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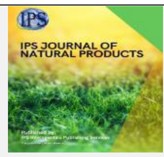

Synergistic Effects of Medicinal Plant Extracts and Lactobacillus Species against Bacterial Pathogens from Smoked Fish and Chicken: An *In Vitro* Study

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Abstract

The rising threat of antibiotic-resistant bacterial pathogens in smoked fish and chicken necessitates exploring alternative natural approaches to control these pathogens. Combining medicinal plant extracts with probiotics may offer a synergistic solution. This study investigates the synergistic effect of medicinal plant extracts and lactobacillus species against bacterial pathogens isolated from smoked fish and chicken providing insights for improving food safety and reducing antibiotic resistance. A total of 280 samples that comprises 40 samples each of native chicken meat, layers chicken meat, broiler chicken meat, *Chupea havengus* (Herring/sawa), *Truchurus trachurus* (Horse Mackerel/Kote), *Scomber Scombrus* (Atlantic Mackerel/Titus) and *Sphyraema barracuda* (panla). The synergistic effect of some selected medicinal plants and *Lactobacillus* species was carried out using the agar-welled diffusion method after combining the biological agents at different concentrations. The data obtained were analyzed using a one-way analysis of variance (ANOVA) and turkey's test as post hoc analysis. The plant extracts; *piper guineense* (PU), *Ocimum gratissimum* (OR) and *Gongronema latifolium* (GA) which contained alkaloids, tannins, phenolics, saponins, flavonoid, glycosides and steroids, and *Lactobacillus* species; *L. acidophilus* strain NC56 (LAN56) and *L. plantarum* stain 2359 (LP2359) significantly ($p \leq 0.05$) inhibited *Escherichia coli* 0157:H7 strain ECP19-598 (ECEC1), *staphylococcus aureus* strain JP18269 (SAJP1), *Listeria monocytogenes* strain LM16 (LMLM1) and *Salmonella enterica* serovar Enteritidis EC20110358 (SEEC2) isolated from the studied samples. The diameter zones of inhibition significantly ($p \leq 0.05$) increased when the biological agents were combined in different doses, and OR+PU+GU and LAN56 + LP2359 combined in 2:1:1 and 2:1 respectively recorded the highest diameter zones of inhibition. Therefore, the medicinal plant extracts and *Lactobacillus* species combined in their optimal doses showed the highest inhibition against ECEC1, SAJP1, LMLM1 and SEEC2 isolated from smoked fish and chicken meat.

Keywords: *Smoked-fish, Chicken-meat, Inhibition, Biological-agents, Extracts, Lactobacillus, probiotics*

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Introduction

Medicinal plants are plants that contain natural substances that enable them to be optimized for disease prevention and control (Ajiloye *et al.*, 2022). These plants are distributed globally but can only be identified by botanists and herbalists in traditional medicine. Several ailments have been treated using local medicinal plants and most of the medicinal plants are processed so that their form can be easily assimilated by patients (Ashok *et al.*, 2014). Though medicinal plants are known and optimized globally, yet, their natural endowment such as prophylactic and therapeutic potentials have been tapped by a few countries such as India, China, Thailand, Japan, Germany, Brazil, Italy, and South Africa.

Research has shown that medicinal plants contain phytochemical compounds, which can be extracted and optimized in crude form or as essential oils for tackling bacterial pathogens (Dahiya and Purkaryastha, 2012). Phytochemical compounds in medicinal plants\ extracts such as flavonoids, saponins, tannins etc. have been revealed to exhibit antibacterial potentials. These extracts are potent to the extent that metabolic processes and structural development in pathogenic bacteria are inhibited and eliminated (Ahmed *et al.*, 2014).

The need for an effective inhibitory compound against pathogenic bacteria isolated from smoked fish and chicken cannot be overemphasized (Bardoe *et al*., 2023). Smoked fish and roasted chicken are mostly consumed by people in Nigeria due to their cost-effectiveness and high utility but the presence of pathogenic bacteria poses a threat to consumers (Camargo *et al*., 2017). When an individual contracts infection due to consumption of unwholesome smoked fish and chicken, treatment requires a potent antibacterial agent, which may be difficult to obtain due to excessive microbial resistance. When there is an inadequate tackling of an infectious agent, the life of the infected patient could be jeopardized and others could be infected as well (Ahmed *et al*., 2014; Bisii-Johnson *et al*., 2017).

