

Original Research Article

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Antibacterial Activity of Bacteria Isolated from Fermenting Cocoa Water against Some Pathogenic Bacteria

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ABSTRACT

The antimicrobial activity of bacteria isolated from fermenting cocoa water at a local farm at Supare-Akoko Ondo State, Nigeria against selected enteropathogenic bacteria was investigated. The fermentation was monitored for three days while the antimicrobial activity of the predominant isolates was evaluated using agar well diffusion method. The selected organisms were *Klebsiella pneumoniae*, *Salmonella paratyphi*, *Staphylococcus aureus*, *Proteus mirabilis*, *Escherichia coli* and *Enterococcus faecalis*. Bacteria that were isolated from the fermenting cocoa juice include *Bacillus licheniformis*, *B. licheniformis*, *B. subtilis*, *B. cereus*, *Lactobacillus plantarum*, *L. brevis* and *Lactococcus lactis*. The total viable count and lactic acid bacteria count of the fermenting juice increased from 7.94×10^3 cfu/ml and 3.98×10^2 cfu/ml to 1.25×10^6 cfu/ml and 3.16×10^5 cfu/ml respectively. The pH of the juice decreased from 5.90 to 4.30 while the temperature increased from 35°C to 41°C. The cell free supernatants of the dominant isolates, *L. plantarum* and *L. brevis*, inhibited all the pathogenic microbes. The highest and the lowest zones of inhibition were observed in *S. aureus* and *E. faecalis* cultures respectively. The antibacterial activity of these bacteria could be due to their ability to produce varieties of antimicrobial substances such as organic acids, hydrogen peroxide, carbon dioxide, and bacteriocins. Consumption of fermented cocoa juice could be an economical alternative approach to the use of antibiotics in treating infections caused by these pathogens.

Keywords

Fermentation,
Cocoa,
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Introduction

Antimicrobial agents are general nomenclature for all drugs or chemical substances that act on microorganisms either to kill or suppress their growth. Antimicrobial agents with selective toxicity are especially useful as chemotherapeutic agents in treating infectious diseases (Prescott *et al.*, 2008). They can exert their effect on microorganisms in any of the

following ways; protein synthesis inhibition, inhibition of cell wall synthesis, inhibition of nucleic acid synthesis (Kimball and Jefferson, 2006). The preservative activities of these microorganisms are due to their ability to produce a variety of antimicrobial substances as a natural competitive means to overcome other microorganisms sharing the same niche (Olivera *et al.*, 2000). Cocoa belongs to the family Malvaceae esterculiacea. It is a

neotropical species native to the humid tropical plains of Central and South America (Whitkus *et al.*, 1998). The raw cocoa beans possess astringent and bitter flavours which have to be processed after harvest before they can be converted to good taste and flavoursome fruit juice (cocoa water). The quality of the final cocoa beans is determined by curing which involves fermentation followed by drying of the cocoa beans (Biehl *et al.*, 1996).

Evidence had it that cocoa pulp is composed of 82%-87% of water, 10%-15% of sugar (Ardhana *et al.*, 2003). The cocoa water is usually milky in colour which can be consumed fresh or after fermentation. The water (cocoa juice) becomes an alcoholic drink due to the fermentative action of the constituent microorganisms. Of the sugar present in fermenting cocoa water, about 60% is sucrose and 39% are mixture of glucose and fructose (Lopez, 2007). During its fermentation, the anaerobic yeasts present in the fermenting cocoa water ferment the sugar to ethanol and also decrease the pH of the cocoa water. During fermentation, the growth of pathogens, as well as other spoilage organisms is frequently inhibited through antimicrobial components produced by fermenting microorganisms (Park *et al.*, 2005). These antimicrobial components produced by lactic acid bacteria include organic acids, hydrogen peroxide, carbon dioxide, acetaldehyde, diacetyl, ethanol and bacteriocins (González *et al.*, 2007). In cocoa fermentation, lactic acid bacteria occur in large number and confer qualities like extended shelf life, aroma and make the product safe for consumption. In fact, the cultures of lactic acid bacteria have the ability to produce antimicrobial metabolites to combat contamination caused by microbial pathogens, especially on cocoa commodities (Motarjemi, 2002).

