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Evaluation of Citrus Fruit-Derived Bacterial Isolates for Natural Antioxidant Potential

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Abstract: The growing demand for biologically derived antioxidants has intensified interest in microorganisms as alternative sources of functional metabolites. This study evaluated the antioxidant potential of bacteria isolated from citrus fruit peels, specifically calamansi (Citrus microcarpa), orange (Citrus sinensis), and lemon (Citrus limon).

Bacterial isolates were obtained using standard microbiological procedures and characterized through Gram staining, catalase, and oxidase tests. Cell-free supernatants were prepared and assessed for antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Among the citrus fruits examined, only calamansi yielded viable bacterial isolates, which were presumptively identified as Lactobacillus spp. This observation highlights Citrus microcarpa as a potentially underexplored reservoir of antioxidant-producing bacteria.

The bacterial supernatant demonstrated statistically significant, concentration-dependent DPPH radical scavenging activity ($p < 0.05$), indicating the production of metabolites with antioxidant properties. These findings suggest that calamansi-derived Lactobacillus spp. may serve as promising natural antioxidant sources with potential applications in food, pharmaceutical, and biotechnological industries. Further molecular identification and expanded antioxidant profiling are recommended to support future functional investigations.

Keywords: *Citrus Microcarpa; Lactobacillus Spp.; Lactic Acid Bacteria; Microbial Antioxidants; DPPH Radical Scavenging; Bioactive Metabolites.*

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I. INTRODUCTION

Oxidative stress is a fundamental biochemical process that occurs when the generation of reactive oxygen species (ROS) exceeds the capacity of biological antioxidant defense systems to neutralize them. Reactive oxygen species, including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and singlet oxygen, are continuously produced in aerobic organisms as by-products of normal cellular metabolism. Major intracellular sources of ROS include mitochondrial oxidative phosphorylation, peroxisomal reactions, and various enzymatic pathways such as those involving NADPH oxidases. In addition to endogenous production, external factors such as ultraviolet radiation, environmental pollutants, heavy metals, smoking, and xenobiotic exposure further contribute to excessive ROS generation. Oxidative stress has been strongly associated with the pathogenesis of numerous chronic diseases, increasing the demand for safe and naturally derived antioxidants (Singh et al., 2023). Microbial antioxidants, particularly those produced by lactic acid bacteria, have gained attention due to their therapeutic potential and biological safety.

Under physiological conditions, ROS play important roles in cell signaling, immune defense, and regulation of gene expression. However, when present in excess, ROS can overwhelm endogenous antioxidant mechanisms and induce oxidative stress, leading to cellular and molecular damage. These microorganisms exert antioxidant effects through mechanisms such as free radical scavenging, metal ion chelation, and enzymatic defense systems (He et al., 2023). Lipid peroxidation compromises membrane integrity and function, protein oxidation disrupts enzymatic activity and structural stability, and oxidative DNA damage may result in mutations and genomic instability. These deleterious effects have been strongly associated with the development and progression of numerous chronic and degenerative diseases, including cardiovascular disorders, diabetes mellitus, neurodegenerative diseases such as Alzheimer's and Parkinson's diseases, inflammatory conditions, accelerated

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aging, and various forms of cancer. As a result, mitigating oxidative stress has become a central focus in biomedical, nutritional, and pharmaceutical research.

Biological systems are equipped with complex antioxidant defense mechanisms designed to counteract ROS. These include enzymatic antioxidants such as superoxide dismutase, catalase, and glutathione peroxidase, as well as non-enzymatic antioxidants including glutathione, uric acid, ascorbic acid, carotenoids, and tocopherols. While these endogenous defenses are effective under normal physiological conditions, they may become insufficient during periods of increased oxidative burden caused by disease, environmental stressors, or lifestyle-related factors. Consequently, exogenous antioxidants derived from dietary and natural sources are often required to supplement intrinsic defense systems.

Synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary-butylhydroquinone (TBHQ) have been widely used in food and pharmaceutical industries to prevent oxidative deterioration and extend shelf life. Despite their effectiveness, concerns have been raised regarding the long-term safety of synthetic antioxidants. Several experimental and toxicological studies have suggested potential cytotoxic, genotoxic, and carcinogenic effects associated with excessive or prolonged exposure. These concerns have led to stricter regulatory controls and a growing consumer preference for natural alternatives. As a result, there has been a marked shift toward the exploration of safe, effective, and sustainable sources of natural antioxidants.

Natural antioxidants are generally perceived as safer and more biologically compatible than synthetic compounds. In addition to free radical scavenging, natural antioxidants often exhibit multifunctional properties, including anti-inflammatory, antimicrobial, and immunomodulatory effects. Among natural sources, fruits and vegetables have received extensive attention due to their high content of phenolic compounds, flavonoids, vitamins, and carotenoids. Citrus fruits, in particular, are among the most widely consumed fruits worldwide and are recognized for their nutritional and health-promoting properties. Fermentation of citrus substrates has been associated with improved phenolic composition and enhanced antioxidant activity, suggesting a synergistic relationship between microbial metabolism and plant-derived compounds (Razola-Díaz et al., 2024; He et al., 2023).

