3) mature Coconut samples were obtained from Angalabiri community market, sagbama, Bayelsa State. The coconuts were identified by the Department of Crop and Pest Management, Faculty of Agriculture, University of Africa.

Preparation of coconut milk

The coconut was cracked open into halves. The split nuts were de-shelled to separate the coconut „meat" (kernel). Coconut meat of 500 g was washed and comminuted using an electric blender (Sanyo SM-B12M) with 700 ml of distilled water. This was then pressed through a sterilized linen cloth and strained to obtain coconut „milk" (Nevin and Rajamohan, 2006).

Microbiology analysis

One gram (1g) of each of the various hours (24hrs, 48hrs, 72hrs) of stored coconut milk samples were put in nine millilitres (9ml) of sterile water contained in a sterile beaker and allowed to stand for 30mins, to dislodge any microorganism in the sample. One (1ml) of the water mixture was serially diluted. The water mixture was serially diluted using ten-fold serial dilution in ten test tubes. After the serial dilution, 0.1ml aliquots of the $10^{-3,}$ $10^{-5,}$ 10^{-8} dilution were inoculated onto sterile plates of Nutrient agar and Saboraud dextrose agar respectively, using the spread plate inoculation method. The plate was incubated at 37℃ for 24 hours while the Saboraud dextrose agar plates were incubated at room temperature (27℃) for 72 hours. The microorganisms enumerated on the culture plate were counted using a colony counter. The microbial isolation obtained was further identified using cultural morphology, gram staining, and biochemical tests in the case of the bacterial isolates and the lactophenol cotton blue staining technique were employed in the case of the fungal isolates (cheesbrough, 2005).

Identification of Bacterial Isolates

The bacterial isolates from the plates were identified by gram staining and another biochemical test.

Gram Staining Techniques

A smear was prepared from each bacterial isolate and fixed through air drying. The smear was then stained with crystal violet for 60 seconds, followed by a quick rinse with water. Next, Lugol's iodine was applied to the smear for 60 seconds and washed off with water. Decolorization was performed using acetone alcohol for 10 seconds, after which the smear was rinsed with water again. Finally, the smear was counterstained with safranin for 2 minutes and washed with clean water. The back of the slide was wiped, and the slide was placed in a drying rack to air-dry before being observed under a microscope using a x100 oil immersion objective lens (Cheesbrough, 2005). Gram-positive bacteria appeared purple, while gram-negative bacteria showed a pink coloration.

Motility Test

A smear of each bacterial isolate was prepared on a glass slide, covered with a coverslip (wet mount preparation), and observed under a microscope to assess bacterial motility (Fawole and Oso, 2004).

Catalase Test

This test distinguishes bacteria capable of producing the enzyme catalase, such as staphylococci. Approximately 2 ml of hydrogen peroxide solution was dispensed

into separate test tubes for each bacterial isolate. Using a sterile wooden stick, a colony of each bacterial isolate was introduced into the hydrogen peroxide solution. The presence of active bubbling within 10 seconds indicated a positive result, while the absence of bubbling indicated a negative result (Cheesbrough, 2005).

Citrate Utilization Test

This test is used to identify members of the Enterobacteriaceae family. Each test organism was inoculated into a sterile Simon citrate agar slope using the stab inoculation technique. The inoculated agar slopes were incubated at 37°C for 24 hours. A bright blue color signified a positive result, while no color change indicated a negative result (Cheesbrough, 2005).

Coagulase Test

A drop of distilled water was placed on each end of a slide for the test organisms. A colony of each test organism was emulsified in the drops to create two suspensions. A loopful of plasma was added to one suspension for each test organism and mixed gently. Clumping within 10 seconds indicated a positive result, while the absence of clumping indicated a negative result (Cheesbrough, 2005).

Indole Test

Certain microorganisms hydrolyze the amino acid tryptophan, producing indole as a byproduct. This biochemical reaction aids in microbial characterization. Test organisms were suspended in 3 ml of sterile peptone solution in test tubes and incubated at 37°C for 48 hours. After incubation, 0.5 ml of Kovac's reagent was added and gently shaken. The appearance of a red color in the surface layer within 10 minutes signified a positive result, while the absence of color change indicated a negative result (Cheesbrough, 2005).

Oxidase Test

Following the method of Cheesbrough (2005), a piece of filter paper was placed in a clean petri dish, and three drops of freshly prepared oxidase reagent were added. A sterile stick was used to transfer a colony of each test organism onto the reagent-soaked filter paper. The appearance of a purple coloration indicated a positive result, while the absence of color change indicated a negative result.

Sugar Fermentation Test

Each test organism was inoculated into a sterile agar slope of triple sugar iron agar using the stab inoculation technique. The inoculated agar was incubated at 37°C for 24 hours. Changes in color, gas production, and hydrogen sulfide (H2S) blackening

were observed, providing information about the type of bacteria present (Cheesbrough, 2005).

