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Metabolomic Profiling of *Lactobacillus plantarum* Isolated from *Ocimum gratissimum* Rhizosphere

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Authors' contributions

This work was carried out in collaboration among all authors. Author RGI carried out this laboratory study as part of her PhD dissertation. Author COA was the main supervisor for the research work. Author MOI co-supervised the work and carried out the ideation and manuscript preparation. Author CUE carried out the data curation and proof-reading. All authors read and approved the final manuscript.

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ABSTRACT

L. plantarum is a known member of the lactic acid bacteria that possesses antimicrobial activities. Organic acids, hydrogen peroxide and bacteriocins have been majorly identified as the contributors to its antimicrobial activity. The present study sought to employ the GC-MS technique to further classify biological compounds that comprise the antimicrobial metabolite produced by *L. plantarum* through sub-merged fermentation. The bacterium was isolated from the rhizosphere of *O. gratissimum* and confirmed using molecular typing. Preliminary antibacterial screening of the organism was done with indicator strains isolated from urinary tract and wound surface, after which sub-merged fermentation was employed for the production of

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secondary metabolites for a 24 h period. The GC-MS technique was employed to identify the volatile bioactive compounds that comprise the secondary metabolite produced. The organism had significant (p<0.05) inhibition of the indicator strains when compared to the ciprofloxacin standard antibiotic. Metabolomics analyses identified Hydroxylamine, O-decyl-, 2,4-Di-tert-butylphenol, and a wide range of organic compounds mainly from the alkane, amine, carboxylic acids and phenol functional groups, as the components of its antibacterial metabolite. GC-MS based metabolomics analyses is a profitable tool for identifying key components of the antibacterial substance produced by *L. plantarum* as this will give a room for its bio-prospecting potentials as alternative and sustainable source of novel antimicrobial compounds and other beneficial medications used by humans.

Keywords: Lactobacillus plantarum; GC-MS; ocimum gratissimum; antimicrobial activity.

1. INTRODUCTION

Rhizosphere is a narrow zone of soil surrounding roots of plants [1], known to be of high microbial diversity ranging from bacteria, fungi to actinomycetes [2]. This is basically as a result of increased nutrient supply from photosynthesis which releases different organic compounds to the root and encourage the existence and proliferation of some of these microbes at the rhizosphere [3].

The genus, *Ocimum*, has been classified as a power plant genus [4] because of its many species known to have several medicinal advantages. This genus of plants has been in the fore-front of alternative provisions to antibiotic therapy as well as for the production of new antimicrobial compounds from the African and Asian context [5]. In addition to its much medical and industrial importance, its environmental importance is not left out as seen from the diversity of medically important microorganisms it harbors in its rhizosphere. *Ocimum gratissimum* and *Ocimum basilicum* are the two most popular species of the plant that have been extensively reported for their richness in rhizosphere organisms that are of importance with regards to antimicrobial activity and bioremediation respectively [5,4,6]. Some bacteria identified to be associated with Rhizospheres of *Ocimum* plants include *Achromobater* spp., *Serratia* spp., *Ochrabactrum* spp., *Bacillus* spp.; basically isolated from *Ocimum gratissimum* and *Ocimum basilicum*. Due to the desire for new antimicrobials to be produced as a means of regulating antibiotic resistance threat currently facing the world, researchers have concentrated efforts in the rhizosphere habitat as a rich source of microorganisms with antibacterial, antifungal and even anti-helminthic activities.

Lactobacillus is a genus of bacteria reported extensively for their antibacterial and probiotic potentials. Species of this genus are facultatively anaerobic, Gram positive, catalase negative rods; found in different habitats such as fermented foods, vagina, colon and other habitats that possess some degree of anaerobiosis, and also degree of mesophilic and thermophilic temperatures [7,8]. The rhizosphere being an anaerobic habitat and also a rich habitat for organisms with antimicrobial potentials is thought to possibly harbor some species of *Lactobacillus*. This study thus had the objectives of isolating *Lactobacillus* species with antibacterial activity from rhizosphere of *Ocimum gratissimum*; production of antibacterial compounds from the choice isolate using sub-merged fermentation; and identifying the volatile bioactive compounds that constitute its antibacterial properties using gas chromatography-mass spectrometric analyses.

2. METHODS

2.1 Isolation of Microorganism from Rhizosphere

Isolation of *Lactobacillus* was performed by serial dilution and plating technique using nutrient agar and De Mann Rogosa and Sharpe agar (MRS) medium. One gram of each soil sample was suspended in 9 ml sterile water in a test tube. Ten fold serial dilution was done up to fifth dilution, and then 1 ml was collected from each 10^{-2} dilution and plated on the agar media. The nutrient agar plates were incubated for 24 h aerobically and anaerobically at 25^{o}C [9].

