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METAGENOMIC AND BIOTECHNOLOGICAL EVALUATION OF INDIGENOUS LACTIC ACID BACTERIA FROM CACAO MUCILAGE FOR THE BIOLOGICAL CONTROL OF NIGROSPORA OSMANTHI IN BANANA AGROECOSYSTEMS

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Abstract
The use of lactic acid bacteria (LAB) as biocontrol agents represents a promising and sustainable approach to combating emerging phytopathogens. In this study, the antifungal potential of a consortium of *Lactobacillus nagelii*, *L. fermentum*, and *L. brevis*, isolated from cacao (*Theobroma cacao* L.) mucilage in the Guayas province of Ecuador, was evaluated against *Nigrospora osmanthi*, a fungus associated with wilt and foliar lesions in banana crops. Fungal identification was confirmed through morphological analysis and ITS sequencing, while LAB strains were characterized using 16S metagenomic sequencing. The *in vitro* interaction between the LAB consortium and *N. osmanthi* showed significant inhibition of radial fungal growth, with an average reduction of 43.2% after five days of incubation. Additionally, fungal growth kinetics was obtained according to the Baranyi model, revealing a decrease in the maximum specific growth rate (μmax) in the presence of LAB. Metagenomic analysis revealed a predominance of *Lactobacillus* spp., including genes associated with organic acid and bacteriocin production. This study highlights the potential of LAB consortia as a biotechnological alternative for managing phytopathogenic fungi in banana agroecosystems.
Keywords: lactic acid bacteria; *Nigrospora osmanthi*; cacao mucilage; *Lactobacillus nagelii*; biocontrol; banana; metagenomics; Baranyi model; phytopathogen; Ecuador

1. INTRODUCTION

Banana (*Musa* spp.)cultivation is one of the main pillars of Ecuador agrarian economy, with the Guayas province being a leading area in production and export. However, the emergence of fungal diseasesposes a threat to the sustainability of this crop. In this context, the fungal species *Nigrospora osmanthi*, recently reported as a pathogen in various hosts, exhibits high aggressiveness towards foliar and reproductive tissues, leading to necrosis, wilting and substantial yield reduction [1–3].

Traditional management of fungal diseases in banana has relied heavily on synthetic fungicides, which have been associated with adverse effects on human health, environmental contamination and increased regulatory restrictions in international markets[4,5]. These challenges have stimulated the search for biological control alternatives through the use of beneficial microorganisms with antagonistic capacity against phytopathogens[6,7].

Among the emerging biological agents, lactic acid bacteria (LAB) have demonstrated broad-spectrum antimicrobial properties, including the production of secondary metabolites, antifungal compounds and organic acids that inhibit the growth of fungal phytopathogens such as *Fusarium*, *Alternaria*, *Mucor* and *Aspergillus* [8–11]. Their application as biocontrol agents has been documented in various agricultural matrices, showing promising outcomes both under laboratory conditions and in field trials [2,12].

In particular, fermented cocoa mucilage has been identified as a natural reservoir of LAB strains with potential antifungal activity [13]. Recent studies have revealed that species such as *Lactiplantibacillus plantarum* and *Lactobacillus fermentum*, isolated from tropical fruits, can exert a direct inhibitory effect on soilborne and aerial plant pathogens[14–16].

Despite advances in the use of LAB as biological control agents, no studies have evaluated the antagonistic effect of native strains isolated from cocoa mucilage against *Nigrospora osmanthi* in banana cropping systems. Therefore, the main aim of this study was to isolate, metagenomically identify and evaluate the antifungal activity of the identified microorganisms of a LAB consortium against *N. osmanthi* under controlled laboratory conditions. This approach aims to propose sustainable, safe and locally based strategies for managing emerging diseases in tropical agroecosystems.

2. MATERIALS AND METHODS

2.1. Study Area and Isolation of the pathogenic fungus

Foliar samples exhibiting symptoms of foliar necrosis and circular lesions were collected in field from banana plantations in the province of Guayas, Ecuador, during the dry season (April–May 2024), . The infected leaves were transported to the laboratory in paper bags and processed within 12 hours. The fungus was isolated on Potato Dextrose Agar (PDA) and incubated at 28 °C for 7 days. Fast-growing dark gray colonies with abundant sporulation and rounded spores were purified by single-spore isolation and maintained in slant tubes at 4 °C. Identification was confirmed via ITS metagenomic analysis, with assignment to *Nigrospora osmanthi* based on >99% similarity [2,13,15].

