Research Article

# Inhibiting *Stenotrophomonas maltophilia*, a Pathogenic Bacterium Responsible for Kernel Rot Disease in Pili nut (*Canarium ovatum* Engl.) with Ionic Liquid-loaded Nanoemulsions

Roberth San Abando Solita and Felmer Sesaldo Latayada
Department of Chemistry, Caraga State University, Ampayon, Butuan City, Philippines

Julius Anthony Magadan Leones
Department of Biology, Caraga State University, Ampayon, Butuan City, Philippines

Elizabeth Pio Parac
Department of Plant and Soil Sciences, Caraga State University, Ampayon, Butuan City, Philippines

Arnold Cafe Alguno
Department of Physics, Mindanao State University-Iligan Institute of Technology, Iligan City, Philippines

Rey Yonson Capangpangan*
Department of Physical Sciences and Mathematics, Mindanao State University at Naawan, Poblacion, Naawan, Misamis Oriental, Philippines
Research on Environment and Nanotechnology Laboratories (REY Labs), Mindanao State University at Naawan, Poblacion, Naawan, Misamis Oriental, Philippines

* Corresponding author. E-mail: rey.capangpangan@msunaawan.edu.ph DOI: 10.14416/j.asep.2024.07.004
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**Abstract**
Pili nut production in the Philippines has grown steadily, but it faces significant challenges from pests and diseases, notably kernel rot. Yield losses due to this pathogen are still not measured, but the damage could extend from the purple immature to the dried postharvest nuts. Therefore, there is a pressing need for safe, effective, and environmentally friendly control measures. This study reports on the successful formulation of various Ionic Liquid-loaded Eucalyptus Essential Oil Nanoemulsions (IL-EEONE) for potential applications against *Stenotrophomonas maltophilia*, a pathogenic bacterium responsible for kernel rot disease in Pili nut (*Canarium ovatum*). Combining eucalyptus essential oil (EO) and Tween 80 in an oil-in-water (O/W) system, followed by stirring and sonication, and the subsequent loading of 1-Butyl-3-methylimidazolium hydrogen sulfate ([Bmim][$HSO_4$]), an ionic liquid, at varying ratios (1:1, 2:1, and 3:1), yielding the formation of IL-EEONE. The nanoemulsion droplets exhibited a size range of 9.4–12.26 nm, highlighting their nanoscale dimensions. The IL-loaded nanoemulsions formulated at varying ratios typically displayed nearly monodisperse characteristics, except for the higher concentration 1:1 ratio of IL:EEONE formulation, as indicated by their Polydispersity Index (PDI) values. Fourier Transform Infrared (FT-IR) analysis further confirmed the successful formulation of the different IL-EEONE nanoemulsion compositions. Significantly, these nanoemulsions demonstrated excellent inhibitory properties against *S. maltophilia*, as indicated by Zone of Inhibition (ZOI) ranging from 11.3 ± 0.58 mm to 32.7 ± 0.58 mm. The antibacterial activity varied from partially active to very active across different formulations, with the 1:1 IL-EEONE ratio formulation standing out as exceptionally effective. This study shows

the potential of IL-loaded nanoemulsions, IL-EEONE, as a potential agent for mitigating *S. maltophilia* causing the kernel rot disease, offering innovative avenues for addressing bacterial infection in agricultural settings.

**Keywords**: Kernel rot disease, Pili nut, *Stenotrophomonas maltophilia*, Ionic liquids, Nanoemulsions

## 1 Introduction

Kernel rot disease in Pili nut, *Canarium ovatum*, is a significant agricultural concern that affects the quality and yield of this valuable crop. Pili nut, native to the Philippines and other Southeast Asian countries, produces nutrient-rich nuts that are not only consumed locally but are also exported worldwide [1], [2]. However, the presence of kernel rot disease poses a threat to the Pili industry. Kernel rot disease is primarily caused by various pathogenic microorganisms, including fungi and bacteria [3]. These pathogens can infiltrate the kernels of Pili nuts, leading to deterioration, discoloration, and reduced market value. Additionally, infected nuts may exhibit changes in taste and texture, making them unsuitable for consumption [3].