2.2 Isolation and Identification of the Test Bacterial Isolates from Urine and Wound Samples

Bacterial isolates were isolated from urine samples according to the modified method of Alshomrani et al. [10]. Clean-catch midstream morning urine specimen was collected using sterile wide mouth glass containers. Until laboratory analysis, the samples were kept cooled in a refrigerator. The time between sample collection and the sample analysis did not exceed one hour. Using sterile wire loops, 0.01 ml urine sample was then plated onto blood agar and MacConkey agar plates, incubated aerobically at 37℃ for 24 h. This was used for the isolation of *E. coli*, *Klebsiella* and *Staphylococcus aureus* from urine samples.

Pseudomonas aeruginosa was isolated from wound sites using method described by Al-Mathkhury and Al-Aubeidi [11]. With sterile swab sticks, wound swabs were taken carefully from the site of infection and placed in tubes containing normal saline to maintain the swab wet during transferring to laboratory. Each specimen was inoculated on cetrimide agar plates supplemented with 1% glycerol and allowed to incubate for 24 h at 28°C.

2.3 Biochemical Characterization for Lactobacillus Species

Isolates were characterized using Gram Staining, catalase, motility and sugar fermentation characteristics as described by Cheesbrough [12].

2.4 Molecular Identification

Isolates were characterized using 16S rRNA sequencing.

2.5 Production of Antibacterial Metabolites from the Bacterial Isolates

About 50 ml of the fermentation medium containing the following (g/L); L-glutamic acid 5.0; KH_2PO_4 0.5; KH_2PO_4 0.5; $MgSO_4.7H_2O$ 0.2; $MnSO_4.H_2O$ 0.01; NaCl 0.01; $FeSO_4.7H_2O$ 0.01; $CuSO_4.7H_2O$ 0.01 ; CaCl.2H2O 0.015; Glucose 10; and PH 7); and 1% glucose, was prepared in 250 ml Erlen-Meyer flask and sterilized. Stock culture of organisms was prepared by inoculation of a loop-full of each bacterium in 10 ml MRS broth. Stock cultures were incubated for 24 h, after which 5 ml of the stock culture was transferred into the sterile 50 ml fermentation medium, incubated in a rotary shaker at 120 rpm for 24 h, anaerobically at 28°C. Broth cultures were filtered firstly with whatman No.1 filter paper and secondly with nitrocelloluse membrane (0.45 μm pore diameter) after incubation (Sethi et al. 2013). The filtrate was kept in the refrigerator until gas chromatography and mass spectrometry analyses.

2.6 Extraction and Identification of Bioactive Compounds Present in the Produced Antibacterial Metabolites Using Gas Chromatography and Mass Spectrometry

A 1ml aliquot of the sample was extracted with 5ml of acetonitrile, the mixture was centrifuge for 1hr and the acetonitrile layer (upper layer) was collected and evaporated to dryness. 1ml of pyridine was added prior to GC analysis.

The GC–MS analysis of bioactive compounds from the different extracts was done using Agilent Technologies GC systems with GC-7890A/MS-5975C model (Agilent Technologies, Santa Clara, CA, USA) equipped with HP-5MS column (30 m in length × 250 µm in diameter × 0.25 µm in thickness of film). Spectroscopic detection by GC–MS involved an electron ionization system which utilized high energy electrons (70 eV). Pure helium gas (99.995%) was used as the carrier gas with flow rate of 1 mL/min. The initial temperature was set at 50 –150 °C with increasing rate of 3 °C/min and holding time of about 10 min. Finally, the temperature was increased to 300 °C at 10 °C/min. One microliter of the prepared 1% of the extracts diluted with respective solvents was injected in ansplitless mode. Relative quantity of the chemical compounds present in each of the extracts of was expressed as percentage based on peak area produced in the chromatogram.

2.7 Identification of Chemical Constituents

Bioactive compounds extracted from different extracts were identified based on GC retention time on HP-5MS column and matching of the spectra with NIST library (Replib and Mainlab data of GC–MS systems).

2.8 Statistical Analyses

Statistical Analyses was done using GraphPad Prism version 8. Mean values were compared

using one way Analyses of Variance (ANOVA), at 95% confidence interval.

3. RESULTS

3.1 Isolation, Characterization and Antibacterial Screening of *Lactobacillus plantarum* from Rhizosphere Samples

Lactobacillus sp. was characterized as shown in Table 1 and was confirmed using molecular typing as *Lactobacillus plantarum*. The isolate was screened for antibacterial activity using the organisms isolated from urine and wound sites as shown in Fig. 1. This organism significantly ($p<0.05$) inhibited the growth of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli* when compared to the ciprofloxacin standard (Fig. 1).