Citrus fruits are rich in vitamin C and contain a diverse array of bioactive compounds such as flavanones, flavones, phenolic acids, carotenoids, and essential oils. Numerous epidemiological and experimental studies have demonstrated that regular consumption of citrus fruits is associated with a reduced risk of chronic diseases linked to oxidative stress and inflammation. In addition to their edible pulp, citrus fruits generate large amounts of peel waste during processing for juice and food products. Citrus peels may constitute up to 50% of the total fruit mass and are often discarded or underutilized, posing environmental and economic challenges.

Recent research has emphasized the valorization of citrus peel waste as a sustainable source of high-value bioactive compounds. Several studies have reported that citrus peels exhibit higher concentrations of phenolic compounds and greater antioxidant activity than the edible portions of the fruit. This has stimulated interest in exploring citrus peels not only as sources of plant-derived antioxidants but also as ecological niches that harbor diverse microbial communities with potential functional significance.

The surface of citrus peels provides a unique microenvironment characterized by acidic pH, high sugar availability, moisture retention, and the presence of antimicrobial phytochemicals. These conditions selectively shape microbial communities capable of surviving and adapting to oxidative and chemical stress. Microorganisms associated with plant surfaces, particularly bacteria, may contribute additional biofunctional properties through the production of secondary metabolites, including antioxidant compounds. However, compared with plant-derived antioxidants, the contribution of citrus-associated microbiota to antioxidant potential remains relatively underexplored.

Lactic acid bacteria (LAB) are a group of Gram-positive, non-spore-forming microorganisms commonly associated with fermented foods, plant materials, and the gastrointestinal tract of humans and animals. Lactic acid bacteria are recognized not only for their role in fermentation but also for producing bioactive metabolites with antioxidant and anti-inflammatory properties (Abdel-Daim et al., 2021; Riaz et al., 2022). Members of the genus *Lactobacillus* are particularly well known for their role in food fermentation and preservation and are generally regarded as safe (GRAS). Beyond their technological applications, LAB have been extensively studied for their probiotic and health-promoting properties, including modulation of gut microbiota, enhancement of immune responses, inhibition of pathogenic microorganisms, and reduction of oxidative stress.

In recent years, increasing attention has been directed toward the antioxidant activity of LAB. These bacteria can exert antioxidant effects through multiple mechanisms, including direct scavenging of free radicals, chelation of pro-oxidant metal ions, production of antioxidant enzymes, and synthesis of bioactive metabolites such as peptides, organic acids, and exopolysaccharides. Several studies have reported antioxidant-producing LAB isolated from fermented foods and plant-based substrates, suggesting that the source of isolation plays a critical role in determining functional potential.

Despite growing interest in microbial antioxidants, most existing studies have focused on LAB isolated from fermented products, while relatively few have investigated LAB directly associated with fresh fruits and fruit peels. Citrus peel–associated LAB represent an underexplored resource with promising biotechnological applications. The chemical composition of citrus peels, including organic acids and phenolic compounds, may influence microbial metabolism and enhance the production of antioxidant metabolites. Moreover, differences in peel structure and composition

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among citrus species may result in distinct microbial communities with varying antioxidant capacities.

In the Philippine context, *Citrus microcarpa* (calamansi) holds significant cultural, culinary, and economic importance. It is widely consumed and commonly used in beverages, condiments, and traditional remedies. Despite its widespread use, limited scientific attention has been directed toward the microbial communities associated with calamansi peels and their potential functional properties. Comparative data on bacterial populations isolated from calamansi and their antioxidant activity remain scarce.

Identifying antioxidant-producing bacteria from locally available citrus fruits offers a sustainable and cost-effective approach to natural antioxidant development. Such efforts align with global trends toward natural product utilization, waste valorization, and circular bioeconomy principles. Furthermore, exploring fruit-associated LAB contributes to a broader understanding of microbial diversity and functional potential in non-fermented plant environments.

Recent scientific trends indicate a growing shift from purely plant-based antioxidants toward microbial-derived bioactive compounds. Advances in microbiology and biotechnology have enabled researchers to recognize microorganisms not only as agents of fermentation but also as active producers of health-promoting metabolites. Lactic acid bacteria, in particular, have attracted significant attention due to their dual role in food preservation and functional health enhancement.

Studies conducted over the past decade have demonstrated that microbial antioxidants may offer advantages in terms of stability, scalability, and controlled production. Unlike plant antioxidants, which are often influenced by seasonal variability and environmental conditions, microbial metabolites can be produced under standardized fermentation parameters. This allows for more predictable yields and facilitates large-scale industrial application.

Furthermore, the exploration of plant-associated microbiota has opened new research pathways in natural product discovery. Microorganisms that inhabit fruit surfaces frequently interact with phytochemicals, leading to metabolic transformations that may enhance antioxidant capacity. Such interactions highlight the importance of examining microbial communities as integral contributors to the biochemical properties of plant materials.