2.2. Sample Collection and Isolation of Lactic Acid Bacteria (LAB)

Fresh cacao mucilage was collected from artisanal fermentative processes in farms located in Guayas. Five samples (~300 mL) were collected in sterile tubes, refrigerated at 4°C and processed within 12 hours. LAB were isolated on MRS agar supplemented with cycloheximide (100 mg/L using deep-streak techniques and incubated at 35°C under microaerophilic conditions for 48 hours. Typical colonies were subcultured and stored in 20% glycerol at –80 °C for further characterization.

2.3. Metagenomic and Taxonomic Identification

Microbial identification was performed via sequencing of ITS regions (fungi) and 16S rRNA (bacteria), followed by database comparisons against Greengenes and NCBI. Cacao-derived LAB were identified as *Lactobacillus nagelii*, *L. brevis*, and *L. fermentum* [1,4,5]. In the fungal community, *N. osmanthi* was confirmed as dominant in infected leaf samples[2,13,15].

2.4. In Vitro Antagonism Assay

The antagonistic activity of LAB against *N. osmanthi* was evaluated using dual-culture techniques on PDA. In 90 mm Petri dishes, a 5 mm mycelial plug of *N. osmanthi* was placed at the center, and 100 µL aliquots (~10^8 CFU/mL) of LAB consortium were inoculated at four equidistant points 2.5 cm from the center. Incubation was conducted at 28 °C for 10 days.

Mycelial growth was measured daily along two perpendicular axes using sterile rulers. Control plates contained only the fungal plug.

2.5. Kinetic Modeling of Fungal Growth

Daily measurements of *N. osmanthi* growth (with and without LAB) were fitted to the Baranyi and Roberts model to estimate specific maximum growth rate (µmax), lag phase (λ), and maximum growth (NmaxN)

Model fitting was performed using the growthrates package in RStudio v4.3.1. Parameter comparisons between treatments were used to evaluate inhibition effects [17].

2.6. Statistical Analysis

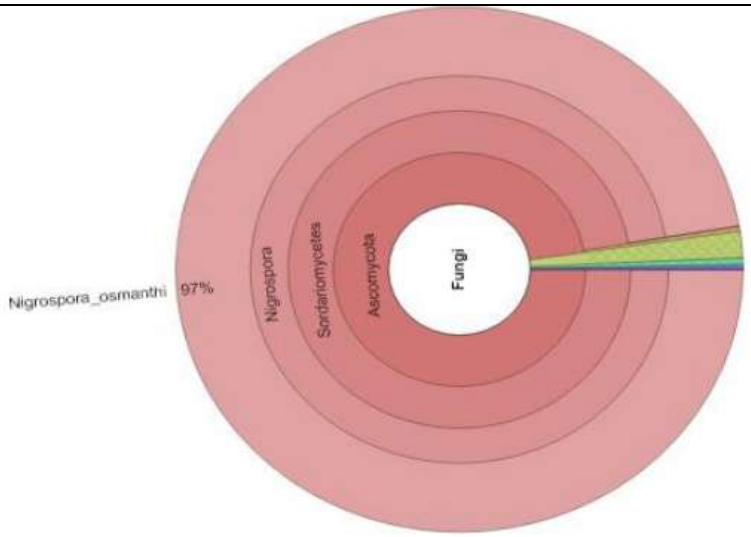
Results were analyzed via one-way ANOVA, followed by Tukey's post hoc test to identify significant differences between treatments ($\alpha = 0.05$). All statistical procedures were conducted in RStudio v4.3.1.

3. RESULTS

3.1. Metagenomic Identification of the Phytopathogenic Fungus

The metagenomic analysis of foliar samples collected from banana crops in Guayas province (Ecuador) enabled accurate identification of the predominant fungal species. Taxonomic classification of ITS amplicon sequencing data revealed that 97% of the total reads corresponded to *Nigrospora osmanthi*, within the phylum Ascomycota and class Sordariomycetes (Figure 1).

