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Enhancing safety and prebiotic functionality of fresh amaranth sprout salad using low-concentration limonene pretreatment

Narumol Matan[1–3]*, **Katthayawan Khunjan**[2]

[1]School of Agricultural Technology and Food Industry, Walailak University, Nakhon Si Thammarat, Thailand; [2]Center of Excellence in Innovation of Essential Oil and Bioactive Compounds, Walailak University, Nakhon Si Thammarat, Thailand; [3]Center of Excellence in Wood and Biomaterials, Walailak University, Nakhon Si Thammarat, Thailand

***Corresponding Author:** Narumol Matan, School of Agricultural Technology and Food Industry, Walailak University, Nakhon Si Thammarat, Thailand. Emails: nnarumol@mail.wu.ac.th; nnarumol@yahoo.com

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RESEARCH ARTICLE

Abstract

Red amaranth sprouts are rich in nutrients and bioactive compounds that promote health-promoting properties. These sprouts are suitable for home growing or commercial production for use in fresh salads. In this study, red amaranth seeds were pretreated with limonene nanoemulsions at concentrations ranging from 5 to 20 µL/mL to improve germination and enhance functionality. At 5 µL/mL, limonene had no negative effect on germination, with sprouts averaging 3.3 cm in length and a 96% germination rate. Higher concentrations reduced germination significantly. Microscopy showed that limonene at 5 µL/mL was absorbed and moved to stems and leaves, forming a thin surface film and encouraging vascular growth. This led to a deep red coloration, with anthocyanin content of 57.1 ΔA/100 g and an a^* value of 1.29, higher than the control. Sprouts also increased phenolics at 53.7 mg GAE and flavonoids to 16.2 mg QE/100 g, along with strong antioxidant activity. Nutritional content supported the growth of beneficial probiotics, including *Lactobacillus plantarum*, *L. acidophilus*, and *Bifidobacterium longum*. Antibacterial activity was more effective against Gram-positive bacteria. This simple seed-soaking method provides a novel approach to producing bioactive-rich, high-quality sprouts that resist microbial contamination, support probiotic growth, and can be sustainably applied in industry using limonene from citrus peels.

Keywords: antibacterial; bioactive compounds; limonene; prebiotic; red amaranth sprouts

Introduction

Red amaranth (*Amaranthus* spp.) has been recognized as a promising crop for both vegetable consumption and health-related nutritional applications. It can be cultivated as edible sprouts within a short growth cycle, and its tolerance to various climatic conditions offers advantages (Idris *et al.*, 2020) for sustainable food production and natural pigment development (Sarker *et al.*, 2022). In addition to its adaptability, red amaranth is valued for its high nutritional content, abundance of phytochemicals, and strong antioxidant activity (Sarker and Oba, 2019). Plants rich in bioactive compounds, including anthocyanins, phenolic acids, and organic acids, have shown considerable nutraceutical and pharmaceutical potential as functional foods. These compounds are reported to reduce the risk of various chronic diseases and contribute to overall health, highlighting their suitability as promising future food sources (Zielińska-Dawidziak, 2021). Despite these benefits, microbial spoilage during germination remains a significant challenge in sprout production. Fresh salads, which are typically consumed without cooking, have the most critical requirement of ensuring adequate microbiological quality to protect consumer health and safety. This quality is influenced by cultivation practices, washing, packaging, storage, transport, distribution, and other processes that may promote or select for microbial growth. Certain microorganisms can lead to premature spoilage of the product, while others pose a direct hazard to human health (Klištincová *et al.*, 2024). In addition, inadequate postharvest management of vegetables was found to increase the risk of contamination by Enterobacteriaceae and environmental species that are present at high levels during fresh produce production (Sanna *et al.*, 2025). When prepared as ready-to-eat salads, red amaranth sprouts have a reduced shelf life and an increased risk of contamination with foodborne pathogens. Bacterial species such as *Listeria monocytogenes*, *Escherichia coli*, *Salmonella* spp., and *Staphylococcus aureus* have occasionally been detected in fresh-cut fruits and vegetables (Zhang *et al.*, 2020). Therefore, the adoption of safe handling procedures, such as application of antimicrobial treatments and proper cold storage, is essential to ensure food safety and prolong the shelf life of fresh sprouts.

Limonene has been identified as the main component of several citrus essential oils, including those derived from orange and tangerine. It is a naturally occurring monoterpene found abundantly in oranges, grapefruits, and lemons (Zancan *et al.*, 2025). This compound can be readily extracted from citrus processing by-products, making it an attractive candidate for food-related applications. Due to its antioxidant and antibacterial properties, d-limonene has been classified as a generally recognized as safe (GRAS) food flavoring agent (Adams *et al.*, 2011) and is considered a promising natural preservative in the food industry (Dos Santos *et al.*, 2024). The use of essential oils as nanoemulsions has been shown to enhance antimicrobial efficacy, enabling effective activity (Xu *et al.*, 2026) and increasing the potential for application at lower concentrations. In general, essential oils, when reduced to nanoscale or encapsulated in microcapsules, exhibited increased stability and could be applied more effectively for food preservation (Zhang *et al.*, 2024). The use of natural compounds to enhance bioactivity during seed germination has garnered growing research interest, although relatively few studies have been conducted. When seeds are soaked in an essential oil nanoemulsion solution, bioactive compounds can penetrate the seeds, and once germination occurs, the resulting plants exhibit enhanced bioactive properties. This provides benefits for consumption, such as fresh salad vegetables with antimicrobial activity. In a study by Matan and Matan (2025), sunflower seeds were germinated using lime oil containing approximately 12% limonene. The presence of limonene was detected in the resulting sprouts, where it was shown to inhibit the growth of *E. coli* and *L. monocytogenes* by disrupting cell walls, inducing nucleic acid leakage, and reducing biofilm formation. Moreover, when essential oils are applied as a seed pretreatment, pathogenic microbes associated with seeds are reduced or eliminated during the growth of sprouts and vegetables. Van der Wolf *et al.* (2008) reported that seeds pretreated with essential oils reduced fungal contamination from 70% to less than 10%, as per blotter tests. These results clearly demonstrate that the application of essential oils to seeds can enhance the safety of crop production.