The increasing global demand for safer and more sustainable antioxidant sources continues to drive interest in microbial alternatives. As consumers and industries shift away from synthetic additives, research focusing on naturally derived microbial metabolites is expected to expand significantly. Investigations into citrus-associated bacteria therefore represent a timely and relevant contribution to this evolving field.

Given the increasing demand for safe and natural antioxidants, the limited information on citrus peel–associated bacteria, and the lack of focused studies on *Citrus microcarpa*, further investigation is warranted. This study aimed to isolate and characterize bacterial strains from the peels of selected citrus fruits and to evaluate their antioxidant potential using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Specifically, the study sought to determine the presence and phenotypic characteristics of bacterial isolates associated with citrus peels and to assess the in vitro antioxidant activity of bacterial metabolites. The findings of this research are expected to contribute valuable insights into the role of citrus-associated bacteria as potential sources of natural antioxidants with applications in food, pharmaceutical, and biotechnological industries.

II. MATERIALS AND METHODS

➢ *Study Design and Experimental Framework*

This study employed a laboratory-based experimental research design aimed at isolating bacterial strains from citrus fruit peels and evaluating their antioxidant potential through an in vitro free radical scavenging assay. The experimental framework was structured to ensure reproducibility, accuracy, and methodological consistency across all procedures. The study focused on citrus fruits that are commonly consumed and readily available, namely calamansi (*Citrus microcarpa*), orange (Citrus sinensis), and lemon (Citrus limon). However, antioxidant evaluation was ultimately conducted only on bacterial isolates successfully obtained from calamansi peels, as no viable bacterial growth suitable for analysis was observed in the other citrus samples. The use of cell-free supernatants in antioxidant assays is well established, as bioactive metabolites released during bacterial growth contribute significantly to radical scavenging activity (Choi et al., 2020).

The overall workflow of the study included: (1) collection and preparation of citrus fruit samples, (2) isolation and purification of peel-associated bacteria, (3) phenotypic and biochemical characterization of bacterial isolates, (4) preparation of bacterial cell-free supernatants, and (5) assessment of antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay.

➢ *Collection of Citrus Fruit Samples*

Fresh, mature citrus fruits were obtained from local public markets within the study area. Fruits were selected based on uniform size, maturity, and the absence of visible physical damage, spoilage, or fungal contamination. To minimize variability, all samples were collected within the same time period and transported to the laboratory on the same day of purchase. Fruits were placed in clean, covered containers and transported at ambient temperature. All samples were processed within 24 hours of collection to preserve microbial viability and prevent post-harvest contamination.

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➢ *Surface Cleaning and Preparation of Citrus Peels*

Upon arrival at the laboratory, citrus fruits were thoroughly washed under running tap water to remove surface debris, dirt, and loosely attached contaminants. This was followed by rinsing with sterile distilled water. Surface sterilization was performed by immersing each fruit in 70% ethanol for approximately one to two minutes. This step was intended to reduce transient surface microorganisms while preserving peel-associated microbial populations.

After ethanol treatment, fruits were rinsed again with sterile distilled water to remove residual alcohol and allowed to air-dry inside a biosafety cabinet. Using sterile knives and forceps, the peels were aseptically separated from the pulp to prevent contamination from internal tissues. The peels were cut into small pieces measuring approximately 1–2 cm^2 and transferred into sterile containers for subsequent bacterial isolation.

➢ *Isolation of Bacteria from Citrus Peels*

Approximately 10 g of prepared citrus peel samples were aseptically transferred into sterile Erlenmeyer flasks containing 90 mL of sterile peptone water. The samples were homogenized using a laboratory blender to facilitate the release of peel-associated microorganisms into suspension. Serial dilutions were prepared up to 10^{-6} using sterile peptone water to obtain countable bacterial colonies.

Aliquots of 0.1 mL from appropriate dilutions were spread-plated onto De Man, Rogosa, and Sharpe (MRS) agar plates, a selective medium commonly used for the cultivation of lactic acid bacteria. All plates were incubated at 37 °C for 24–48 hours under anaerobic conditions using anaerobic jars with gas-generating sachets. After incubation, plates were examined for bacterial growth, and colonies with distinct morphological characteristics were selected for further purification.

➢ *Purification and Maintenance of Bacterial Isolates*

Selected colonies were purified by repeated subculturing on fresh MRS agar plates using the streak plate technique. Subculturing was continued until uniform colony morphology was observed, indicating culture purity. Purity was further confirmed through microscopic examination following Gram staining.

Pure bacterial isolates were maintained on MRS agar slants and stored at 4 °C for short-term preservation. Subculturing was performed periodically to maintain bacterial viability. Each isolate was assigned a unique code to ensure traceability and minimize bias during subsequent analyses.

➢ *Phenotypic and Biochemical Characterization of Isolates*

Phenotypic characterization of bacterial isolates was conducted based on colony morphology, including size, shape, color, elevation, and margin. Microscopic characterization was performed using Gram staining to determine Gram reaction and cellular morphology. Only Gram-positive isolates exhibiting rod-shaped or coccoid morphology were considered presumptive lactic acid bacteria and selected for further testing.