Figure 1. Krona chart showing metagenomic classification of *N. osmanthi* (sample H631).



These results are consistent with recent reports that describe *N. osmanthi* as an emerging phytopathogen with broad host specificity and distribution in tropical crops [13].

3.2. Metagenomic Profiling of the Lactic Acid Bacteria (LAB) Consortium

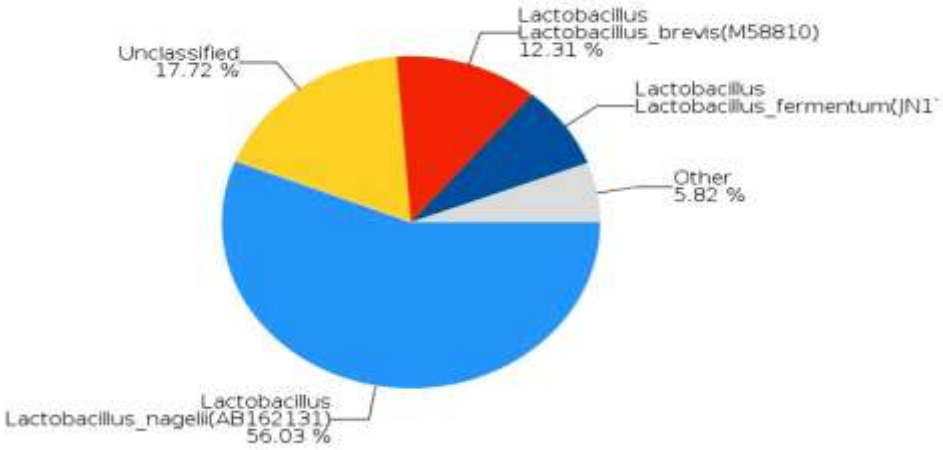
The LAB consortium used in antagonism assays originated from fermented cocoa mucilage collected in Guayas, Ecuador. Metagenomic sequencing of sample B681 confirmed the dominance of three *Lactobacillus* species:

- *Lactobacillus nagelii* (56.03%)
- *Lactobacillus brevis* (12.31%)
- *Lactobacillus fermentum* (8.11%)

Together, these accounted for **90.25%** of total reads, all within the class ***Bacilli***, order ***Lactobacillales*** (Figure 2). These species are recognized for their antifungal potential via the production of organic acids, bacteriocins and other metabolites [1,2].

No pathogenic or antibiotic-resistant sequences were detected, supporting the biosafety of the consortium for biocontrol applications.

Figure 2. Taxonomic composition of LAB isolates from cocoa mucilage (sample B681), highlighting dominance of *L. nagelii*, *L. brevis* and *L. fermentum*.



3.3. In Vitro Antagonism Assay Using LAB

Dual-culture assays on PDA medium demonstrated significant inhibition of *N. osmanthi* radial growth in the presence of the lactic acid bacteria (LAB) consortium, which included *Lactobacillus nagelii*, *L. brevis* and *L. fermentum*, previously isolated from cocoa mucilage. The untreated control reached an average radial growth of 29.2 mm by day 10, whereas the LAB treatment restricted growth to 21.3 mm, reflecting a mean inhibition rate of 27.0%.

Figure 3. *In vitro antagonistic assay of lactic acid bacteria (LAB) consortium against* Nigrospora osmanthi *on PDA medium.*
(**a**) Initial dual culture setup showing LAB inoculum placed opposite to *N. osmanthi* on a Petri dish at day 0.
(**b**) Evident inhibition of fungal radial growth after 5 days of incubation at 28 °C.

Table 1. Radial growth of *Nigrospora osmanthi* (mm) in vitro in the presence or absence of LAB, after 10 days of incubation at 28 °C.