Cocoa has become an important ethnomedicinal plant since due to its unique chemical composition which is more than 500 different compounds. Among the reported contributions of cocoa to human health, are the antioxidant (Jalil and Ismail, 2008), anti-inflammatory (Cragg *et al.*, 2005), anticarcinogenic (Maskarinec, 2009) and antimicrobial activities (Fapohunda and Afolayan, 2012). The intake of fermenting cocoa water reduces anxiety while also promoting alertness. A cup of cocoa juice can provide the same energy as a cup of coffee. They are considered to be nature's anti-depressant. Cocoa contains dopamine, phenylethylamine (PEA) and serotonin, all of which are used to promote positive mental health and moods. It was shown that the polyphenols in cocoa beans might improve sensitivity to insulin. Scientists are currently studying the connection between obesity and condition known as insulin resistance syndrome (Cragg *et al.*, 2005).

The aim of this project is to study the effect of the cell free culture isolates of bacteria involved in fermentation of cocoa beans water (cocoa juice) as an antimicrobial feature to inhibit growth of selected pathogenic bacteria such as *Klebsiella pneumoniae*, *Salmonella paratyphi*, *Staphylococcus aureus*, *Proteus mirabilis*, *Escherichia coli* and *Enterococcus faecalis*.

Materials and Methods

Collection of samples and screening of test organisms

Fermented cocoa water samples were aseptically collected from a local farm in Supare-Akoko Ondo state into sterile bottles and immediately transported to the Microbiology laboratory of Adekunle Ajasin University, Akungba-Akoko, Nigeria. The cocoa water was fermented for 3 days in a

sterile bucket and was hermetically covered. The morphological and biochemical test were carried out to ascertain proper identification of the isolates. They were thereafter sub cultured into nutrient agar slants and stored at 4°C in the laboratory refrigerator.

Klebsiella pneumoniae, *Salmonella paratyphi*, *Staphylococcus aureus*, *Proteus mirabilis*, *Escherichia coli* and *Enterococcus faecalis* were collected from the Microbiology laboratory of Ondo State Specialist Hospital (OSPH), Ikare, Ondo State, Nigeria.

Isolation and enumeration of microorganisms from the cocoa water sample

Ten ml of the sample was added to 90 ml of normal saline to make the initial dilution. This suspension was homogenized by gentle manual agitation and serially diluted from 10^{-1} to 10^{-10} . Isolation of the bacteria was done using pour plate method on de Mann Rogosa Sharpe (MRS) and Nutrient media. Bacterial cultures were incubated at 35°C for 1-2 days. MRS plates were incubated under anaerobic conditions simulated using a H_2/CO_2 generating kit (Oxoid) according to the manufacturer's instructions. Enumeration and isolation were done on daily basis during the fermentation period. Counts were expressed in colony-forming unit per ml of sample. The isolates were subcultured repeatedly until pure isolates were observed. The slants of the respective pure isolates were prepared and kept in the refrigerator at 4°C for subsequent tests (Alexopoulos and Mims, 1988).

Identification of the microorganisms from the samples

Morphology of the colonies such as shape, colour, size, edge, elevation and surface texture were observed on the plates after 18-24 hours of incubation after subsequent

streaking on solidified plates. Gram staining and biochemical characteristics of the isolates carried out according to Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1999).

Determination of total titration acidity (TTA)

The TTA was determined by titrating 0.2M of NaOH against a known amount of the fermenting cocoa water for each day, 25 ml of sodium hydroxide was pipetted into a conical flask; a drop of phenolphthalein indicator was added to give a deep pink colouration. The fermented cocoa water was placed in 50 ml capacity of burette and titrated against the base in conical flask until it brings a light pink colour showing that end point is reached (TTA = Volume of NaOH used (ml) \times 0.009 \times 100/ volume of sample used) according to Owuamanam *et al.*, (2011).

pH and Temperature measurement

The pH and temperature of the fermenting cocoa water were determined daily using the pH meter and thermometer, respectively. The pH was measured by dipping the electrode connected to the meter into a buffer solution to standardize the meter, the electrode was then inserted into the sample and the reading was taken for each day. The temperature was determined by dipping the thermometer into the sample from fermenting cocoa water and the reading was taken by the movement of the mercury in the thermometer (Prescott *et al.*, 2008).