Biochemical characterization included catalase and oxidase tests. Catalase activity was assessed by placing a small amount of fresh bacterial culture onto a clean glass slide and adding a drop of 3% hydrogen peroxide. The absence of bubble formation was recorded as a negative catalase reaction, consistent with lactic acid bacteria. Oxidase activity was evaluated using standard oxidase reagent, with the absence of color change indicating a negative reaction. These tests served as preliminary identification methods prior to functional antioxidant analysis.

➢ *Preparation of Bacterial Cell-Free Supernatants*

To obtain bacterial metabolites for antioxidant testing, selected bacterial isolates were cultured in MRS broth and incubated at 37 °C for 24 hours. Following incubation, bacterial cultures were centrifuged at 10,000 rpm for 10 minutes at 4 °C to separate bacterial cells from the supernatant.

The resulting supernatants were carefully decanted and filtered through sterile 0.22 µm membrane filters to remove residual cells and particulate matter. The filtered cell-free supernatants were stored at 4 °C and used within 24 hours to minimize degradation of bioactive compounds. These supernatants were assumed to contain extracellular metabolites produced by the bacteria during growth and were used directly for antioxidant activity evaluation.

➢ *DPPH Radical Scavenging Assay*

The antioxidant activity of bacterial cell-free supernatants was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. A freshly prepared 0.1 mM DPPH solution in methanol was used for all experiments and protected from light to prevent photodegradation.

Equal volumes (1 mL) of bacterial supernatant and DPPH solution were mixed thoroughly and incubated in the dark at room temperature for 30 minutes. A control sample consisting of DPPH solution mixed with methanol was prepared concurrently. After incubation, absorbance values were measured at 517 nm using a UV–visible spectrophotometer.

The percentage of DPPH radical scavenging activity was calculated using the following formula:

$$DPPH\ Scavenging\ Activity\ (\%) = ((Acontrol - Asample) / Acontrol) \times 100$$

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➢ *Experimental Replication and Statistical Analysis*

All experiments were conducted in triplicate to ensure reproducibility and reliability of results. Data were expressed as mean ± standard deviation. Statistical analyses were performed using appropriate statistical software. One-way analysis of variance (ANOVA) was applied to determine significant differences in antioxidant activity among samples. A p-value of less than 0.05 was considered statistically significant.

➢ *Rationale for Method Selection and Assay Reliability*

The selection of appropriate experimental methods is critical to ensuring the reliability and interpretability of antioxidant studies. In this research, the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay was chosen as the primary method for evaluating antioxidant activity due to its simplicity, reproducibility, and widespread acceptance in antioxidant research. The DPPH assay is based on the ability of antioxidant compounds to donate hydrogen atoms or electrons to neutralize free radicals, resulting in a measurable decrease in absorbance at 517 nm.

The use of the DPPH assay is particularly suitable for preliminary screening of antioxidant activity in biological samples such as bacterial cell-free supernatants. Unlike more complex cellular or in vivo assays, the DPPH method allows for rapid and direct assessment of free radical scavenging potential without interference from cellular metabolism. This makes it an effective tool for evaluating extracellular metabolites produced by microorganisms, which was the primary focus of this study.

The reliability of the assay was ensured through strict standardization of experimental conditions. All reagents were freshly prepared, protected from light, and used within recommended time frames to prevent degradation. Control samples were included in every experimental run to establish baseline absorbance values, and all measurements were performed in triplicate to minimize experimental variability. The consistent absorbance readings observed in control samples across replicates confirmed assay stability and methodological reliability.

Furthermore, the use of spectrophotometric analysis at a fixed wavelength allowed for objective quantification of antioxidant activity. The observed dose-dependent scavenging activity further supports the validity of the assay, as such trends are characteristic of genuine antioxidant behavior. Although the DPPH assay evaluates only one mechanism of antioxidant action, its methodological robustness makes it an appropriate first-line assay for assessing the antioxidant potential of microbial metabolites.

➢ *Quality Control and Laboratory Safety*

Quality control measures were implemented throughout all experimental procedures. Culture media and reagents were prepared according to manufacturer instructions and inspected for contamination prior to use. Sterility controls consisting of uninoculated media were incubated under the same conditions as test samples to confirm aseptic handling.

All laboratory procedures were performed in accordance with standard biosafety and microbiological practices. Personal protective equipment, including laboratory coats, gloves, and protective eyewear, was worn at all times. All biological waste was autoclaved prior to disposal.

➢ *Ethical Considerations*

This study involved only microbial isolates obtained from food materials and did not include human or animal subjects. As such, ethical approval was not required.

III. RESULTS AND DISCUSSION

➢ *Overview of Experimental Findings*

This study investigated the antioxidant potential of bacterial isolates obtained from the peels of selected citrus fruits using the DPPH free radical scavenging assay. Among the citrus fruits examined—*Citrus microcarpa* (calamansi), Citrus sinensis (orange), and Citrus limon (lemon)—only calamansi peels yielded viable bacterial isolates suitable for antioxidant evaluation. These isolates were presumptively identified as *Lactobacillus spp.* based on phenotypic and biochemical characteristics. The antioxidant activity of the calamansi-derived bacterial metabolites was assessed and compared against a control and a standard antioxidant. Emerging research indicates that strain-specific fermentation can significantly improve antioxidant potential and overall product quality (Wu et al., 2025; Yang et al., 2025).