In a study conducted by Holkem *et al*. (2022), it was observed that extracts from medicinal plants are best optimized when combined with another extract, probiotics, or conventional antibiotic. The combined efficacy of two or more extracts produces effects that could be difficult to resist by pathogenic bacteria. Similarly, *Lactobacillus* species are known for their probiotic potentials, and the ability of these species to produce inhibitory substances has been documented (Goh *et al*., 2014; Sookkhee *et al*., 2024). This shows that combining extracts from medicinal plants with *Lactobacillus* species could result in enhancement or acceleration of the bioactive compounds produced by the bacteria as reported by Ziarno *et al*. (2021).

Several researchers have worked on the synergistic effects of extracts from medicinal plants and *Lactobacillus* species against bacterial pathogens such as Ziarno *et al*. (2021), Holkem *et al*. (2022), and Sookkhee *et al*. (2024) but few studies are available on the synergism of extracts from medicinal plants and *Lactobacillus* species against pathogenic bacteria isolated from smoked fish and chicken. Hence, this study aims to elucidate the synergism of extracts from medicinal plants and *Lactobacillus* species against pathogenic bacteria isolated from smoked fish and chicken.

Materials and Methods

Sample Collection: A total of 280 samples which comprises 120 roasted chicken meat samples and 160 smoked fish were used for this study (Iheukwumere *et al*., 2018a). The roasted chicken meat samples included 40 samples of each of Native chicken meat samples, old layer meat samples and broiler chicken meat samples. The smoked fish samples included 40 samples of each of *Clupea harengus* (Sawa/Herring), *Truchurus trachurus* (Kote/Horse Mackerel), *Scomber scombrus* (Titus/Atlantic Mackerel) and *Sphyraema barracuda* (Panla). Ready-to-eat samples were aseptically separated using a sterile stainless spoon (Hamada) and collected into a sterile aluminium foil through hand picking (Iheukwumere *et al*., 2018b). Before the sampling, the hands were washed thoroughly with soap and cleaned water and then rinsed with 70 % ethanol. Sampling was done at different selling locations in different towns in Anambra State. The samples were placed into a cooler containing ice blocks wrapped in a sterile polythene bag and were used for sample transportation. The temperature of the cooler was checked and adjusted to 28^{o}C-30^{o}C by reducing the quantity of ice inside the cooler to reduce or prevent microbial shock. The samples were carefully and aseptically arranged inside the cooler without direct contact with the ice bag. The cooler was then covered and the drain plug was securely taped with packing tape to prevent accidental opening of the cooler. The cooler was then sagely carried to the Laboratory for analysis within 2 hours of sample collection. The same procedure was repeated for other collection times (Iheukwumere *et al*., 2018c).

Sample Preparation: The samples were prepared using the routine laboratory technique. The meat samples were ground using a sterile blender (LXB 242). Then 1.0 g of each of the ground samples was aseptically weighed into a 10 mL test tube (Pyrex) each respectively. Three milliliter of sterile peptone water was aseptically added into each test tube and these were shaken thoroughly and then made up to 10.0 mL using the sterile peptone water for each test tube as described in Chesbrough (2010)

In Vitro Synergistic Antibacterial Activities of the Plant Extracts and *Lactobacillus* Species

Preparation of test isolate: The test isolates were prepared using the method described by Okpalla *et al*. (2012), Iheukwumere *et al*. (2017), Iheukwumere *et al*. (2018d) and Cheesbrough (2010). The isolates were aseptically subcultured into a broth culture and incubated at 35+ 2^{0}C for 24 h. The broth culture of each isolate was centrifuged using an electric centrifuge. The sediment from each culture was diluted to a turbidity that matched 0.5 MacFarland standard that was prepared by mixing 0.5 mL of 1.175% $BaCl_2$ $2H_2O$ and 99.5 mL of 1% Conc. H_2SO_4. The prepared isolates were standardized by comparing the absorbance with that of 0.5 McFarland standards at 640 nm using a UV/visible spectrophotometer.