*S. maltophilia*, a bacterial species [4], has been identified as a prominent contributor to kernel rot disease in Pili nuts for the first time. As reported in our paper currently under review, this bacterium serves as the primary causative agent of the disease. Thriving within the microenvironment of Pili nuts, it exploits the available nutrients, resulting in substantial damage. Effective control of *S. maltophilia* activity is imperative to halt the spread of the disease and preserve the quality of Pili nuts. Efforts to understand and combat kernel rot disease in Pili are essential for sustaining this valuable agricultural resource, ensuring the continued production of high-quality nuts, and supporting the livelihoods of farmers and communities dependent on the Pili industry.

To address this challenge, researchers have been exploring numerous methods and technologies for combating bacterial infections, ranging from traditional antibiotics [5] to natural extracts [6]–[8], metal nanoparticles (e.g., Ag, Cu, ZnO) [9]–[14], quaternary ammonium compounds [15], [16], essential oils [17], [18], and probiotics [19], [20]. One emerging field showing promise in providing superior performance against a wide spectrum of bacteria is nanoemulsions [21], [22], owing to their myriad advantages. Nanoemulsions, with their unique properties and advantages, offer a promising avenue for antibacterial applications [23], [24]. These stable oil-water (O/W) systems, composed of small droplets surrounded by surfactants, possess a high surface area that enhances their interaction with bacterial cell membranes, making them effective carriers for various antibacterial agents, including antibiotics and natural extracts [25]. Their small droplet size facilitates better penetration into bacterial biofilm, increasing agent efficacy. Additionally, nanoemulsions are versatile, allowing for tailored compositions to optimize stability and antibacterial activity, while also offering benefits, such as sustained release, reduced toxicity, and potential for targeted drug delivery [25], [26]. This versatility makes them a valuable tool in developing innovative antibacterial therapies with improved efficacy and reduced side effects.

This study explores the potential of harnessing the synergistic effects arising from the combination of ionic liquids (ILs) and eucalyptus essential oil within a nanoemulsion, offering a versatile and potent solution for inhibiting the activity of a kernel rot disease-causing bacterium, *S. maltophilia*. Ionic liquids, known for their unique solvation properties and membrane-disrupting capabilities, harmonize with the inherent antimicrobial attributes of eucalyptus essential oil. The synergetic effect of eucalyptus essential oil and [Bmim][$HSO_4$], an IL, against *S. maltophilia* likely arises from the combined antimicrobial properties of the two compounds, enhancing their efficacy in inhibiting bacterial growth. Likewise, the precise tailored composition of the nanoemulsion allows for fine-tuned adjustments in physicochemical properties, optimizing antimicrobial activity while minimizing potential adverse effects. In essence, this innovative potential leverages the strengths of ILs and eucalyptus essential oil to provide an efficient, sustainable, and adaptable solution for diverse antimicrobial applications.

## 2 Materials and Methods

### 2.1 *Chemicals and reagents used*

Eucalyptus pure essential oil (Majestic Pure) was acquired from Amazon, while Polysorbate 80 (Tween

80), Nutrient Agar (NA), Mueller Hinton Agar (MHA) were procured from Sigma-Aldrich. McFarland Standard was sourced from Dalynn Biologicals, Inc., and the ionic liquid 1-Butyl-3-methylimidazolium hydrogen sulfate, ([Bmim][$HSO_4$]) was generously provided by the Material Science and Polymer Chemistry (MSPC) Laboratory at Caraga State University. The pathogen, *S. maltophilia* was cultured by the Department of Biology at Caraga State University, and the standard antibiotic for positive control, Ampicillin (500 mg), was obtained from a local pharmacy.

All chemicals and reagents were utilized as received without any purification unless otherwise specified. Similarly, unless indicated otherwise, all experiments were conducted at room temperature.

### 2.2 *Formulation of the nanoemulsions*

For nanoemulsion formulation, an oil-in-water (O/W) system was employed, following the previously reported protocols [26], with modifications. A 1:1 ratio of eucalyptus essential oil (EO) and Tween 80, with each component measuring 6.4 mL, was combined under magnetic stirring at 600 rpm for 30 min to create the oil phase. Subsequently, 27.2 mL of distilled water (aqueous phase) was introduced drop-by-drop into the oil phase, followed by magnetic stirring at 1000 rpm for 1 h. To further refine the emulsion, 15 mL of the prepared mixture underwent sonication at 80 W utilizing a sonicator device (20 kHz Qsonica, Q55) for 20 cycles. Each cycle consisted of 30 s of sonication with a 5-second interval between rounds. The resulting eucalyptus essential oil nanoemulsion (EEONE) was set aside for use in subsequent experiments.