Prebiotics have been increasingly recognized as functional food components due to their high content of beneficial dietary fiber. Vegetables are considered natural sources of prebiotics and have been extensively studied for their potential to support gut health (Maqsood *et al.*, 2025). The intake of prebiotics has been associated with the promotion of probiotic growth, particularly strains such as *Lactobacillus* and *Bifidobacterium*, leading to improved digestion and modulation of gut microbiota composition. Additionally, probiotic activity has been shown to influence the immune system by enhancing immune cell function and stimulating the production of anti-inflammatory cytokines (Zhou *et al.*, 2024). Once established in the gut, certain probiotic strains have been reported to synthesize antioxidant compounds (Chadathong *et al.*, 2025). This study is the first to report on limonene nanoemulsion seed pretreatment that enhances both bioactive compound accumulation and prebiotic functionality in red amaranth sprouts. In addition, this study provides benefits in terms of food safety by producing ready-to-eat sprouts that are safe for consumption, while also enhancing their function through prebiotic enrichment when cultivated using this method. Therefore, the objective of this study was to apply limonene during the germination of red amaranth seeds to enhance the bioactive compound content in the resulting sprouts. Improvements in both antioxidant levels and antioxidant activity were specifically targeted in the edible sprouts. Additionally, the feasibility of utilizing red amaranth sprouts as a potential prebiotic source and extending the shelf life of fresh sprouts was investigated to support their application in health-oriented food industries.

Materials and Methods

Red amaranth seed, chemical, and medium

Seeds of red amaranth (*Amaranthus caudatus* L.) in the family Amaranthaceae were purchased from Nam Thye Chiang Agriculture Enterprise Co., Ltd. (Bangkok, Thailand). All standards for bioactive compounds and chemical reagents were obtained from Sigma-Aldrich (Bangkok, Thailand). Nutrient broth (NB), nutrient agar (NA), plate count agar (PCA), malt extract agar (MEA), eosin methylene blue (EMB), Baird-Parker agar (BA), Xylose Lysine Deoxycholate agar (XLD), Fraser broth, PALCAM agar, MRS agar, and buffered peptone water (BPW) were supplied by Merck (Bangkok, Thailand). Compact Dry EC for *E. coli*, Compact Dry LS for *L. monocytogenes*, Compact Dry SL for *Salmonella*, and Compact Dry SA for *S. aureus* were purchased from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan).

Preparation of limonene nanoemulsion

Limonene was purchased from Sigma-Aldrich (Bangkok, Thailand). It was dissolved in deionized water to prepare concentrations of 5, 10, 15, and 20 µL/mL in a total volume of 100 mL. Tween 80 (0.1 g) was then added to the solution. The mixture was subjected to ultrasonic homogenization using a Tefic Biotech ultrasonic homogenizer (Shaanxi, China) under the following conditions: probe diameter of 6 mm, 150 W power, and 25 kHz frequency, for 20 min. The limonene nanoemulsion remained homogeneous for over 7 days, with no phase separation detected during storage at 25°C. The resulting homogeneous nanoemulsion was used immediately for the experiments.

Effect of Limonene pretreatment on the growth of red amaranth

Red amaranth seeds (200 seeds, 5 g) were soaked in 100 mL of limonene nanoemulsion at concentrations of 0 (control), 5, 10, 15, and 20 µL/mL in 200 mL glass bottles. Each bottle was closed with a screw cap and stored in the dark for 8 h at 30°C. After soaking, the seeds were covered with a layer of cheesecloth moistened with deionized water and incubated in a germination cabinet (Binder, Tuttlingen, Germany) at 35°C and 100% relative humidity for 48 h. The germinated seedlings were then transplanted into planting soil and maintained under natural light at ambient room temperature (30°C) for 2 weeks to allow sprout development. Red amaranth seeds and sprouts were subsequently sampled for the assessment of growth-related quality parameters.

Germination of red amaranth seeds

Seed germination was evaluated by counting the number of red amaranth seeds that showed root emergence (sprouts) after 48 h of incubation. The germination percentage was determined by dividing the number of sprouts by the total number of seeds initially, and the result was expressed as a percentage (%).

Disease incidence of red amaranth

Disease incidence was evaluated by the visual inspection of red amaranth sprouts after 48 h of germination. Sprouts exhibiting signs of microbial growth, small, underdeveloped stems, and stunted development were classified as diseased. Disease incidence was calculated by dividing the number of diseased sprouts by the total number of germinated sprouts, and the result was expressed as a percentage (%).

Length and weight of red amaranth sprouts

The length of red amaranth sprouts after 2 weeks of growth in soil (100 sprouts per treatment) was measured in centimeters (cm) using a Vernier Caliper (Mitutoyo Thailand Co., Ltd., Bangkok, Thailand). The weight of 100 sprouts per treatment was measured using a digital scale (Shimadzu Corporation, Kyoto, Japan) and reported in grams (g).

Appearance of red amaranth seeds during growth to sprouts

The appearance of red amaranth seeds after incubation at 1, 24, and 48 h, and sprouts after growth in soil for 1 and 2 weeks, was recorded using a digital camera.

Effect of limonene on the color, morphological, and chemical functional properties of red amaranth

Red amaranth sprouts grown in soil for 2 weeks, including both the control group and the limonene-treated group at 5 µL/mL, were selected for evaluation.