Preparation of plant materials: The fresh seeds *of Piper guineense*, leaves of *Ocimum gratissimum* and leaves of *Gongronema latifolium* were collected and they were appropriately authenticated. The fresh seeds and the leaves were dried under shade at room temperature for 14 days. The dried samples were ground to powdered form using a sterile electric grinder (LE Max/ LXB 242). Twenty grams of the ground samples each was macerated with ethanol for 72 h. Whatman No 1 filter paper was used to filter the mixture. The extracts were concentrated by evaporating to dryness at room temperature in a steady air current.

Preparation of plant extracts: The weight obtained was weighed using an electronic weighing balance, 10 mL of peptone water (diluents) was added and made up to 50 mL using the diluents. Then 50 mL mixture (1:1) of OR+PU, OR+GA, PU+GA and 1:1:1 mixture of OR+PU+GA were prepared. All these were tested for the best activity as described in the study published by Iheukwumere *et al*. (2017). Also, 50 mL mixtures of U (OG+PU+GA) at optimum concentration (250 mg/mL) were prepared as follows: U (1:1:1), U (1:1:2), U (1:2:1) and U (2:1:1), and tested for the best activity (Iheukwumere and Ejike, 2016; Chukwurah and Iheukwumere, 2013 and Ejike *et al*., 2017)

Isolation of *Lactobacillus* species: One milliliter (1.0 ml) sample (Yoghurt and banana) was aseptically transferred into a sterile test tube (Pyrex) containing 9.0 ml of the diluent (sterile normal saline). One milliliter of the diluted sample (10$^-$

[1]) was plated on Petri dishes (60 mm OD × 55 mm ID × 13mm high) containing deMann Rogosa Sharpe medium (MRSBiotech) using pour plate method. All the plates in triplicates were incubated inverted in microaerophilic condition for 48 h (Cheesbrough, 2010). The pure isolates were characterized using the morphological, biochemical and molecular characteristics as described in the study published by Cheesbrough (2010) and Frank and Robert (2015).

Preparation of *Lactobacillus* broth culture: The test isolates were prepared using the method described by Cheesbrough (2010). The isolates were aseptically subcultured into a broth culture and incubated at 35+ 2^0C for 24 h. Then 50 mL of different mixtures of LAN56 and LP2359 were prepared as followed: V (1:1), V (1:2) and V (2:1) and tested for the best activity.

***In vitro* susceptibility study of the biological agents using agar well diffusion method:** This was carried out by the modified method described by Iheukwumere *et al*. (2020). The test organisms were seeded in Muller Hinton Agar (MHA/BIOTECH) plates. The plates were appropriately labelled. A sterile cork borer of 5 mm diameter was used to make the wells on the medium. A one-tenth millilitre of various extracts was dropped into each labelled well and then placed vertically in the Bacteriological incubator and incubated at 37±2°C for 24 h. The susceptibility patterns were determined by measuring the diameter of the zones of inhibition (mm) produced after incubation. The procedure was repeated using the mixture of *Lactobacillus* species broth culture.

Statistical Analysis

The data obtained from this study were represented in Tables, Figures and as mean ± standard deviation. The significance of the study was carried out using a one-way Analysis of Variance (ANOVA) at 95 % confidence level. The pair-wise comparison was done using the Turkey test as described by Iheukwumere *et al*. (2018a).

Results

The molecular characteristics revealed the presence of *Escherichia coli* O157:H7 strain ECP19-598 (ECEC1), *Staphylococcus aureus* strain JP 18269 (SAJP1), *Listeria monocytogenes* strain LM16 (LMLM1) and *Salmonella enterica* subspecies *enterica* serovar Enteritidis strain EC20110358 (SEEC2) as shown in Table 1. The molecular identities of the *Lactobacillus* species revealed the presence of *Lactobacillus acidophilus* strain NC56 (LAN56) and *Lactobacillus plantarum* strain 2359 (LP2359) as shown in Table 2.

The *in vitro* biotreatment of the pathogenic bacterial isolates are shown in Table 3. The study revealed the pronounce activities of the plant extracts and *Lactobacillus* species. The activities of the biological agents were most against ECECE1, and OR showed the highest activities against the test isolates. The activities of CPX were significantly ($p<0.05$) higher than the activities of GA, LAN56 and LP2359 against ECEC1, and non-significantly ($p>0.05$) higher than the activities of the biological agents against SAJP1 and SEEC2 except that but its activities were lower than the activities of OR and PU against LMLM1 as shown in Table 3. The activities of the biological agents against the test isolates were statistically significant ($p<0.05$) whereas the variations among the activities of the biological agents were statistically non-significant ($p>0.05$).