The prepared EEONE was loaded with [Bmim][$HSO_4$] ionic liquids at varying ratios (3:1, 2:1, 1:1), while maintaining a consistent total volume of 15 mL. For the 3:1 ratio, 3.75 mL of [Bmim][$HSO_4$] was added to 11.25 mL of EEONE; for the 2:1 ratio, 5 mL of [Bmim][$HSO_4$] was introduced into 10 mL EEONE, and for the 1:1 ratio, 7.5 mL of [Bmim][$HSO_4$] was loaded into 7.5 mL EEONE. In each case, [Bmim][$HSO_4$] was gradually added into the EEONE, followed by magnetic stirring at 1000 rpm for 1 hour. Subsequently, each mixture underwent an additional 10 min sonication treatment.

### 2.3 *Characterization of the formulated nanoemulsions*

Dynamic Light Scattering (DLS) analysis, conducted with an Anton Paar, Litesizer 500 instrument, was employed to determine the droplet size and the corresponding size distribution of both the prepared EEONE and IL-loaded EEONE. The resulting particle size measurements were expressed in nanometers (nm), and the polydispersity index (PDI) was subsequently determined. For functional group analysis, the FT-IR spectra of the formulated nanoemulsions were recorded using a PerkinElmer FT-IR spectrometer (UATR Two). Additionally, the absorption spectrum was obtained through the use of a PerkinElmer EnSight Multimode Plate Reader.

### 2.4 *Kirby-Bauer disk diffusion test*

The antibacterial screening of the formulated nanoemulsions was conducted following the method previously reported [27], specifically utilizing the Kirby-Bauer Disk Diffusion Test [28]. In brief, 6.9 grams of Nutrient Agar (NA) and 11.4 grams of Mueller Hinton Agar (MHA) were individually dissolved in separate reagent bottles, each containing 300 mL of distilled water. The mixtures were heated in a microwave oven until the NA and MHA powders were completely dissolved. All necessary materials, including prepared agar, test tubes, petri dishes, paper disks, and distilled water, underwent sterilization by autoclaving at 121 °C for 20 min. The NA was poured into test tubes and slanted until it solidified to create NA slant cultures, which were then inoculated with a small amount of bacteria and incubated for 24 h. The MHA was liquefied, poured into Petri dishes, and allowed to solidify. The bacterial suspension was prepared by dissolving cultured bacteria in distilled water and compared to a 0.5 M McFarland standard solution until achieving a matching suspension color. An applicator stick was used to streak the bacterial suspension onto the MHA plates. Sterilized paper disks were soaked in the prepared solutions, including positive control (Ampicillin, 20 mg/mL), negative control (sterilized distilled water), EEONE, and IL-loaded EEONE at three different ratios. These soaked paper disks, along with the positive and negative controls, were placed in the medium with streaked bacterial suspension. The petri dish was then incubated for

24 h to observe the development of a zone of inhibition (ZOI). The ZOI was visually determined as the clear area around the paper disks where bacterial growth had been inhibited. To quantify the ZOI, the diameter of this clear zone was measured in millimeters using a ruler.

### 2.5 *Data analysis*

The results from various trials (3 trials) were computed through simple averaging, and similarly, the standard deviation was calculated based on the data obtained from these multiple trials.

## 3 Results and Discussion

### 3.1 *Formulated nanoemulsions*

The formulation of eucalyptus essential oil nanoemulsions (EEONE), and IL-loaded nanoemulsions in varying ratios followed a sequential process. The oil phase was initially prepared by combining a 1:1 ratio of the surfactant and eucalyptus essential oil. This mixture was subjected to magnetic stirring, which facilitated the integration of the oil and surfactant components. Subsequently, the aqueous phase was gradually added dropwise into the oil phase, adhering to the water-in-oil emulsification process. This method resulted in the development of a creamy white mixture characterized by the dispersion of aqueous phase droplets within the oil phase, forming a stable emulsion [29]. To achieve a nanoscale form, sonication was applied, employing a sonicator device that applied sound waves to the emulsion solution [30]. Remarkably, a noticeable change in color was observed after sonication, transitioning from a creamy white appearance to a pale white one, indicating the transformation into nanoemulsions (Figure 1).

In the EEONE formation process, surfactants like Tween 80, play a pivotal role in ensuring the creation of stable emulsions by effectively reducing the interfacial tension between the two immiscible phases (disperse and continuous), thus enabling their mixing and the formation of small droplets [31]. Additionally, the introduction of energy, such as sonication, serves to further reduce the droplet sizes, breaking down larger droplets into nanometer-scale dimensions, ultimately yielding a stable nanoemulsion characterized by extremely small droplet sizes (Figure 2).