3.2 Molecular Identification of Isolate

The bacterial isolate was identified as *Lactobacillus plantarum* strain MF1298.

3.3 Production and GC-MS Evaluation of Antibacterial Substances from *Lactobacillus plantarum*

Gas chromatography and mass spectrometry analyses showed forty-two bioactive compounds present in the metabolites. The top five bioactive compounds, their paks and retention time that possibly contributed predominantly in the antibacterial activities of *Lactobacillus plantarum* are shown in Fig. 2 and Table 2.

Table 1. Biochemical characteristics of *Lactobacillus plantarum*

Biochemical tests	Results
Gram stain	+
Cell morphology	rod
Catalase reaction	-
Motility	-
Glucose fermentation	+
Gas from glucose	-
Lactose fermentation	+
Sucrose fermentation	+
Galactose fermentation	+
Probable Organism	*Lactobacillus* sp.

Table 2. Predominant metabolites produced by *Lactobacillus plantarum*

Peak numbers	Retention time	Area%	Metabolite names
11	9.428	8.19	a) Docosane, 2,21-dimethyl- b) Hydroxylamine, O-decyl- c) Carbonic acid, decyl vinyl ester
36	22.133	7.74	a) 2,4-Di-tert-butylphenol b) Phenol, 3,5-bis(1,1-dimethylethyl)
17	10.158	5.09	a) 1-Decanol, 2-hexyl- b) Carbonic acid, nonyl prop-1-en-2-yl ester c) Hexadecane, 7-methyl-
15	9.815	4.89	a) Undecane b) Decane, 2-methyl- c) Heptadecane, 2,6,10,14-tetramethyl
40	26.072	4.36	a) Cyclohexene, 6-butyl-1-nitro- b) 8-Heptadecyne, 1-bromo- c) 9-Oxabicyclo [6.1.0]nonane