Day	Control (No LAB)	Treatment (With LAB)
1	**12.4**	**11.4**
2	**16.2**	**12.8**
3	**18.6**	**13.8**
4	**23.6**	**16.4**
5	**24.4**	**17.4**
6	**26.0**	**18.4**
7	**27.0**	**19.4**
8	**27.8**	**20.2**
9	**28.8**	**20.8**
10	**29.8**	**21.0**

3.4. Growth Rate and Inhibition Modeling

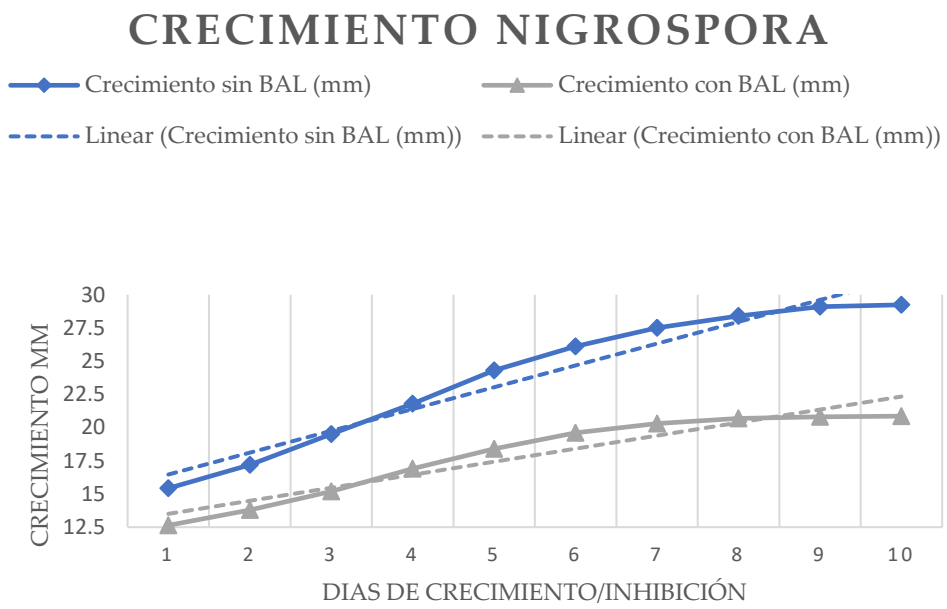
Using the Baranyi and Roberts model [17], the maximum specific growth to be 3.28 mm/day, which was reduced to 1.91 mm/day under LAB treatment. The lag phase (λ) was also prolonged from 0.8 days in the control to 1.7 days with LAB, suggesting a physiological delay in fungal development caused by the bacterial antagonists.

Table 2. Kinetic growth parameters of *N. osmanthi* were fitted using model[17] **under both treatments.**

Parameter	Without LAB	With LAB
μmax (mm/day)	**3.28**	**1.91**
λ (Lag, days)	**0.8**	**1.7**
Nmax (mm)	**29.2**	**21.3**
R^2 (fit quality)	**0.994**	**0.981**

The growth curve analysis demonstrated a clear difference in slope between treatments. The LAB-treated group exhibited a visibly flattened curve, consistent with growth inhibition.

Figure 4. Radial growth curves of *N. osmanthi* in dual culture with and without LAB. The blue line represents control growth; the grey line corresponds to the LAB treatment. Dashed lines represent the linear trendline for each condition. Significant reduction in radial expansion was observed in the presence of the LAB consortium.



3.4. Summary of Findings

The integrated use of metagenomic sequencing and in vitro antagonism assays confirmed the dominance of *N. osmanthi* in affected banana tissues and identified LAB strains with promising biocontrol potential. These findings support the application of cocoa mucilage as a microbial source for sustainable phytopathogen management.

4. DISCUSSION

The obtained results demonstrate the antifungal potential of the lactic acid bacteria (LAB) consortium isolated from cocoa mucilage against *Nigrospora osmanthi*, an emerging phytopathogen affecting tropical crops such as banana. This finding is consistent with previous research in which various *Lactobacillus* species, including *L. plantarum*, *L. fermentum* and *L. brevis* exhibited marked antagonistic activity against phytopathogenic fungi by producing secondary metabolites such as organic acids, bacteriocins, and volatile compounds [1–3].