The findings demonstrate that bacterial metabolites derived from *Citrus microcarpa* exhibit measurable and statistically significant antioxidant activity, supporting the hypothesis that citrus peel–associated bacteria may serve as potential sources of natural antioxidants. The observed antioxidant activity aligns with previous reports demonstrating that Lactobacillus strains can produce exopolysaccharides and peptides with strong radical scavenging capacity (Li et al., 2021; Meira et al., 2022).

➢ *Bacterial Isolation from Citrus Peels*

The isolation results revealed notable differences among the citrus fruit samples. Viable bacterial growth was observed exclusively in calamansi peel samples, while orange and lemon peels did not yield bacterial isolates suitable for further analysis. Instead, the latter samples exhibited minimal bacterial growth and occasional fungal contamination.

This selective bacterial presence may be attributed to differences in peel characteristics among citrus species. Calamansi peels possess relatively higher moisture content, thinner cuticle structure, and favorable nutrient composition, which may support bacterial adherence and survival. In contrast, orange and lemon peels are known to contain higher concentrations of antimicrobial essential oils such as limonene and citral, which have been reported to inhibit bacterial growth. These observations are consistent with previous studies indicating that citrus peel chemistry plays a significant role in shaping surface-associated microbial communities. Microbial fermentation has been shown to enhance the bioavailability of antioxidant compounds, further supporting the functional

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relevance of bacteria-derived metabolites (Fang & Bhandari, 2021).

The exclusive recovery of bacterial isolates from calamansi peels justified the subsequent focus of antioxidant analysis on *Citrus microcarpa*–derived bacteria.

- *Phenotypic and Biochemical Characteristics of Bacterial Isolates*

The bacterial isolate obtained from calamansi peels exhibited Gram-positive, rod-shaped morphology and tested negative for catalase and oxidase activity. These phenotypic and biochemical characteristics are consistent with members of the genus Lactobacillus, a group of lactic acid bacteria commonly associated with plant materials and fermented products. Similar antioxidant behaviors have been documented in probiotic bacterial metabolites, reinforcing their potential as natural alternatives to synthetic antioxidants (Sannasimuthu & Selvaraj, 2021).

The identification of *Lactobacillus spp.* is particularly relevant in the context of antioxidant research, as numerous studies have demonstrated the antioxidant potential of lactic acid bacteria and their metabolites. The generally recognized as safe (GRAS) status of Lactobacillus further enhances the potential applicability of these isolates in food, nutraceutical, and pharmaceutical industries.

- *DPPH Radical Scavenging Activity of Bacterial Supernatants*

The antioxidant activity of the calamansi-derived bacterial isolate was evaluated using the DPPH radical scavenging assay. The control sample exhibited consistently high absorbance values at 517 nm, confirming the stability of the DPPH radical in the absence of antioxidant compounds. This supports the reliability of the assay conditions.

In contrast, bacterial cell-free supernatants demonstrated a reduction in absorbance relative to the control, indicating the ability of bacterial metabolites to scavenge DPPH free radicals. Although the magnitude of absorbance reduction was lower than that observed for the standard antioxidant, the decrease was statistically significant ($p < 0.05$), confirming genuine antioxidant activity.

The observed scavenging activity suggests that extracellular metabolites produced by the Lactobacillus isolate are capable of donating electrons or hydrogen atoms to stabilize free radicals. This behavior suggests that the isolate may possess metabolically active antioxidant defense pathways, reinforcing the emerging view that plant-associated lactic acid bacteria function as adaptive contributors to oxidative stability rather than passive surface colonizers.

- *Comparison with Standard Antioxidant*

As expected, the standard antioxidant exhibited the highest percentage of DPPH radical scavenging activity, reflecting its well-characterized and potent reducing capacity. The bacterial isolate showed lower but significant scavenging activity in comparison.

This difference is biologically reasonable and should be interpreted within the appropriate context. While the standard antioxidant represents a purified single compound, the bacterial supernatant consists of a complex mixture of metabolites produced under biological conditions. The antioxidant activity of the bacterial isolate may therefore result from the synergistic effects of multiple compounds rather than a single dominant molecule.

Such biological complexity may offer advantages in practical applications, including improved stability, sustained antioxidant effects, and additional health benefits when incorporated into functional products.

- *Concentration-Dependent Antioxidant Activity*

The DPPH scavenging activity of the bacterial supernatant exhibited a concentration-dependent trend, with higher concentrations resulting in greater free radical inhibition. This dose–response relationship is a hallmark of antioxidant systems and provides further validation of the functional activity of the bacterial metabolites.

The decrease in scavenging activity at lower concentrations may be attributed to reduced availability of antioxidant molecules capable of interacting with DPPH radicals. This observation is consistent with established antioxidant kinetics and supports the reliability of the experimental results.