Lactophenol Cotten Blue Staining Technique

Fungal isolates were identified based on their morphology on Sabouraud Dextrose Agar (SDA) and microscopic examination using the lactophenol cotton blue staining technique. Each fungal isolate was collected using a sterile wooden stick, teased onto a drop of lactophenol cotton blue stain on a clean glass slide, and prepared as a wet mount. The sample was then examined under a microscope to observe branched and unbranched hyphae (Fawole and Oso, 2004).

Results and Discussion

The result below shows the different PH of the stored coconut milk on the different days (24hours, 48hours, and 72hours) which are represented in Table 1. The result also shows the microbial load of the stored coconut milk sample represented in Table 2. The total viable bacteria count ranges from 2.4×10^1 to 4.12×10^2 cfu/g. No coliform count was detected. Table 3 shows the bacteria isolate from the stored coconut milk, the total of six bacteria were isolated from the stored coconut milk sample on different days and include *Bacillus spp, Staphylococcus aureus, Bifidobacterium bifidum, Pseudomonas aeruginosa, Lactobacillus spp* and *Aeromonas caviae* the result for the identification of bacterial isolates while table 4 is the result for the identification of the fungal isolates. The fungal isolated from the stored coconut milk sample include *Aspergillus niger*, *Trichophyton metagrophyts*, *Aspergillus flavus, Aspergillus fumigatus*, *Acremonium spp* and *Candida spp*.

Table 1. PH values of the stored coconut milk on the various days

Number of days sample	Morning PH value/time: 10:00 am	Evening PH value/time: 4:00 pm	Average PH value for the day/hours	Temperature of sample
Coconut milk actual PH	0.00	6.10	6.10	26℃
Day 1(24hours) coconut milk sample	4.60	4.37	4.48	26℃
Day 2(48hours) coconut milk sample	4.40	4.01	4.20	28.5℃
Day 3(72hours) coconut milk sample	4.21	3.77	3.99	26℃

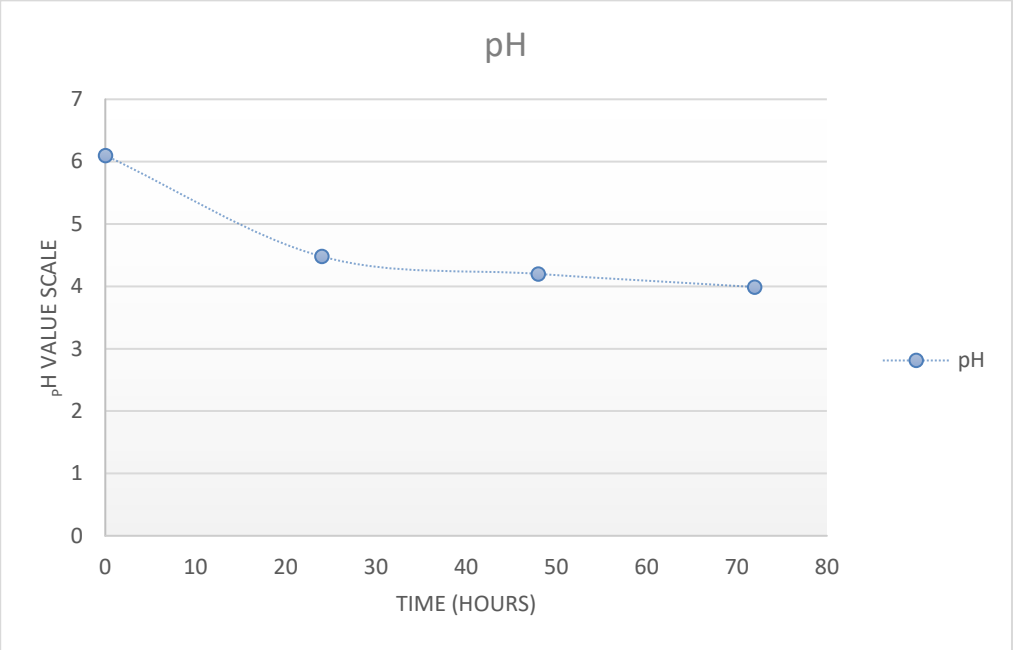

Figure 1. A graphical representation of the PH of the coconut milk sample of the various hours (24hrs, 48hrs, and 72hrs)

Table 2. Total microbial load of coconut milk sample of the various days (24hrs, 48hrs, and 72hrs)