Color measurement

Color analysis was performed using a CIE colorimeter (Hunter Associates Laboratory, Inc., Reston, USA). The L^* value indicated lightness (+) or darkness (–), the a^* value ranged from green (–) to red (+), and the b^* value ranged from blue (–) to yellow (+).

Scanning electron microscopy (SEM)

Red amaranth sprouts were used for morphological examination by SEM microscopy (Zeiss/Merlin Compact, Carl Zeiss Microscopy GmbH, Munich, Germany). Sprout cells were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, followed by 1% osmium tetroxide in distilled water. The samples were then dehydrated through a graded ethanol series, coated with gold, and examined under the SEM.

Fourier transform infrared (FTIR) spectroscopy

FTIR spectroscopy measurements were conducted using an FTIR instrument (PerkinElmer, Inc., Massachusetts, USA) equipped with a zinc selenide internal reflection crystal set at a 45° angle of incidence. Spectra were acquired at a resolution of 4 cm^{-1} over 16 scans, within the range of 4000 to 500 cm^{-1}. Spectral data were analyzed using Spectrum One software.

Effect of limonene on nutritional and bioactive compounds of red amaranth

Red amaranth sprouts cultivated in soil for 2 weeks, treated with limonene at 5 μL/mL, and untreated control samples were selected for nutritional and bioactive compounds.

Proximate compositions

Proximate compositions of red amaranth sprouts, including protein (981.10), fat (984.15), total dietary fiber (985.29), and total sugars (977.20), were analyzed using methods described by the Association of Official Analytical Chemists (AOAC, 2019). Ash content was determined using AOAC method 940.26 (2023). All results were expressed as grams per 100 g of fresh weight.

Bioactive compounds

All bioactive compound analyses were performed using the method by Chaidech *et al.* (2024), with some modifications.

Total phenolic content

One gram of red amaranth sprouts was extracted with 20 mL of 80% methanol. The extract was mixed with Folin–Ciocalteau reagent, sodium carbonate, and deionized water, and incubated at 30°C for 90 min. Absorbance was measured at 760 nm using a UV–VIS spectrophotometer (Unico Co., Ltd., New Jersey, USA). Gallic acid was used as the standard, and TPC was expressed as mg gallic acid equivalents (GAE) per 100 g.

Total anthocyanins

Total anthocyanins in red amaranth sprouts were extracted using methanol at 4°C for 24 h, centrifuged, and filtered. The content was measured using the pH differential method at absorbance 530, 620, and 650 nm, and results were expressed as ΔA/100 g.

Total carotenoid content

Red amaranth sprouts were extracted using 10 mL acetone, homogenized (30 s, 25°C), and centrifuged at 4000 × g for 10 min. Absorbance of the supernatant was measured at 470, 645, and 663 nm (Thermo Fisher Scientific, USA), and carotenoid content was calculated using the equation from Chaidech *et al.* (2024).

Total flavonoid content

Red amaranth sprouts were extracted using 80% methanol and reacted with 5% sodium nitrite, 10% aluminum chloride, and 1 M sodium hydroxide. Absorbance was measured at 510 nm (Thermo Fisher Scientific, USA), and results were expressed as mg quercetin equivalents per 100 g (mg QE/100 g).

Antioxidant activity

Red amaranth sprouts were extracted in 10 mL of methanol. For the ABTS assay, the stock solution was prepared by mixing potassium persulfate with ABTS and incubating the mixture at 30°C for 12 h in the dark. For the DPPH assay, 2 mL of 0.1 mM DPPH solution was mixed with 2 mL of extract and incubated in the dark for 30 min. Absorbance was measured at 517 nm, and results were expressed as mg Trolox equivalent antioxidant capacity per 100 g (mg TEAC/100 g).

Effect of limonene on prebiotic potential assay

The prebiotic potential of red amaranth sprouts was evaluated following the method by Chaidech *et al.* (2024), with modifications. *Lactobacillus plantarum* TISTR 2071, *Lactobacillus acidophilus TISTR 236,* and *Bifidobacterium longum* TISTR 2129 were obtained from the TISTR Culture Collection, Thailand, and cultured in MRS broth under anaerobic conditions at 37°C for 24 h. Gut pathogens (*E. coli*, *Salmonella* Enteritidis, *S. aureus*, and *L. monocytogenes*) were provided by the Food Microbiology Laboratory, Walailak University, and cultured in nutrient broth at 35 ± 2°C for 24 h. All bacterial suspensions were adjusted to 3.0 $\log_{10}$ CFU/mL before use, and viability using NA was confirmed immediately after inoculation into the experimental media.

Red amaranth sprouts, either treated with limonene or untreated (control), were added (10 g) to 100 mL of NB. Each bacterial strain (1 mL) was inoculated into the broth, and the pH was adjusted to 6.8. All flasks were incubated at 37°C. Samples were collected at 1, 12, 24, 36, and 48 h. Ten-milliliter aliquots were serially diluted in sterile peptone water, and 100 μL of each dilution was plated onto selective media: MRS agar for *L. plantarum*, *L. acidophilus* and *B. longum*; EMB agar for *E. coli*; XLD agar for *Salmonella* Enteritidis; BA agar for *S. aureus*, and PALCAM agar for *L. monocytogenes*. Plates were incubated at 37°C for 48 h, and bacterial counts were expressed as $\log_{10}$ CFU/mL. Each treatment was performed in triplicate, and the experiment was repeated three times.

Effect of limonene on microbiological quality during cold storage of sprouts

Red amaranth sprouts from the control (without limonene) and the treated group (grown with limonene) were harvested, cleaned, dried, and packed in perforated polypropylene bags (12 × 18 cm, Ø 2 mm pores). The bags were stored at 4 ± 1°C for 5 days. Microbiological analyses were conducted on Days 1, 3, and 5. A 25 g sample of sprouts was homogenized in 225 mL of peptone water and serially diluted with sterile saline solution. Total viable counts (TVC) and psychrophilic bacterial counts were determined on PCA, incubated at 35 ± 2°C for 24 h and at 7°C for 10 days, respectively. Total yeast and mold (TYM) counts were assessed on MEA, incubated at 25°C for 3 days.