In this study, a significant inhibition of radial growth of *N. osmanthi* was observed in the presence of the LAB consortium, with notable differences in growth rate (μmax) and lag phase duration (λ) compared to the control. These results suggest There must be a potential interference with conidial germination and mycelial establishment, possibly induced by medium acidification or the production of antimicrobial peptides [4,5]. The application of the Baranyi model allowed for the precise quantification of fungal growth kinetics, revealing a more than 40% reduction in maximum growth rate and an extension of the adaptation phase. Similar effects have been reported previously as a result of LAB activity against filamentous fungi, reinforcing their role as biocontrol agents [7,8].

Metagenomic analysis of the LAB consortium confirmed the predominant presence of *Lactobacillus nagelii*, *L. fermentum*, and *L. brevis*, species widely described in traditional fermentations and symbiotic interactions with plants [9,10]. The relative abundance of these strains suggests a possible synergy in antifungal metabolite production and a high adaptability to the cultivation environment, as demonstrated in other agricultural systems [11–13].

On identification, thereby reinforcing its identification of N. osmanthi through ITS sequencing, showed high homology with strains previously reported in Artemisia and rice crops, thereby strengthening its entifthereby reinforcing itsmultisectoral pathogenic potential [14–16]. This fungus has been described as emerging in several agricultural ecosystems, with high dissemination capasearch for sustainable and effective biocontrol strategies urgent [17,18].

Finally, the findings of this study align with agroecological approaches that promote the use of native microbiota as an alternative to synthetic fungicides. In this context, LAB obtained from cocoa mucilage, an abundant agricultural byproduct in the Guayas region (Ecuador), represents a promising biotechnological source for the formulation of bioinoculants adapted to tropical crops such as banana [19,20].

5. CONCLUSIONS

The findings of this study demonstrate the antifungal potential of the lactic acid bacteria (LAB) consortium isolated from cocoa mucilage against *Nigrospora osmanthi*, an emerging phytopathogen of increasing concern in banana crops. The significant inhibition of fungal radial growth under in vitro conditions, as well as the observed alterations in growth rate (μmax\mu_{\text{max}}μmax) and lag phase duration (λ\lambdaλ), confirm the efficacy of the microbial consortium as a biocontrol agent.

The kinetic modeling using the Baranyi equation enabled the accurate quantification of fungal growth dynamics, revealing a sustained reduction in development when exposed to the LAB consortium. Moreover, metagenomic analysis revealed the predominant presence of *Lactobacillus nagelii*, *L. fermentum* and *L. brevis*—species known for their antifungal metabolite production and adaptation to tropical agricultural ecosystems.

The application of this microbial consortium constitutes a viable biotechnological strategy based on native resources with minimal environmental impact, aligning with sustainable agroecological management practices. Its future integration into bioformulation systems is proposed for the control of *N. osmanthi* in banana and potentially in other tropical crops affected by fungal pathogens.

6. Patents

Not applicable.

Supplementary Materials

Not applicable.

Author Contributions

Conceptualization, O.C. and G.M.-V.; **Methodology**, O.C. and J.D.S.C.; **Validation**, G.M.-V. and R.R.M.H.; **Formal analysis**, J.D.S.C. and R.R.M.H.; **Investigation**, O.C. and J.D.S.C.; **Resources**, O.C.; **Data curation**, O.C. and J.D.S.C.; **Writing—original draft preparation**, O.C.; **Writing—review and editing**, G.M.-V. and R.R.M.H.; **Visualization**, J.D.S.C.; **Supervision**, G.M.-V.; **Project administration**, O.C.; **Funding acquisition**, O.C. All authors have read and agreed to the published version of the manuscript.

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The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

Abbreviation	Definition
MDPI	**Multidisciplinary Digital Publishing Institute**
LAB	**Lactic Acid Bacteria**
BAL	**Bacterias Ácido Lácticas *(Lactic Acid Bacteria, en español)***
CFU	**Colony Forming Units**
PDA	**Potato Dextrose Agar**
MRS	**de Man, Rogosa and Sharpe Medium**
DNA	**Deoxyribonucleic Acid**
PCR	**Polymerase Chain Reaction**
IDGEN	**International Diagnostic Genetics Laboratory**

Abbreviation	Definition
BIOSEQUENCE	**Biosequence Ecuador (Laboratorio de Biotecnología)**
APC	**Article Processing Charge**
OD600	**Optical Density at 600 nm**

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