Sample code	TVC (cfu/g)	TCC (cfu/g)	TFC (cfu/g)
Day 1 (24hrs) coconut milk sample	4.12×10^2	-	1.2×10^2
Day 2 (48hrs) coconut milk sample	6.0×10^1	-	1.0×10^1
Day 3 (72hrs) coconut milk sample	2.4×10^1	-	1.0×10^2

Note: TVC = Total viable count, TCC = total coliform count, TFC = total fungal count

Table 3. Biochemical characteristics of bacterial isolates

Sample	Morphology	Oxidase	Indole	Catalase	Coagulase test	citrate	TSI TEST					Gram stain	Motility	Probable microorganism
							G	L	S	G	H2s			
Day1:Na1, Na2, Na3 and Day2: Na2, Na3	Milk flat dry smooth edges	+	-	+	+	+	+	+	+	-	-	+	+	*Bacillus spp.*

Sample	Morphology	Oxidase	Indole	Catalase	Coagulase test	citrate	TSI TEST					Gram stain	Motility	Probable microorganism
							G	L	S	G	H2s			
Day1: Na4, Day2: Na1, Na5	Milkish raised no mucoid colour	-	-	+	-	+	+	+	+	-	-	+	-	*Staphylococcus aureus*
Day2: Na4	Milkish flat rough dry edge	-	-	-	+	-	+	+	+	-	-	+	-	*Bifidobacterium bifidum*
Day3: Na1	Milkish raised rough dry edge	+	-	+	+	+	-	-	-	-	-	-	+/-	*Pseudomonas spp.*
Day3: Na2, Na3, Na4	Milkish raised rough dry edge	-	-	-	+	-	+	+	+	-	-	+	-	*Lactobacillus spp*
Day3: Na5	Milkish raised smooth dry edge	+	+	+	-	+	+	-	+	-	-	-	+/-	*Aeromonas caviae*

Note: **+** = positive, **-** = negative, **G**= glucose, **L**= Lactose, **S**= Sucrose, **G**= Gas production, **H_2S**= Hydrogen Sulphide production, **$Na_{1,2,3,4,5}$** = nutrient Agar (subculture)

Table 4. Identification of fungal isolates

Sample code	Macroscopic appearance	Microscopic appearance	Possible fungi
Day 1(24hrs), Day 2 (48hrs) coconut milk sample	Whitish brown like a cottony colony with a white edge and yellow on the reverse plate	Septate Hyphae with conidia bearing sterigmata	*Aspergillus niger*
Day 1(24hrs) coconut milk sample	Colonies are generally flat, white to cream in colour, with a powdery to the granular surface and a yellow-brown on the reverse plate.	No conidial, only hyphae	*Trichophyton mentagrophytes*
Day 1(24hrs) coconut milk sample	White cottony colony with yellow on the reverse plate	Conidial with visible Hyphae	*Aspergillus flavus*
Day 1(24hrs) and Day 3(72HRS) coconut milk sample	Blue-green cottony colony with white edge and yellow on the reverse plate	Smooth conidial with visible hyphae	*Aspergillus fumigatus*

Sample code	Macroscopic appearance	Microscopic appearance	Possible fungi
Day 1(24hrs) and Day 3(72hrs) coconut milk sample	Raised, circular, white, and pale yellow on the reverse plate	Yeast form unicellular cells measuring 2-4 micrometre in diameter	*Candida spp*
Day 2(48hrs) coconut milk sample	White orange cottony colony with yellow-orange on the reverse	Joint septate hyphae visible and no conidia	*Acremonium spp.*

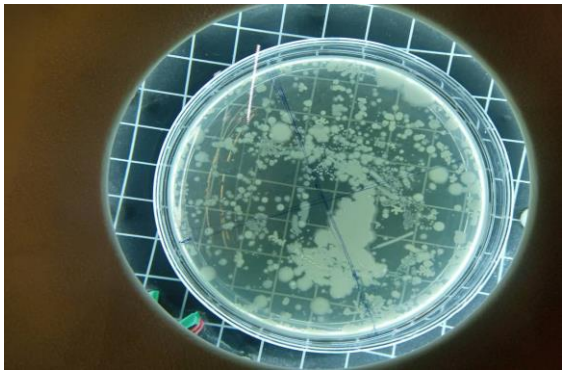

Figure 2. Plate 1: microbial growth of the coconut milk sample on the Nutrient agar plate