- *Statistical Analysis and Data Reliability*

Statistical analysis confirmed significant differences between the control and bacterial supernatant samples ($p < 0.05$). The low standard deviation values observed across triplicate measurements indicate good experimental precision and reproducibility. These findings suggest that the antioxidant activity observed was not due to random variation but rather to the intrinsic properties of the bacterial metabolites.

- *Mechanistic Insights into Antioxidant Activity of Citrus-Associated Lactic Acid Bacteria*

Elucidating the mechanisms underlying the antioxidant activity of citrus-associated lactic acid bacteria is critical for determining their functional relevance within biological systems. The radical scavenging behavior observed in this study suggests that the *Lactobacillus* isolate may engage metabolically regulated defense pathways capable of mitigating oxidative stress. Such activity supports the growing recognition of microbial antioxidants as dynamic participants in redox balance rather than merely incidental metabolic by-products. These biochemical interactions interrupt oxidative chain reactions, thereby reducing cellular damage associated with reactive oxygen species.

Lactic acid bacteria are known to synthesize a diverse range of antioxidant molecules during metabolic processes. These include organic acids, bioactive peptides, exopolysaccharides, and enzymatic antioxidants that collectively contribute to redox balance. Some strains have also been reported to enhance the availability of phenolic compounds through enzymatic biotransformation, converting complex plant polyphenols into more biologically active

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forms. Considering that citrus peels are naturally rich in flavonoids and phenolic acids, it is plausible that microbial metabolism enhanced the antioxidant potential of these substrates.

Another possible explanation for the observed antioxidant activity is the presence of intracellular defense systems within lactic acid bacteria. Certain strains produce glutathione and possess superoxide dismutase–like activity, both of which are critical in mitigating oxidative stress. Although intracellular components were not directly measured in this study, their potential contribution cannot be excluded, particularly if partial cell lysis occurred during metabolite extraction.

Environmental adaptation may also play a role in the antioxidant behavior of peel-associated bacteria. Citrus surfaces present challenging conditions, including acidity, fluctuating moisture levels, and exposure to ultraviolet radiation. Microorganisms capable of surviving in such environments often develop protective biochemical strategies, including antioxidant production. This adaptive response may explain the functional activity observed in the calamansi-derived isolate.

Importantly, the antioxidant effect demonstrated by the bacterial supernatant likely reflects a synergistic interaction among multiple metabolites rather than the action of a single compound. Biological antioxidant systems frequently rely on cooperative mechanisms in which different molecules target distinct reactive species. Such synergy may provide broader protective effects compared to isolated chemical antioxidants.

These mechanistic considerations reinforce the concept that microbial antioxidants represent a biologically dynamic alternative to conventional antioxidant sources. While purified compounds may exhibit stronger immediate radical scavenging activity, microbial metabolites offer advantages such as sustained production, metabolic adaptability, and potential compatibility with living systems.

Future mechanistic studies employing metabolomic profiling and enzyme analysis would help clarify the specific pathways responsible for antioxidant production in citrus-associated Lactobacillus strains.

- *Synthesis of Findings*

Overall, the results of this study demonstrate that bacterial isolates derived from *Citrus microcarpa* peels possess statistically significant antioxidant activity. Although their scavenging capacity is lower than that of standard antioxidants, the biological relevance and sustainability of microbial-derived antioxidants highlight their potential value.

These findings support the hypothesis that citrus peel–associated bacteria can serve as natural sources of antioxidants and provide a strong foundation for further research into their applications in food, pharmaceutical, and biotechnological industries.

- *Comparative Perspective with Existing Antioxidant Research*

The antioxidant activity observed in the calamansi peel–derived *Lactobacillus* isolate aligns with a growing body of literature recognizing lactic acid bacteria as functional producers of bioactive metabolites. Previous investigations have reported that certain *Lactobacillus* strains exhibit measurable free radical scavenging activity comparable to naturally occurring antioxidant compounds, supporting their potential role in oxidative stress mitigation.

Compared with plant-derived antioxidants, microbial antioxidants present unique advantages. While plant extracts often depend on environmental conditions such as soil quality, climate, and harvest timing, microbial metabolites can be produced under controlled laboratory conditions, allowing for greater standardization and reproducibility. This characteristic is particularly valuable for industrial applications where consistency is essential.

Studies involving lactic acid bacteria isolated from fermented foods have demonstrated antioxidant mechanisms including metal ion chelation, inhibition of lipid peroxidation, and enzymatic neutralization of reactive oxygen species. Although the present study focused on DPPH radical scavenging activity, the observed results suggest that citrus-associated bacteria may share similar protective mechanisms. This expands the current understanding of antioxidant-producing microorganisms beyond traditional fermented matrices and highlights fresh fruit surfaces as promising reservoirs of functional bacteria. Antioxidant metabolites produced by lactic acid bacteria have been widely reported to enhance cellular defense mechanisms against oxidative damage (Ahn et al., 2022; Wang et al., 2021).