Screening and selection of Lactobacilli with antimicrobial activity

Screening and selection of *lactobacilli* colonies of *Lactobacillus plantarum* and *Lactobacillus brevis* with antimicrobial activity was done using enteropathogenic

organisms. The detection was done using the MRS broth. The broth culture was centrifuged at 14,000 rpm for 15 minutes, the supernatants were filtered through a membrane filter of 0.2 µm pores and the resulting cell free supernatant was tested against the selected entero-pathogenic bacteria using agar diffusion assay as described by Onwuakor *et al.*, (2014).

Screening of cell-free supernatant and cell-free filtrate for antibacterial activities

Agar well diffusion method was used. Three wells of 5.00 mm in diameter each were made on solidified Mueller-Hinton agar plate seeded with the test bacteria using a sterile cork borer. Equal volume of cell free supernatant the broth cultures was introduced into the wells made on the agar, sterile distilled water and standard antibiotics (ciprofloxacin 20mg/ml) were used as the negative and positive control, respectively. Experiment was carried out in triplicates, zones of inhibition after 24 hours were calculated. Zones of inhibition that are less than 15, between 16 and 22 and between 23 and above were recorded as low susceptibility, moderate susceptibility and high susceptibility, respectively (Adegbehingbe and Bello, 2014).

Results and Discussion

Two genera of bacteria were identified from the fermenting cocoa water sample during the three-day fermentation period. The genera are *Lactoacillus* species which comprised *Lactobacillus brevis*, *L. lactis* and *L. plantarum* and the *Bacillus* species which were *B. cereus*, *B. licheniformis* and *B. subtilis*. The predominant microorganisms from the fermenting sample were *Lactobacillus brevis* and *L. plantarum* which appeared throughout the fermentation period. *Bacillus subtilis* and *B. licheniformis* appeared at the first and the days of fermentation while

B. cereus was isolated only on the second day of fermentation (Table 1).

The microbial counts of the fermenting cocoa water increased during the course of fermentation. The total viable counts increased from 7.94×10^2 cfu/ml to 1.25×10^4 cfu/ml while the lactic acid bacteria counts increased from 3.98×10^2 cfu/ml to 3.16×10^3 cfu/ml (Figure 1).

Figure 2 shows the pH, TTA and the temperature of the fermenting cocoa juice. The pH decreased from 5.90 to 4.10 while the total titratable acidity increased from 3.62% to 4.39%. The temperature of the fermenting cocoa juice increased from 35°C to 41°C at the third day of fermentation.

Cell free filtrates and supernatants of *L. brevis* and *L. plantarum* were inhibitory against all the test isolates; *Klebsiella pneumoniae*, *Salmonella paratyphii*, *Staphylococcus aureus*, *Proteus mirabilis*, *Escherichia coli* and *Enterococcus faecalis*. The results revealed variable zones of inhibition with *L. plantarum* having the stronger antibacterial property than *L. brevis*. The most susceptible among the pathogens was *S. aureus* with zones of inhibition 12.20 mm and 9.00 against *L. plantarum* and *L. brevis* respectively. *Escherichia coli* and *P. mirabilis* had the same zones of inhibition against each of the isolates with values of 8.40 mm and 8.00 mm against *L. plantarum* and *L. brevis* respectively while the least susceptible among them was *E. faecalis* having 6.50 mm and 5.70 mm respectively (Table 2).

Two genera of bacteria were identified from the fermenting cocoa water sample during the three-day fermentation period. The genera are *Lactobacillus* species which comprised *Lactobacillus brevis*, *L. lactis* and *L. plantarum* and the *Bacillus* species which were *B. cereus*, *B. licheniformis* and *B. subtilis*.