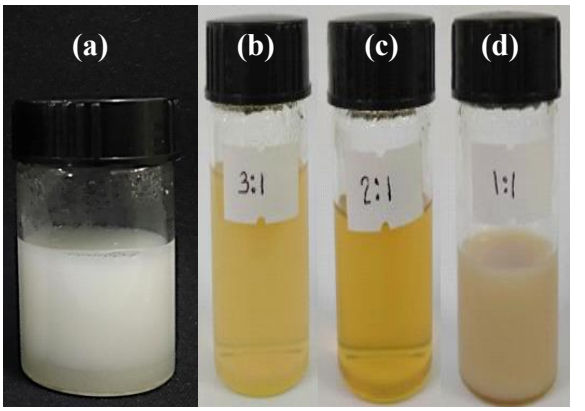


**Figure 1**: The nanoemulsions were formulated in (a) their initial state before ionic liquid loading and at different ILs:EEONE ratios, namely (b) 3:1, (c) 2:1, and (d) 1:1.

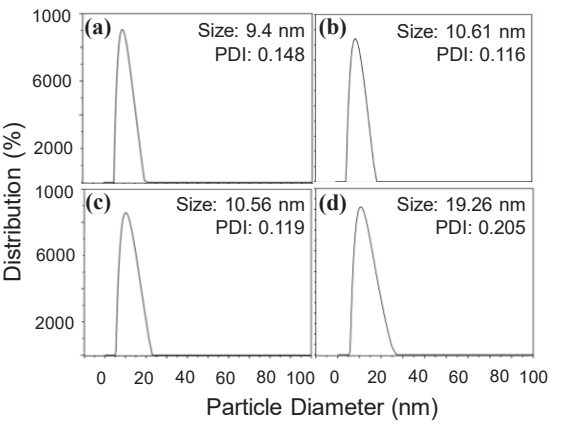


**Figure 2**: The size distribution, falling within the nanometer range, and the PDI values of the formulated nanoemulsions, including (a) EEONE; and IL-loaded EEONE with varying ratios (b) 3:1; (c) 2:1 and (d) 1:1.

Incorporating ionic liquids (ILs) within the nanoemulsions brings versatile functionality to the formulated nanoemulsions. ILs' tunable solvation properties and exceptional solubilization capabilities make them ideal for encapsulating hydrophobic substances within nanoemulsions [32]. When ILs are introduced into the oil phase of the nanoemulsion, they create a compatible environment that efficiently disperses and stabilizes IL-loaded droplets [33]. This IL loading process, as exemplified by the choice of [Bmim][$HSO_4$], enhances the solubility and bioavailability of the bioactive components, leading

to improved efficacy in targeted applications, such as enhancing antibacterial properties. Notably, the selection of [Bmim][$HSO_4$] as the preferred IL is underpinned by its stability and compatibility with EEONE components ensuring seamless integration and maintaining the nanoemulsion's integrity and stability. The synergy between ILs and nanoemulsions offers a versatile platform for the development of innovative delivery systems with enhanced performance in various fields [34].

### 3.2 *Characterization of the formulated nanoemulsions*

#### *3.2.1 Dynamic Light Scattering (DLS)*

Figure 2 illustrates the size distribution and polydispersity index (PDI) values of the formulated nanoemulsions. Notably, it reveals a discernible increase in the hydrodynamic size of nanoemulsion droplets with increasing concentration, exemplified by the 1:1 ratio. The variation in the DLS data for IL-loaded nanoemulsions with different ratios is likely due to differences in the composition and interactions between the components, influencing droplet size and stability. The observable increase in the hydrodynamic diameter of nanoemulsion droplets with increasing concentration, as demonstrated by the 1:1 ratio, can be primarily attributed to various factors such as Ostwald ripening, droplet coalescence, and phase separation at higher concentrations, leading to a broader distribution of droplet size. Ostwald ripening is a phenomenon driven by thermodynamic instability [35]. Smaller droplets, characterized by higher surface energy, tend to dissolve and transfer their material to larger droplets in order to minimize their overall energy, resulting in the growth of larger droplets over time, leading to an increase in their hydrodynamic diameter [35]–[37]. Additionally, elevated concentrations increase the likelihood of droplet coalescence, where smaller droplets come in contact and merge to form larger ones [38]. The DLS data confirms the attainment of nanoemulsions with nanometer-sized droplets. Furthermore, Figure 2 presents the PDI values, reflecting droplet size distribution uniformity. Most formulated nanoemulsions, including EEONE and 3:1 and 2:1 IL-loaded EEONE, exhibit nearly monodisperse characteristics, as their PDI values are close to 0.1. The PDI value can range from 0 to 1, with values less than or equal to 0.1 indicating monodisperse particles and values greater than 0.1 indicating polydisperse particle size distributions [39]. However, the 1:1 formulation demonstrates a PDI value exceeding 0.1, indicating polydispersity. This higher PDI value for 1:1 formulation can be attributed to several factors, including Ostwald ripening, coalescence [40], and the possibility of phase separation at higher concentrations, where the formulation becomes saturated, resulting in the separation of excess material into larger droplet or clusters [41].