Notably, the antioxidant capacity recorded in this study was lower than that of the standard antioxidant. However, such differences are expected when comparing complex biological extracts with purified chemical compounds. Rather than indicating reduced value, this distinction emphasizes the multifactorial nature of microbial antioxidant systems, which often rely on synergistic interactions among metabolites.

The findings therefore support the emerging perspective that microbial antioxidants should not be viewed solely as replacements for conventional antioxidants but as complementary agents capable of enhancing overall oxidative stability. Continued investigation into citrus-associated lactic acid bacteria may reveal strains with optimized antioxidant production suitable for functional food development, probiotic formulations, and therapeutic research.

- *Biological Significance and Applied Implications*

Traditionally, the antioxidant properties of citrus fruits have been primarily attributed to plant-derived compounds such as vitamin C, flavonoids, and phenolic acids. However, the findings of this study suggest that citrus peel–associated bacteria may also play a meaningful role in contributing to overall antioxidant potential. The presence of antioxidant-producing microorganisms on calamansi peels highlights a synergistic relationship between plant substrates and microbial

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metabolism, wherein microbial biotransformation of phytochemicals may enhance functional antioxidant activity.

The acidic and phytochemically rich environment of calamansi peels likely promotes adaptive bacterial responses, including the production of metabolites capable of mitigating oxidative stress. Such ecological pressures may encourage the development of protective biochemical pathways, providing a plausible explanation for the DPPH scavenging activity observed in the Lactobacillus isolate. These findings support the emerging perspective that plant-associated microorganisms are not merely passive inhabitants but active biochemical contributors within antioxidant systems.

The demonstration of antioxidant activity in calamansi-derived Lactobacillus spp. carries important implications for the development of natural antioxidant sources. Microbial antioxidants represent a promising alternative or complement to plant-derived and synthetic antioxidants, particularly in applications requiring biological compatibility, metabolic adaptability, and multifunctional properties. Unlike purified chemical antioxidants, microbial metabolites may offer sustained activity through dynamic biological processes.

From an industrial standpoint, antioxidant-producing lactic acid bacteria present valuable opportunities across food, pharmaceutical, and biotechnological sectors. In food systems, these microorganisms may be incorporated into fermented products or functional beverages to enhance oxidative stability, extend shelf life, and reduce reliance on synthetic additives while simultaneously delivering probiotic benefits. Probiotics have demonstrated substantial roles in disease prevention and health promotion, largely attributed to their metabolic activities and functional compounds (Liu et al., 2020; Plaza-Díaz et al., 2020). Given the generally recognized as safe (GRAS) status of Lactobacillus spp., their integration into food applications is particularly feasible.

In pharmaceutical and nutraceutical contexts, microbial antioxidants offer additional therapeutic promise due to their potential immunomodulatory and anti-inflammatory effects, both of which are closely linked to oxidative stress–related disorders. The incorporation of antioxidant-producing bacteria into probiotic formulations may therefore provide synergistic health benefits beyond conventional antioxidant supplementation.

Moreover, the utilization of bacteria isolated from locally available citrus fruits supports sustainable research practices and aligns with efforts to valorize agricultural by-products. Citrus peels are abundant yet frequently discarded despite their rich biochemical and microbial composition. Harnessing peel-associated bacteria for antioxidant production reflects principles of circular bioeconomy and promotes cost-effective, environmentally responsible strategies for functional ingredient development.

Collectively, these findings reinforce the growing recognition of microorganisms as viable sources of bioactive compounds and position citrus-associated lactic acid bacteria as promising candidates for future antioxidant research. While additional studies are necessary to optimize production pathways and characterize specific metabolites, the present results provide a strong scientific foundation for continued exploration of microbial antioxidants across multiple applied fields. These results contribute to the expanding paradigm that plant-associated microorganisms represent an underexplored reservoir of functional bioactive compounds with significant scientific and industrial relevance.

IV. LIMITATIONS OF THE STUDY

While the findings of this study provide valuable insights into the antioxidant potential of citrus peel–associated bacteria, several limitations should be acknowledged. Recognizing these limitations is essential for proper interpretation of the results and for guiding future research in this area.

First, the antioxidant activity was evaluated using only a single in vitro assay, the DPPH radical scavenging method. Although this assay is widely accepted and reliable for assessing free radical scavenging activity, it represents only one aspect of antioxidant behavior. Antioxidants may act through multiple mechanisms, including metal ion chelation, inhibition of lipid peroxidation, and enhancement of endogenous antioxidant enzymes. The exclusive use of the DPPH assay limits the ability to fully characterize the antioxidant profile of the bacterial metabolites.

Second, the study focused on a single bacterial isolate obtained from *Citrus microcarpa* peels. While this isolate demonstrated significant antioxidant activity, the results may not be representative of the full diversity of citrus peel–associated microbiota. Other bacterial strains or species present on citrus peels may possess higher or complementary antioxidant capacities. The limited number of isolates restricts broader generalization of the findings.

Third, bacterial identification was based solely on phenotypic and biochemical characteristics. Although these methods are useful for preliminary classification, they do not provide definitive species-level identification. Molecular techniques such as 16S rRNA gene sequencing would offer greater accuracy and allow for more precise taxonomic classification of the isolate.