For pathogenic bacteria, 1 mL of each dilution was plated on Compact Dry EC (for *E. coli*), SL (for *Salmonella*), SA (for *S. aureus*), LS (for *L. monocytogenes*), and BC (for *B. cereus*). Samples for *Salmonella* and *L. monocytogenes* were enriched in BPW and Fraser broth, respectively, before plating. All Compact Dry plates were incubated at 35 ± 2°C for 48 h. Analyses were carried out in a biological safety cabinet, and results were expressed as $\log_{10}$ CFU/g.

Statistical analysis

All data were analyzed in triplicate and presented as mean ± SD (n = 3). One-way ANOVA followed by Duncan's test ($P < 0.05$) was performed to determine the germination rate, disease incidence, sprout length, weight, and prebiotic activity. Independent t-tests ($P < 0.05$) were applied for nutritional value, bioactive compounds, and color. All analyses were performed using StatSoft software (Oklahoma, USA).

Results

Germination and sprout quality of red amaranth seeds pretreated with limonene

The germination performance of red amaranth seeds treated by dipping in limonene solutions at concentrations of 5–20 μL/mL, along with an untreated control, is shown in Figure 1. As illustrated in Figure 1A, germination following treatment with 5 μL/mL limonene was not significantly different from the control, with germination rates reaching 95–96% after 48 h of incubation at 100% relative humidity. However, when the limonene concentration was increased to 10 μL/mL and above 15 μL/mL, germination rates were markedly reduced to 22% and 1%, respectively. At concentrations exceeding 15 μL/mL, germination was effectively inhibited. After 2 weeks of sprout development, disease incidence in the control group reached approximately 20%, whereas sprouts derived from treated seeds (5 μL/mL limonene) exhibited no visible signs of fungal infection or morphological abnormalities. Measurement of sprout length after 2 weeks revealed no significant difference between the control and the 5 μL/mL limonene treatment, with mean lengths ranging from 3.3 to 3.5 cm. In contrast, seeds treated with 10 μL/mL limonene not only showed reduced germination but also yielded shorter sprouts with a fresh weight of only 2.11 g, substantially lower than that of the control and 5 μL/mL groups, which had weights between 8.16 and 8.18 g. At concentrations of 5 μL/mL and above, germination was nearly completely inhibited, and the resulting sprouts were extremely short and underdeveloped (Figure 1B).

Morphology and functional group analysis of red amaranth sprouts treated with limonene

The morphology of red amaranth sprouts is shown in Figure 3. Sprouts from the control group, harvested 2 weeks after planting in soil (Figures 3A and 3E), exhibited typical external characteristics in terms of root, stem, and leaf development. SEM revealed that the leaf surfaces of control sprouts appeared normal, displaying well-defined plant cell grids and evenly distributed stomata for transpiration (Figure 3B). In contrast, leaves from sprouts treated with 5 μL/mL limonene exhibited a smoother surface, with a thin film partially covering the stomata (Figure 3F). When stem morphology was compared, the control sprouts showed segmented stems with distinct internal partitions and uniform cellular structure (Figure 3C). However, the stems of limonene-treated sprouts appeared swollen, with a thicker structure and shallower cellular grooves (Figure 3G). Root morphology in the control group was characterized by smooth, tubular surfaces (Figure 3D), while the roots of treated sprouts appeared wrinkled and exhibited a rougher texture (Figure 3H).

A broad absorption peak of functional groups was observed on the surface of red amaranth sprouts in the 3700–3000 cm^{-1} region, particularly at 3348 cm^{-1}, corresponding to the stretching vibration of hydroxyl groups and the presence of moisture content in the sprouts (Kamalpour *et al.*, 2025). Slight differences in absorption between 1121 cm^{-1} and 1240 cm^{-1} were attributed to C–O ether groups and C–O–C vibrations, indicating the presence of glycosidic bonds between monosaccharide units (Kamalpour *et al.*, 2025). Minor spectral shifts were also observed in treated samples within the regions of 2917–2850 cm^{-1} and 1414–1635 cm^{-1}. These shifts were associated with aromatic