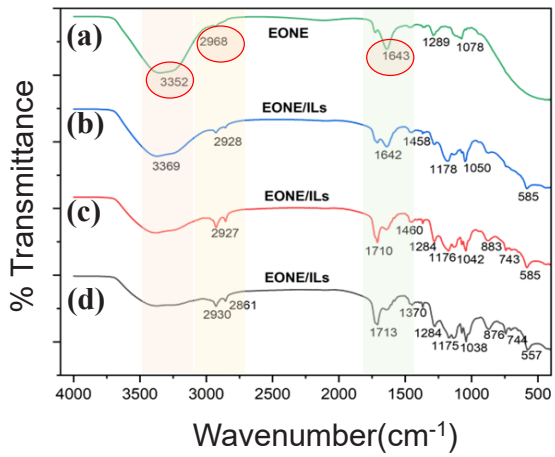


**Figure 3**: FT-IR spectra of (a) EEONE, (b) 3:1 IL-EEONE, (c) 2:1 IL-EEONE, and (d) 1:1 IL-EEONE.

#### *3.2.2 FT-IR Characterization*

The FT-IR spectrum of EEONE (Figure 3(a)) exhibits a characteristic band at 3352 $cm^{-1}$, corresponding to the N-H stretching vibration, and 2968 $cm^{-1}$ for the C-H vibration, along with the prominent peak at 1643 $cm^{-1}$ indicative of strong C=C stretching, in accordance with established literature [42], [43]. Notably, in the FT-IR spectra of IL-loaded EEONE, an additional peak emerges, attributed to the strong S=O stretching vibration. Specifically, peaks are observed at 1458 $cm^{-1}$ (3:1 ratio, Figure 3(b)), 1460 $cm^{-1}$ (2:1 ratio, Figure 3(c)), and 1370 $cm^{-1}$ (1:1 ratio, Figure 3(d)). Similarly, distinctive peaks at 1178 $cm^{-1}$ (3:1 ratio), 1176 $cm^{-1}$ (2:1 ratio), and 1175 $cm^{-1}$ (1:1 ratio) correspond to S-OH bending vibrations. These findings are consistent with existing literature [44] and unequivocally confirm the successful formulation of IL-loaded nanoemulsions.

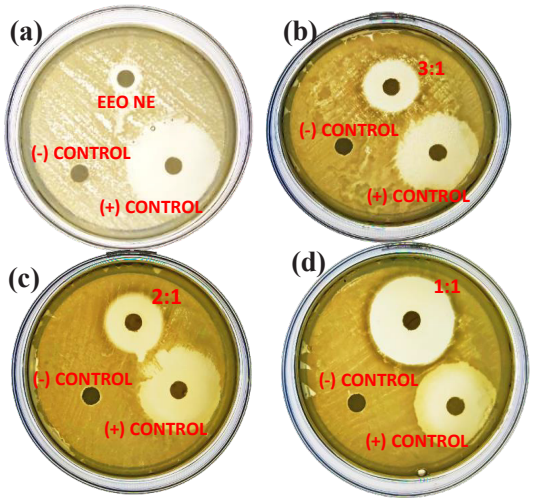


**Figure 4**: The images showing the ZOI for various formulations of nanoemulsions including (a) EEONE, (b) 3:1 IL-EEONE, (c) 2:1 IL-EEONE, and (d) 1:1 IL-EEONE.