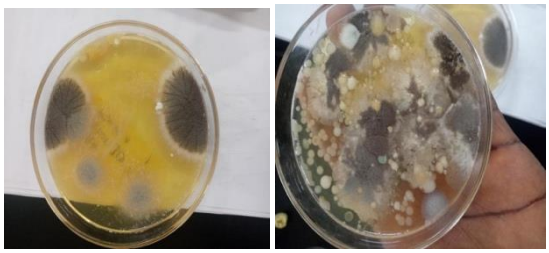

Figure 3. Plate 2: fungi growth in the culture plate

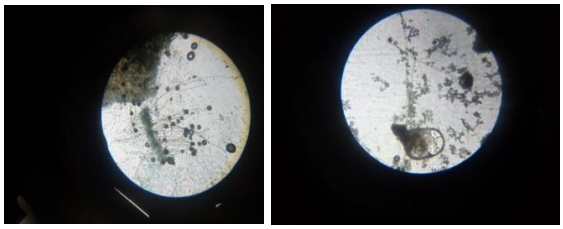

Figure 4. Plate 3: microscopic appearance of fungi growth

Coconut milk is very nutritious and has an increasing role in the fast-developing functional food market as they provide a key essential element that helps increase the immunity of the body system. Coconut milk also contains nutrients necessary for microbial growth and metabolism which makes it susceptible to microbial contamination. It is therefore necessary that this liquid food must be free from contamination as much as possible to ensure safety from health problems after consumption and to promote or enhance quality assurance of food consumable products. From the result of the analysis carried out, the bacteria isolate includes Bacillus spp, Staphylococcus aureus,

Bifidobacterium bifidum, pseudomonas spp, Lactobacillus spp and Aeromonas Caviae, and the fungi isolated include Aspergillus niger, Trichophyton mentagrophytes, Aspergillus Flavus, Aspergillus fumigatus, Acremonium spp, Candida spp. The 24hours (Day 1) and the 48hours (Day 2) coconut milk sample for the bacteria and the 24hours (Day 1) and 72hours (Day3) for fungi has the highest microbial load. According to Nurul et al. (2017), the source of contamination happened along the milking chain and it was mostly due to the unhygienic milking environment and contaminated milking equipment. These can be attributed to the level of exposure of the sample to the environment or practice of handling the material as well as the raw materials and equipment. Sari and Muhlisin, (2019) detected Staphylococcus aureus in traditional coconut milk Drinks and their report is similar to these studies. The presence of Staphylococcus aureus, Bacillus spp, and Aeromonas caviae, suggests the possibility of fecal contamination of the product (coconut milk) due to sanitation practices. The presence of Pseudomonas aeruginosis in coconut milk is contaminated due to the environment (Becerra,2014).

The presence of Lactobacillus is an important bacteria that serves as a probiotic and is used for the fermentation of food. According to Satheesh and Prasad, (2013) Lactobacillus can convert sugars in coconut milk into lactic acid which decreases the pH of fermenting milk to acidic which is in line with the value of 4.48 to 3.99 which we observed in the low pH of the coconut milk in the cause of the store hours. In acidic conditions, coconut milk undergoes denaturation and destabilization of proteins, causing the release of water and clusters of oil droplets (Pathil, 2018)

Thanuja et al., (2016) reported that the fermentation of coconut cream occurred when the enzymatic starter had been employed for processing. The presence of Aspergillus spp in coconut milk has been attributed to the microbial activity of the production of a toxin known as aflatoxin which is poisonous when consumed (Yoon et al., 2014). Aspergillus spp is associated with the ability to produce cellulase enzymes.

Contamination of coconut milk can be due to cross contaminations from the soil, water, air, human activities, storage, and distribution facilities. As earlier reported by Benyagoub et al., (2013) that high ambient temperatures also can lead to the growth of bacteria during the sales and transport of milk.

Many people had experienced sickness caused by the consumption of food produced under unsafe conditions. Other factors that lead to food contamination

include lack of hygiene, education, improper food storage condition and lack of clean environment, etc. several hazards are known to associate with food which includes microbiological, nutritional, food additives, and environmental (Fleetwood et al., 2019).

Conclusion

In conclusion, different bacteria and fungi isolated from coconut milk samples are members of the family Enterobacteriaceae namely *staphylococcus aureus*, *Bacillus spp*, and fungi such as *Aspergillus niger*, *Trichophyton mentagrophytes*, *Aspergillus Flavus*, *Aspergillus fumigatus*, *Acremonium spp* and *Candida spp*. The presence of staphylococcus aureus could be the fact that it is a skin microflora. Staphylococcus aureus is known to cause Enterotoxigenicity due to the production of enterotoxin.

Some Bacillus spp are food poisoning bacteria. Therefore, the presence of these organisms in coconut milk is of public health significance since these food products are marketed without proper supervision by the regulatory authorities like National Agency for Food and Drug and Control (NAFDAC), Standard Organization of Nigeria (SON), National Codex Committee, Federal Ministry of Agriculture and Nigeria Institute of standard (NIS).

Furthermore, it is more advisable that coconut milk made should be consumed within 24hours, and if to be preserved for further consumption or use it should be added or preserved with preservative and should be stored in a very cold condition to avoid microbial influence.

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