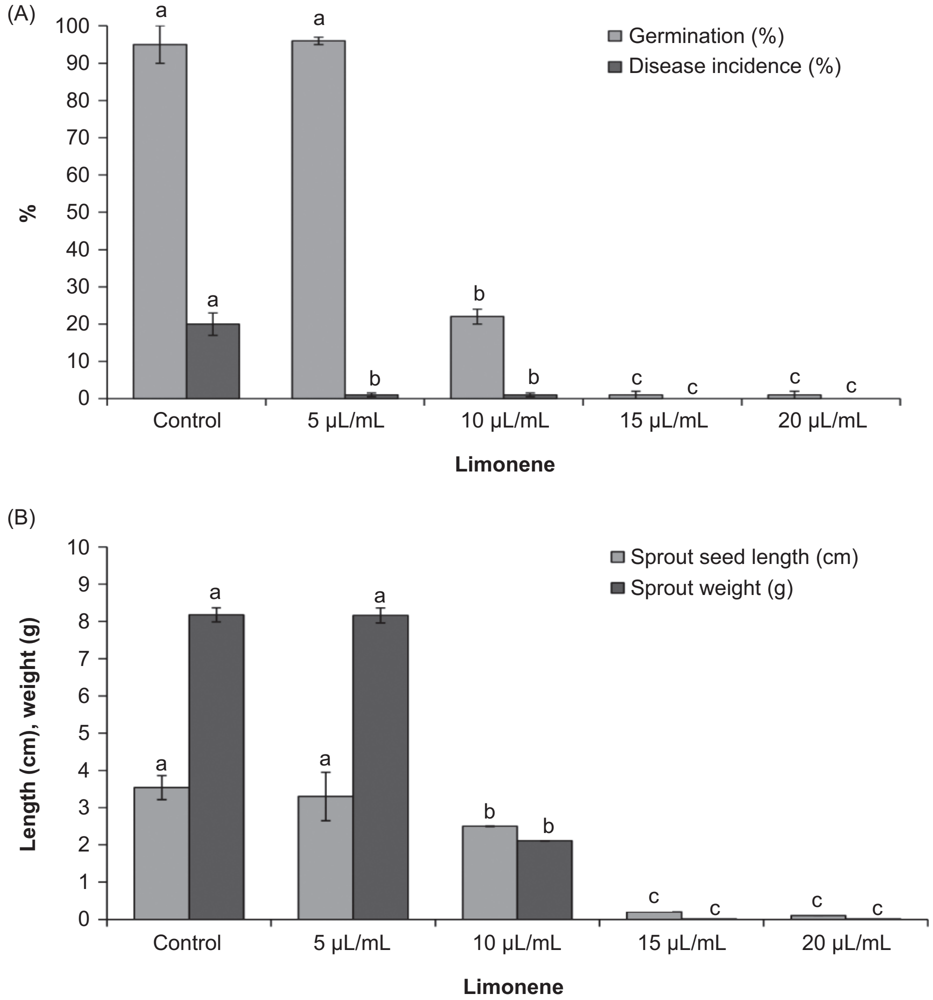

Figure 1. Effect of limonene at 0 (control), 5, 10, 15, and 20 µL/mL on germination (%) of red amaranth seeds at 48 h (A), and disease incidence (%) of red amaranth sprouts at 2 weeks of growth (B) Data are presented as mean ± standard deviation. $^{a–c}$Different superscript letters indicate significant differences among treatments ($P < 0.05$).

structures containing oxygenated functional groups and carbonyl compounds derived from limonene. Vibrational signals from C–H and CH=CH bonds further confirmed the incorporation of limonene-associated compounds (Feng *et al.*, 2025). The limonene's characteristic absorption peaks at 2917 cm^{-1} and 2850 cm^{-1} indicated the C–H bond. Some peaks of limonene also appeared at 1645 cm^{-1} (-CH=CH) (Figure 4).

Color, nutritional composition, and bioactive compounds of red amaranth sprouts treated with limonene

Sprouts treated with 5 μL/mL limonene exhibited a brighter red appearance compared to the control group. Specifically, higher values of lightness (L^* = 3.18) and redness (a^* = 1.29) were recorded, compared to the control (L^* = 2.92, a^* = 1.00), while yellowness was slightly reduced (Table 1). In terms of nutritional composition, sprouts germinated in the presence of 5 μL/mL limonene contained slightly higher levels of protein (1.88 g/100 g), carbohydrate (2.23 g/100 g), and ash (1.50 g/100 g) than the control group (1.73 g/100, 0.99 g/100, and 1.44 g/100 g, respectively). Furthermore, high dietary fiber levels, ranging from 14.9 to 16.9 g/100 g, were observed in both control and limonene-treated red amaranth sprouts (Table 1).

Analysis of bioactive compounds revealed that limonene-treated sprouts exhibited elevated levels of total phenolics (53.7 mg GAE/100 g), total anthocyanins (57.1 ΔA/100 g), and total flavonoids (16.2 mg QE/100 g), along with increased antioxidant activity, as determined by ABTS (11.73 mg TEAC/100 g) and DPPH (20.3 mg TEAC/100 g) assays, relative to the