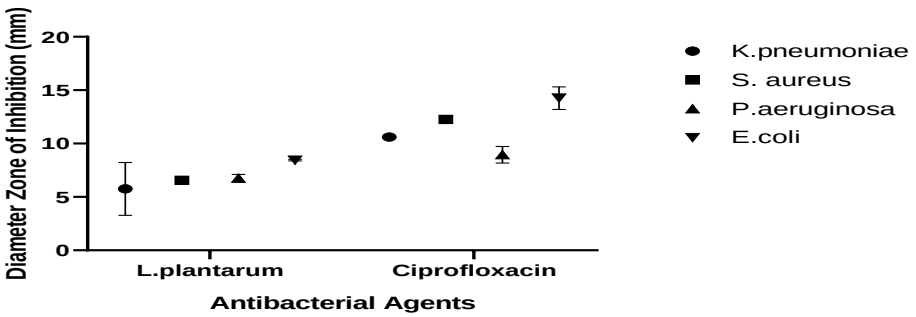

Fig. 1. Antibiotic screening of *L. plantarum* against selected organisms

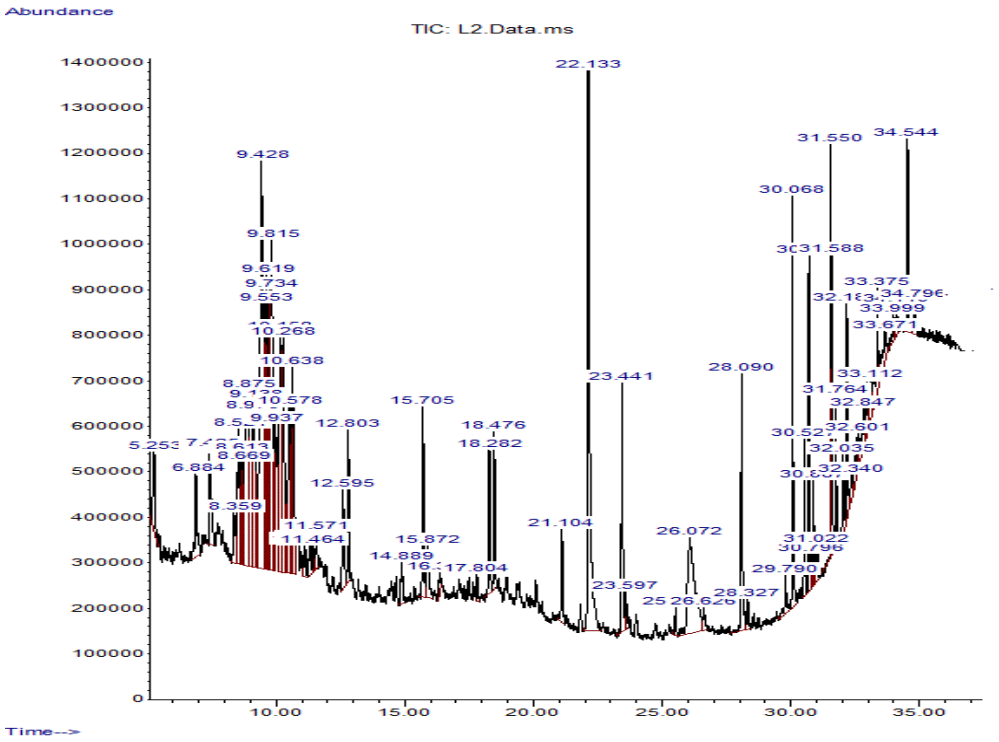

Fig. 2. Gas chromatogram showing elution peaks of identified bioactive compounds produced by *L. plantarum*

4. DISCUSSION

L. plantarum is a well–researched lactic acid bacterium known for its various antimicrobial characteristics both bacteriocin-mediated and non-bacteriocin mediated. Various food sources are known to be normal habitats where this microorganism can be isolated from. This study however, isolated this microorganism as part of the rhizosphere organisms for *Ocimum gratissimum*, commonly known as 'scent leaf' in Nigeria.

Antibacterial screening of this organism against urinary tract pathogens and surface wound pathogens using the agar well diffusion assay of the cell-free supernatant showed significant ($p<0.05$) inhibition of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Escherichia coli*. This finding partly corresponds with the reports of De Giani et al. [13] and Hu et al. [14]. Lin and Pan [15] used similar indicator strains used in this study and had a similar report as that found in this finding. However, a striking finding from this study that differs from theirs is that *S. aureus* strain they used was susceptible to both the cell-free supernatant and bacteriocin tested from *L. plantarum*, but in this study, *S. aureus* showed resistance to the cell-free supernatant of *L. plantarum*. Comparing the inhibitory capacity of *L. plantarum* with that of ciprofloxacin, it was observed that their inhibition differed significantly ($p<0.05$) with *E. coli* and *P. aeruginosa* in this study. According to the review report of Dinev et al. [16], *L. plantarum* inhibitory properties are basically organic acid -, hydrogen peroxide-, bacteriocin-mediated, which constitute volatile and non-volatile inhibitory properties.

A host of some scientists [13,15,17,18,19] have put in efforts in this regard to identify further the bioactive components in antimicrobial metabolites produced by *L. plantarum*. Some of the probing methods they have employed include the Fourier Transform-Infra Red (FT-IR) analyses [18], Electro spray ionization (ESI) mass spectrometry [13], High performance liquid chromatography (HPLC) analyses (Lin and Pan, 2017), Nuclear magnetic resonance (NMR) profiling (Lin and Pan, 2017) and also the GC-MS analyses. The present study carried out untargeted metabolomics of *L. plantarum* volatile inhibitory substances using the GC-MS analyses. A total of 64 bioactive compounds were identified from culture supernatant in this study while Huang et al. [19] identified 43 metabolites, and Chaudhary et al. [17] identified 10 compounds from *L. plantarum* DB2 also using GC-MS. They

also used GC-MS to analyze metabolites produced by *L. plantarum* alongside other lactic acid bacteria, and their metabolite findings partly correspond with the metabolites identified in this study. Dawwam et al. [20] identified 22 bioactive antimicrobial compounds from *L. plantarum* using GC-MS, some of their reported compounds partly corresponded with those found in this study. Assessing the top five predominant metabolites identified and shown in Table 2, Hydroxylamine, O-decyl- is known to be toxigenic to some bacteria by having mutagenic effects on them as reported by Zhang et al. [21]; 2,4-Di-tert-butylphenol has been reported by Vinati Organics (2021) as a secondary metabolite with a wide range of toxicity to various microorganisms; Hexadecane, 7-methyl- is a petroleum compound reported for its antimicrobial activity by Yu et al. [22]. Other hydrocarbon compounds identified by the GC-MS analysis possess antimicrobial activities and thus contribute to the antibacterial activity expressed by *L. plantarum* used in this study.

5. CONCLUSION

This metabolomics study of *L. plantarum* has shown that a wide range of organic compounds, mainly from the alkane, amine, carboxylic acids and phenol functional groups predominantly make up the volatile antibacterial compounds produced by this vastly studied lactic acid bacteria which also doubles as a probiotic microorganism.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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