### 3.3 *Inhibiting the activity of S. maltophilia*

The results demonstrate varying degrees of antibacterial activity exhibited by both EEONE and the IL-loaded EEONE against *S. maltophilia* (Table 1, Figure 4). EEONE displayed partially active based on the reported ratings [45]. In contrast, IL-loaded EEONE exhibited remarkable bacterial activity, earning a "very active" rating compared to the negative control, with the 1:1 ratio demonstrating the highest average ZOI. This enhanced activity can be attributed to a synergistic effect resulting from the balanced combination of eucalyptus essential oil (EO) and the IL within this formulation. EO inherently possesses antimicrobial properties due to its bioactive compounds [46]–[48], and when combined with the IL in a 1:1 ratio, the IL enhances the solubility and bioavailability of EO's antimicrobial components, enhancing their overall efficacy against *S. maltophilia*. This formulation ensures optimal dispersion and interaction of both EO and IL within the nanoemulsion, enabling a controlled and sustained release of bioactive compounds. Additionally, the concentration-dependent nature of IL-loaded EEONE's bioactivity is observed, aligning with the equivalent volume of essential oil and ionic liquids present in the 1:1 ratio. This observation is consistent with previous literature [49]–[53].

**Table 1**: Summary of the Zone of Inhibition (ZOI) for the formulated nanoemulsions.

| Sample | Average ZOI (in mm) | Interpretation* |
|---|---|---|
| (–) Control | 0 | Inactive |
| (+) Control | 32.3 ± 0.58 | Very active |
| EEONE | 11.3 ± 0.58 | Partially active |
| 3:1 | 22.7 ± 1.53 | Very active |
| 2:1 | 24.3 ± 0.58 | Very active |
| 1:1 | 32.7 ± 0.58 | Very active |

**Note**: *Rating: 0–10 mm (inactive); 11–13 (partially active); > 13 (very active) [45].

The underlying mechanism behind the highly active antibacterial effect of IL-loaded EEONE against *S. maltophilia* is likely to involve the disruption of the bacterial cell wall, ultimately leading to bacterial cell death [54]. In all nanoemulsions formulations, the IL component likely plays a crucial role in destabilizing the bacterial cell wall due to its membrane-disrupting properties [55]. This destabilization increases the permeability of the cell membrane, resulting in the leakage of vital cellular components, disruption of ion gradients, and ultimately, bacterial cell death [56]. Furthermore, the release of antimicrobial compounds present in eucalyptus essential oil [57], facilitated by the nanoemulsions, can further enhance the antibacterial effect by targeting intracellular processes or structures [58], [59]. The synergistic action of IL and eucalyptus essential oil within the 1:1 IL-loaded EEONE formulation amplifies their antibacterial potency, rendering it highly effective in causing bacterial cell wall damage and subsequent bacterial death.

## 4 Conclusions

In conclusion, this study offers a promising approach to combat Kernel Rot Disease in Pili, caused by the pathogenic bacterium *Stenotrophomonas maltophilia*, through the utilization of Ionic Liquid-loaded Nanoemulsions (IL-EEONE). The nanoemulsions, formulated across varying ratios including 1:1, 2:1, and 3:1, successfully achieved droplet sizes within the nanometer range and exhibited nearly monodisperse characteristics based on their PDI values, except for the 1:1 ratio. Remarkably, the IL-loaded EEONE demonstrated highly active and potent antibacterial properties, showcasing its potential as a formidable

tool to inhibit the activity of *S. maltophilia*. In particular, the 1:1 ratio formulation stood out as exceptionally effective, achieving antibacterial activity comparable to that of the antibiotic-positive control. These findings underscore the potential of IL-loaded nanoemulsions as a promising avenue for developing innovative antimicrobial strategies, with the potential to significantly impact the management of Kernel Rot Disease in Pili nuts. Moreover, reducing bacterial infections in Pili nuts could significantly increase their value, emphasizing the broader agricultural and economic impacts of this study.

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**Author Contributions**

R.Y.C.: conceptualization, investigation, reviewing, and editing; R.S.A.S.: investigation, methodology, writing an original draft; R.Y.C., A.C.A., R.S.A.S., F.S.L., E.P.P., J.A.M.L.: research design, data analysis; R.Y.C., A.C.A., F.S.L., E.P.P., J.A.M.L.: conceptualization, data curation, writing—reviewing and editing, funding acquisition, project administration. All authors have read and agreed to the published version of the manuscript.

**Conflicts of Interest**

The authors declare no conflict of interest.

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