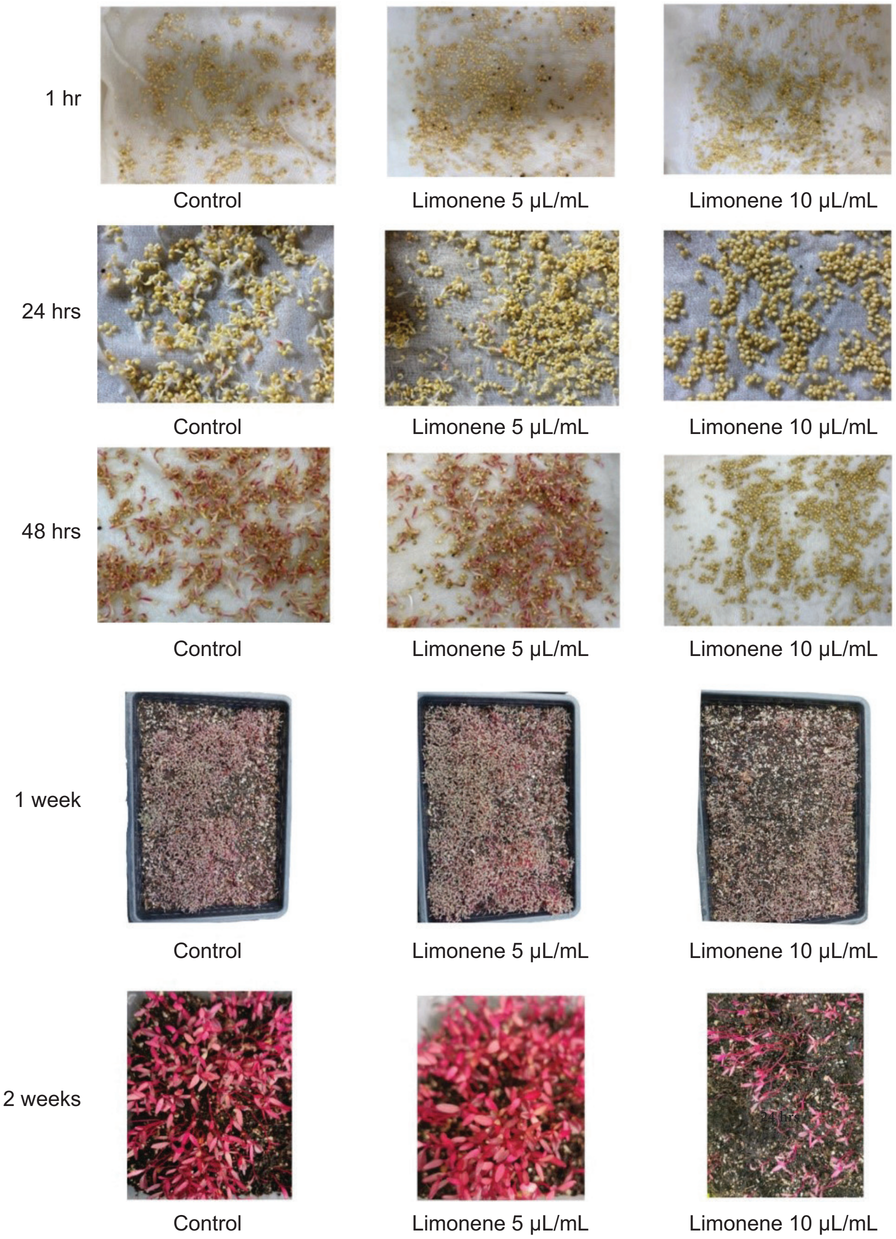

Figure 2. Limonene at 5 and 10 μL/mL applied to activated seed germination and reduced microbial risk during early development, as observed after 1, 24, and 48 h, and after 1 and 2 weeks of growth.

control group. However, the carotenoid content in the treated sprouts (28.0 mg/100 g) was lower than that of the control (34.8 mg/100 g) (Table 1).

Prebiotic potential of limonene-treated red amaranth sprouts

The prebiotic potential of red amaranth sprouts treated with limonene was evaluated by monitoring the growth of probiotic and pathogenic bacteria in NB supplemented with the treated sprouts (Figure 5).

In the control group, all tested microorganisms, including probiotic and pathogenic strains, grew from an initial level of 3 $\log_{10}$ CFU/mL to approximately 5 $\log_{10}$ CFU/mL within 48 h. On the other hand, in the group containing limonene-treated sprouts, the probiotic strains *L. plantarum* and *B. longum* exhibited moderate growth, reaching 4.1 and 3.6 $\log_{10}$ CFU/mL, respectively, after 48 h. *L. acidophilus* showed a slight increase up to 36 h, followed by stabilization at 2.9 $\log_{10}$ CFU/mL at 48 h.

The pathogenic gram-negative bacterium *E. coli* and *Salmonella* Enteritidis showed a gradual decline from