The mean diameter zone of the combination of the biological agents against the pathogenic bacterial isolates are shown in Table 4. The study revealed that there were significant synergistic activities among the combination, and the activities (U) were non-significant ($p>0.05$) mostly against the test isolates and ECEC1 was mostly inhibited. Also, the activities of the combination of LAN56 and LP2359 (V) against the test isolates were synergistic and higher than the individual activities.

The study further revealed that the dose mixtures of the biological agents showed pronounced activities against the test isolates (Table 5). The dose mixture (2:1:1) of the plant extract and (1:1) of the *Lactobacillus* species showed the highest activities against the test isolates. Their activities were statistically significant ($p<0.05$) whereas the activities among the dose mixtures were statistically non-significant ($p>0.05$).

Table 1: Molecular characteristics of the bacterial isolates

Parameter	Isolate P	Isolate Q	Isolate R	Isolate S
Max score	38284	34485	25078	26613
Total Score	38284	34485	60734	26613
Query cover (%)	100	100	100	100
E-value	0.0	0.0	0.0	0.0
Identity (%)	100	100	100	100
Accession Number	CP066753.1	CP097114.1	CP027029.1	CP007260.1
Description	*Escherichia coli* 0157:H7 strain ECP19-598 C.G (ECEC1)	*Staphylococcus aureus* strain JP18269 complete genome (SAJP1)	*Listeria monocytogenes* strain LM16 chromosome (LMLM16)	*Salmonella enterica* subsp *enterica* serovar Enteritidis strain EC 20110358 complete genome (SEEC2)

Table 2: Molecular characteristics of the *Lactobacillus* species

Parameter	Isolate K	Isolate M
Max score	40501	35179
Total score	40501	68428
Query cover (%)	100	100
E – value	0.0	0.0
Percent identity	100	100
Accession number	CP106868.1	CP145812.1
Description	*Lactobacillus acidophilus* strain NC56 chromosome complete genome (LAN56)	*Lactobacillus plantarum* strain 2359 chromosome complete genome (LP2359)

Table 3: *In vitro* biotreatment of pathogenic bacterial isolates

Biotreatment		Mean Diameter (mm) using 5mm corkborer		
	ECEC1	SAJP1	LMLM1	SEEC2
OR (250mg/ml)	17.86 ± 0.11	13.57 ± 0.12	17.48 ± 0.11	17.22 ± 0.17
PU (250mg/ml)	16.22 ± 0.11	12.86 ± 0.22	15.77 ± 0.33	15.48 ± 0.11
GA (250mg/ml)	14.44 ± 0.11	11.08 ± 0.17	13.86 ± 0.14	12.78 ± 0.11
LAN56 (10^8cells/ml)	14.26 ± 0.11	10.84 ± 0.21	12.36 ± 0.33	12.92 ± 0.22
LP2359 (10^8cells/ml)	15.84 ± 0.81	15.83 ± 0.41	14.28 ± 0.17	16.22 ± 0.11
CPX (5mg/ml)	24.56 ± 0.12	14.22 ± 0.11	15.48 ± 0.14	19.36 ± 0.12

Table 4: *In vitro* activities of combination of the biological agents against the test isolates.

Biological agents	Mean zone Diameter (mm) of 5mm cork borer			
	ECEC1	SAJP1	LMLM1	SEEC2
OR + PU	21.86 ± 0.82	18.21 ± 0.11	23.14 ± 0.12	18.33 ± 0.17
OR + GA	19.76 ± 0.51	16.18 ± 0.14	20.74 ± 0.33	17.11 ± 0.11
PU + GA	17.26 ± 0.12	14.86 ± 0.13	17.23 ± 0.14	15.22 ± 0.22
OR + PU + GA (U)	24.14 ± 0.22	19.86 ± 0.21	24.22 ± 0.11	19.86 ± 0.12
LAN56 + LP2359 (V)	16.84 ± 0.14	16.22 ± 0.81	15.11 ± 0.11	17.86 ± 0.33
CPX	24.56 ± 0.12	14.22 ± 0.11	15.48 ± 0.14	19.36 ± 0.12