Fig.1 Bacterial counts of the fermenting cocoa juice

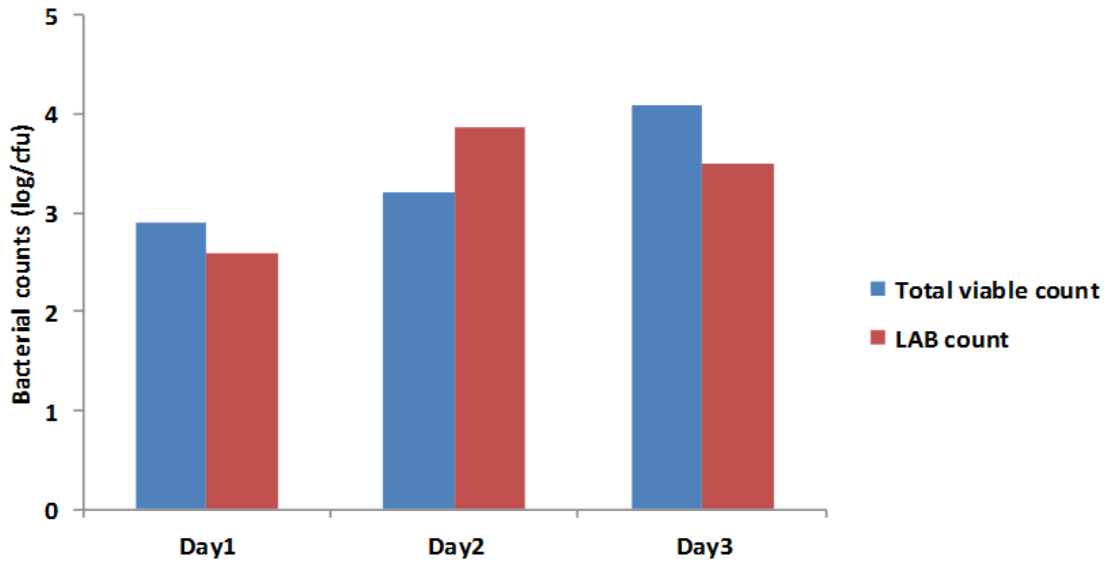
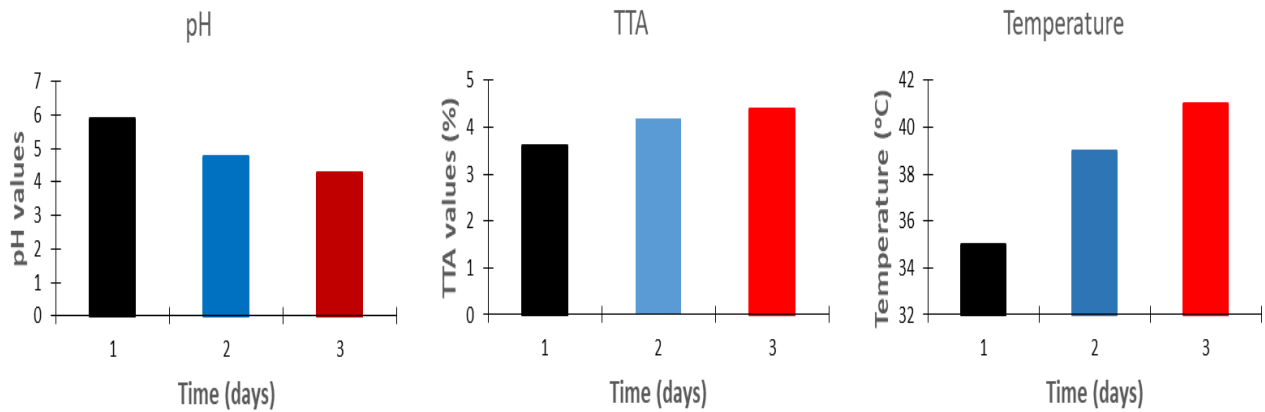


Fig.2 Physico-chemical properties of fermenting cocoa water during fermentation



TTA = total titratable acidity.