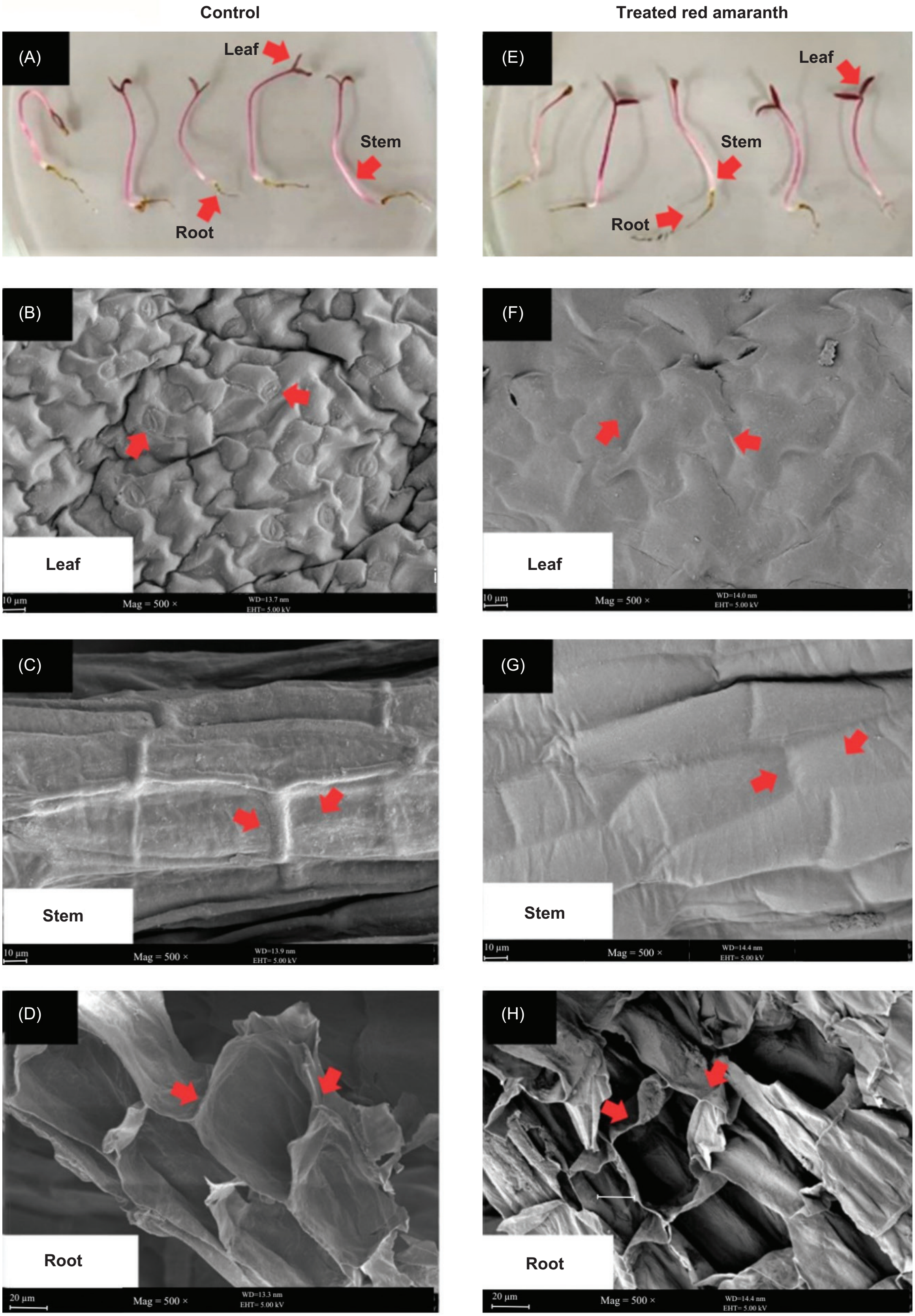

Figure 3. Appearance of red amaranth sprouts after 2 weeks of growth without limonene (A) and morphology of leaf (B), stem (C), and root (D); and with limonene (E) and morphology of leaf (F), stem (G), and root (H), showing clear cellular differences that suggest limonene enhances self-protection against microbial infection after harvest.

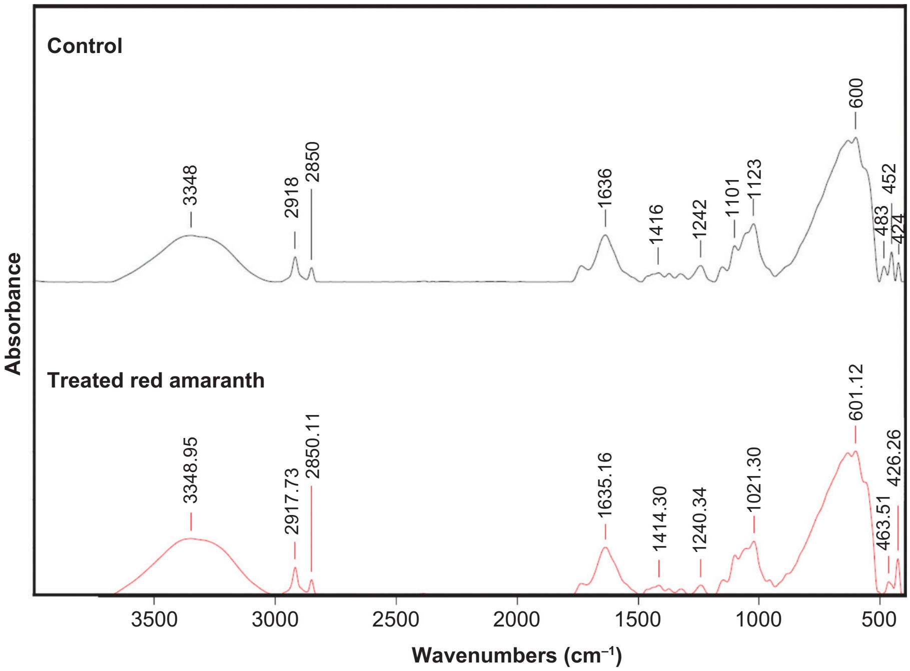

Figure 4. FTIR spectra of control red amaranth sprouts, and red amaranth sprouts treated with limonene.

Table 1. Nutritional composition, bioactive compound content, and color of red amaranth with and without limonene treatment.

Measurement	Control	Treated red amaranth
Color		
*L**	2.92 ± 0.08^{b}	3.18 ± 0.31^{a}
*a**	1.00 ± 0.16^{b}	1.29 ± 0.15^{a}
*b**	0.58 ± 0.01^{a}	0.36 ± 0.02^{b}
The nutrients (g/100 g)		
Protein	1.73 ± 0.02^{b}	1.88 ± 0.20^{a}
Fat	0.20 ± 0.00^{a}	0.21 ± 0.00^{a}
Ash	1.44 ± 0.00^{b}	1.50 ± 0.00^{a}
Fiber	16.9 ± 0.33^{a}	14.9 ± 0.37^{b}
Carbohydrate	0.99 ± 0.01^{a}	2.23 ± 0.01^{a}
Total sugar	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Bioactive compounds		
Total phenolic content (mg GAE/100 g)	45.2 ± 0.80^{b}	53.7 ± 0.55^{a}
Total anthocyanins (ΔA/100 g)	47.9 ± 5.65^{b}	57.1 ± 3.62^{a}
Total carotenoid	34.8 ± 2.28^{a}	28.0 ± 3.92^{b}
Total flavonoid (mg QE/100 g)	14.7 ± 0.40^{b}	16.2 ± 0.61^{a}
ABTS (mg TEAC/100 g)	9.55 ± 0.13^{b}	11.73 ± 0.05^{a}
DPPH (mg TEAC/100 g)	14.9 ± 0.85^{b}	20.3 ± 0.53^{a}

Data are presented as mean ± standard deviation. $^{a\text{–}b}$Different letters indicate significant differences between treatments ($P < 0.05$).

12 to 48 h, with final counts of 1.9 and 2.2 $\log_{10}$ CFU/mL, respectively. Gram-positive pathogens, including *S. aureus* and *L. monocytogenes*, exhibited a more pronounced reduction, with counts decreasing to 1.1–0.9 $\log_{10}$ CFU/mL at 48 h. These results indicated that red amaranth sprouts treated with 5 μL/mL limonene selectively supported the growth of probiotic bacteria while inhibiting pathogenic strains, demonstrating potential prebiotic properties.