Additionally, the study did not attempt to identify or quantify the specific bioactive compounds responsible for the observed antioxidant activity. The antioxidant effects observed may result from a combination of metabolites rather than a single compound. Without chemical characterization, the exact mechanisms underlying the antioxidant activity remain speculative.

Finally, the study was conducted entirely under in vitro laboratory conditions. While in vitro assays are essential for initial screening, they do not necessarily reflect biological activity in living systems. Factors such as bioavailability, metabolism, and interactions with host tissues may influence antioxidant efficacy in vivo.

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Despite these limitations, the study provides a strong preliminary foundation for further investigation and highlights the potential of citrus-associated bacteria as natural antioxidant sources.

- *Significance of the Study and Scientific Contribution*

This study contributes to the expanding field of natural antioxidant research by highlighting the potential of citrus peel–associated bacteria as alternative sources of bioactive compounds. While most antioxidant studies focus on plant-derived extracts, this research shifts attention toward the microbial communities associated with citrus peels, emphasizing their functional and biotechnological relevance.

Scientifically, the study adds novel insight into the antioxidant potential of bacteria isolated from *Citrus microcarpa*, a citrus species that remains underexplored in microbial antioxidant research. The identification of antioxidant activity in peel-associated *Lactobacillus spp.* supports the growing recognition of microorganisms as active contributors to antioxidant systems rather than passive inhabitants of plant surfaces.

From a methodological standpoint, the study demonstrates the applicability of the DPPH radical scavenging assay in evaluating microbial-derived antioxidants. By adapting a widely accepted chemical assay to bacterial metabolites, the research provides a practical framework for preliminary screening of antioxidant-producing microorganisms. This approach may be adopted by future researchers aiming to explore microbial antioxidants from other plant-associated environments.

The study also holds significance within the context of sustainable science. Citrus peels are commonly treated as agricultural waste despite their rich biochemical and microbial composition. By utilizing bacteria isolated from calamansi peels, this research promotes value-added utilization of agro-industrial by-products and aligns with principles of waste reduction and circular bioeconomy.

In the field of medical technology and laboratory sciences, the findings underscore the interdisciplinary relevance of microbiology, biochemistry, and functional food research. The study encourages further exploration of microbial antioxidants for potential integration into diagnostic research, therapeutic development, and preventive health strategies. The findings of this study contribute to the expanding paradigm that microorganisms associated with plant surfaces represent an underexplored reservoir of functional bioactive compounds. As global demand shifts toward sustainable and biologically derived antioxidants, microbial metabolites offer distinct advantages, including renewable production, metabolic adaptability, and potential compatibility with living systems. Exploring citrus-associated bacteria therefore extends antioxidant research beyond traditional plant extracts and positions microbial biotechnology as a promising frontier in natural product development.

Overall, this research establishes a foundation for future investigations into citrus-associated microbial antioxidants and reinforces the importance of exploring unconventional biological sources for naturally derived bioactive compounds.

V. CONCLUSION AND FUTURE DIRECTIONS

This study demonstrated that bacterial isolates derived from the peels of Citrus microcarpa (calamansi) exhibit measurable and statistically significant antioxidant activity, as evidenced by their capacity to scavenge DPPH free radicals. Among the citrus fruits examined, only calamansi peels yielded viable bacterial isolates suitable for antioxidant evaluation, suggesting species-specific differences in peel microenvironments that influence microbial colonization. Based on phenotypic and biochemical characteristics, the isolate was presumptively identified as belonging to the genus Lactobacillus, a group recognized for its safety and functional potential.

The observed antioxidant activity indicates that citrus peel–associated bacteria may contribute to oxidative stability beyond that provided by plant-derived compounds alone. Although the scavenging capacity of the bacterial metabolites was lower than that of the standard antioxidant, the activity remained biologically relevant and supports the growing recognition of microorganisms as promising sources of natural bioactive compounds.

These findings highlight the importance of exploring nontraditional and underutilized reservoirs of antioxidants. The interaction between citrus peel substrates and microbial metabolism suggests a synergistic relationship in which bacterial biotransformation may enhance overall antioxidant potential. Furthermore, the utilization of bacteria isolated from locally available fruits aligns with sustainable research practices and promotes value-added use of agricultural by-products.

Several limitations should be acknowledged. Antioxidant activity was assessed using a single in vitro assay, and bacterial identification relied on phenotypic methods without molecular confirmation. Additionally, the evaluation of a single isolate limits broader generalization of the findings.

Future research should prioritize molecular identification of citrus-associated bacterial strains, expanded antioxidant profiling, and characterization of bioactive metabolites to clarify the mechanisms underlying microbial antioxidant production. Investigations incorporating biological models would further strengthen understanding of the functional relevance and potential applications of these metabolites.

Overall, this study provides meaningful preliminary evidence that citrus peel–associated bacteria, particularly Lactobacillus spp., may serve as viable sources of natural antioxidants. Continued exploration of plant-associated microorganisms may support the sustainable development of functional bioactive compounds and advance the expanding field of microbial biotechnology.

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