Table.1 Occurrence of the microorganisms from the fermenting cocoa water

Microorganisms	Days of fermentation		
	1	2	3
<i>Lactobacillus plantarum</i>	+	+	+
<i>Lactobacillus lactis</i>	-	+	+
<i>Lactobacillus brevis</i>	+	+	+
<i>Bacillus subtilis</i>	+	+	-
<i>Bacillus licheniformis</i>	+	+	-
<i>Bacillus cereus</i>	-	+	-

+ = present

- = absent

Table.2 Antibacterial activity of lactic acid bacteria from fermenting cocoa water against selected pathogenic bacteria

(Inhibition zones in millimeter)						
Isolates	<i>E. coli</i>	<i>S. aureus</i>	<i>Klebsiella pneumoniae</i>	<i>Proteus mirabilis</i>	<i>Salmonella paratyphi</i>	<i>Enterococcus faecalis</i>
<i>L. plantarum</i>	8.40	12.20	7.20	8.40	8.20	6.50
<i>L. brevis</i>	8.00	9.00	6.00	8.00	7.40	5.70

The predominant microorganisms from the fermenting sample were *Lactobacillus brevis* and *L. plantarum* which appeared throughout the fermentation period. *Bacillus subtilis* and *B. licheniformis* appeared at the first and the days of fermentation while *B. cereus* was isolated only on the second day of fermentation (Table 1). De Vuyst *et al.*, (2010) isolated *L. plantarum* and *L. brevis* during the fermentation of cocoa. Galvez *et al.*, (2007) reported the presence of *Bacillus cereus*, *Bacillus licheniformis* and *Bacillus subtilis* during the fermentation of cocoa juice. The disappearance of *Bacillus* species after the second day of fermentation was probably due to high concentrations of antibacterial substances such as bacteriocins produced by the lactic acid bacteria.

Figure 1 shows the pH, TTA and the temperature of the fermenting cocoa juice. The pH decreased from 5.90 to 4.10 while the total titratable acidity increased from 3.62% to 4.39%. It is well known that lactic acid bacteria are acid tolerant (Atter *et al.*, 2014). The temperature of the fermenting cocoa juice increased from 35°C to 41°C at the third day of fermentation. The increase in temperature might be due to the metabolic activities of the fermenting microorganisms leading to heat production. This collaborated with the findings of Olivera *et al.*, (2000) and Lima *et al.*, (2011).

The supernatants of *L. brevis* and *L. plantarum*, which were the predominant isolates, were inhibitory against all the test

isolates; *Klebsiella pneumoniae*, *Salmonella paratyphi*, *Staphylococcus aureus*, *Proteus mirabilis*, *Escherichia coli* and *Enterococcus faecalis*. The results revealed variable zones of inhibition with *L. plantarum* having the stronger antibacterial property than *L. brevis*.

The most susceptible among the pathogens was *S. aureus* with zones of inhibition 12.20 mm and 9.00 against *L. plantarum* and *L. brevis* respectively. *Escherichia coli* and *P. mirabilis* had the same zones of inhibition against each of the isolates with values of 8.40 mm and 8.00 mm against *L. plantarum* and *L. brevis* respectively while the least susceptible among them was *E. faecalis* having 6.50 mm and 5.70 mm respectively (Table 2). The selected lactic acid bacteria had been peculiar with the production of antibacterial substances such as acetic acid, bacteriocin, lactic acid and hydrogen peroxide (Fapohunda and Afolayan, 2012; Onwuakor and Ukaegbu-Obu, 2014).

This research work has showed that antimicrobial producing bacteria can be isolated from fermenting cocoa (cocoa juice). The preservative activity of these microorganisms is due to their ability to produce varieties of antimicrobial substances. It was observed that these microorganisms compete and overcame other bacteria in the same community. This will be an innovative approach as alternative to antibiotics in treating infections caused by these pathogens.

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