Table 5: *In vitro* activities of different dose mixture of the biological agents against the test isolates

Dose Mixture	Mean Zone Diameter (mm) of 5mm corkborer			
	ECEC1	SAJP1	LMLM1	SEEC2
U (1:1:1)	21.86 ± 0.82	18.21 ± 0.11	23.14 ± 0.12	18.33 ± 0.17
U (1:1:2)	21.33 ± 0.12	18.02 ± 0.12	22.88 ± 0.42	18.21 ± 0.11
U (1:2:1)	21.72 ± 0.11	18.12 ± 0.11	23.72 ± 0.33	18.86 ± 0.17
U (2:1:1)	22.08 ± 0.22	18.76 ± 0.11	23.72 ± 0.33	18.86 ± 0.17
V (1:1)	16.84 ± 0.14	16.22 ± 0.81	15.11 ± 0.11	17.86 ± 033
V (1:2)	16.38 ± 0.11	16.01 ± 0.21	14.87 ± 0.19	17.33 ± 0.17
V (2:1)	16.92 ± 0.81	16.74 ± 0.33	15.42 ± 0.12	18.08 ± 0.11
CPX	24.56 ± 0.12	14.22 ± 0.11	15.48 ± 0.14	19.36 ± 0.12

Discussion

The significant mean bacterial counts, majorly total heterotrophic bacterial counts (THBC), total coliform counts (TCC), total faecal coliform counts (TFCC), total *Salmonella* counts (TSC), total *Shigella* counts (TSHC), total *Staphylococcus aureus* counts (TSAC) and total *Listeria monocytogenes* counts (TLMC) supported The findings of many researchers (Adeyanju and Ishola, 2014; Mashak, 2018; Ishihava *et al.*, 2020; Karisma *et al.*, 2021). Similar deductions were made by Iheukwumere *et al*. (2017).

The pronounced activities of the plant extracts against the pathogenic bacterial isolates supported the findings of many researchers (Ahmed *et al.*, 2014; Radaelli *et al.*, 2016; Ugur *et al.*, 2016; Heer *et al.*, 2017; Iheukwumere *et al.*, 2017; Mostafa *et al.*, 2018; Hetta *et al.*, 2020; Ghavam *et al.*, 2020; Oliveira *et al.*, 2024; Meshaal *et al.*, 2021). The most significant inhibition of the extracts against ECEC1 agrees with the findings of Iheukwumere *et al*. (2017) and Ghavam *et al*. (2020). The highest activities of OR against the studied pathogenic bacterial isolates could be attributed to the potency of the extract. Similar deductions were made by Iheukwumere *et al*. (2017). The pronounced activities of *Lactobacillus* *acidophilus* strain NC56 (LAN56) and *Lactobacillus plantarum* strain 2359 (LP2359) against the studied pathogenic bacterial isolates corroborated with the findings of many researchers (Nouri *et al.*, 2010; Darsanaki *et al.*, 2012; Tonekabon, 2013; Dec *et al.*, 2014; Bratz *et al.*, 2015; Dec *et al.*, 2016). The slight increase in the activities of LP2359 when compared to that of LAN56 could be attributed to the potency and level of bacteriocins produced by the *Lactobacillus* species.

Conclusion

The study has shown that *Piper guineense* (PU), *Ocimum gratissimum* (OR) and *Gongronema latifolium* (GAmedicinal plant extracts and *L. acidophilus* strain NC56 (LAN56) and *T. plantarum* stain 2359 (LP2359) combined in their optimal doses showed the highest inhibition against *Escherichia coli* 0157:H7 strain ECP19-598 (ECEC1), *staphylococcus aureus* strain JP18269 (SAJP1), *Listeria monocytogenes* strain LM16 (LMLM1) and *Salmonella enterica* serovar Enteritidis EC20110358 (SEEC2) isolated from smoked fish and chicken meat

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Ethical approval: Not applicable

Authors Contributions: All contributed towards the study design, experiment execution, data analysis, and manuscript drafting.

Availability of Data and Materials: All datasets analyzed and described during the present study are available from the corresponding author upon reasonable request.

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