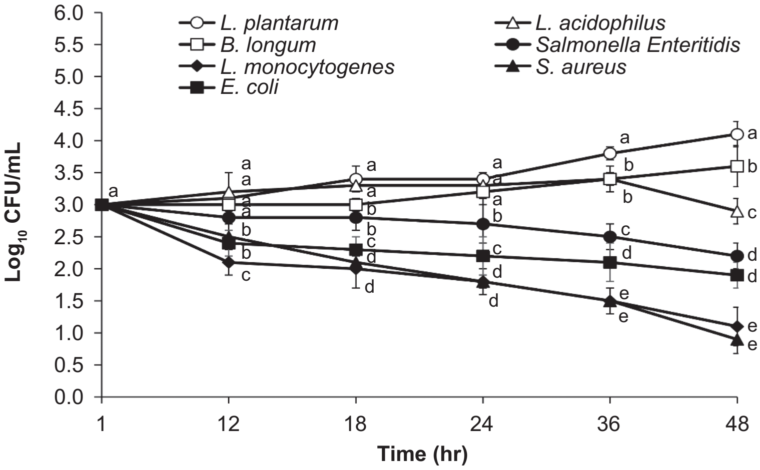

Figure 5. Growth of probiotic bacteria (***Lactobacillus plantarum***, ***Lactobacillus acidophilus***, ***Bifidobacterium longum***) and foodborne pathogens (***Escherichia coli, Salmonella*** **Enteritidis**, ***Listeria monocytogenes***, ***Staphylococcus aureus***) **in nutrient broth containing red amaranth sprouts treated with limonene. Data are presented as mean ± standard deviation.** $^{a\text{–}e}$**Different superscript letters indicate significant differences among treatments ($P < 0.05$).**

Table 2. Microbial counts of red amaranth with and without limonene treatment during storage at 4°C for 5 days.

Microbial (Log_{10} CFU/g)	1 day		3 days		5 days	
	Control	Treatment	Control	Treatment	Control	Treatment
Total visible bacteria	2.1	0.8	4.3	1.2	>6.0	2.2
Psychrophilic bacteria	3.5	0.6	5.5	1.5	>6.0	3.3
Total yeast and mold	2.5	ND	3.2	ND	>6.0	1.8
Escherichia coli	1.1	ND	3.2	ND	5.5	ND
Salmonella spp.	ND	ND	ND	ND	ND	ND
Staphylococcus aureus	2.3	ND	4.9	ND	>6.0	ND
Listeria monocytogenes	ND	ND	ND	ND	ND	ND

ND: not detected.

Microbiological quality of limonene-treated red amaranth sprouts during cold storage

Changes in the microbial quality of red amaranth sprouts after harvest and during cold storage are shown in Table 2. Initial microbial counts in freshly harvested treated sprouts complied with standard limits for ready-to-eat fresh vegetables (Mclauchlin *et al.*, 2022). After 5 days of storage at 4°C, total viable counts measured 2.2 $\log_{10}$ CFU/g, psychrotrophic bacteria reached 3.3 $\log_{10}$ CFU/g, and yeast and mold counts remained at 1.8 $\log_{10}$ CFU/g. All values fell below the commonly accepted safety threshold of 5 $\log_{10}$ CFU/g for fresh produce. Moreover, foodborne pathogens, including *E. coli*, *Salmonella* spp., *S. aureus*, and *L. monocytogenes*, remained undetected in limonene-treated samples throughout the 5-day storage period. By comparison, the control group exhibited contamination by *E. coli* (1.1 $\log_{10}$ CFU/g) and *S. aureus* (2.3 $\log_{10}$ CFU/g), failing to meet microbiological safety criteria and resulting in a shelf life of less than 1 day for fresh consumption. These results indicate that cultivation of red amaranth sprouts with 5 μL/mL limonene serves as an effective postharvest approach to reduce microbial load, extend shelf life, and ensure microbiological safety.

Discussion

A low concentration of limonene at 5 μL/mL was found to be effective for cultivating red amaranth sprouts. Seeds soaked in limonene solution for 8 h absorbed the compound, as the high-water content of the nanoemulsion did not negatively affect germination. The combination of water and limonene penetrated the seed coat, resulting in a germination rate comparable to that of the control group. Consequently, the sprouted seeds showed no significant differences in appearance, height, or weight compared to the control. Moreover, the low-concentration limonene solution influenced plant morphology. The germinated roots absorbed water and developed into stems and leaves. However, the internal cellular structures differed in several aspects when compared to the control group. Limonene treatment appeared to promote the formation of a thin surface film and smoother leaf texture. Swelling of the vascular tissues in limonene-treated plants was also observed, enhancing nutrient transport to the leaves.

Seeds are generally regarded as ripened ovules formed through successful fertilization. A mature seed contains a thick, firm coat and an embryo, which is the young plant enclosed within the protective layer. The application of appropriate pretreatment methods improved seed germination and increased yield (Patade *et al.*, 2025). Soaking seeds in a low concentration of limonene enabled essential compounds to gradually pass through the seed coat and accumulate during maturation. In contrast, higher concentrations of limonene inhibited germination, possibly due to penetration into sensitive internal tissues (Assogbadjo *et al.*, 2011), potentially resulting in embryo damage. At an optimal concentration, limonene treatment triggered morphological changes such as increased thickness, reduced moisture loss, and the formation of a thin film on the leaf surface. These changes promoted a greater accumulation of bioactive compounds. Therefore, limonene concentration was identified as a critical factor influencing both developmental and biochemical processes during germination.

An increased accumulation of bioactive compounds in red amaranth sprouts was associated with limonene treatment. Seeds soaked in a low concentration of limonene developed into sprouts with thick stems and leaves that appeared to be coated with a thin film. This phenomenon was related to the enhanced production of bioactive compounds, as significantly higher levels of phenolics, anthocyanins, and flavonoids were detected compared with the control. These findings were consistent with the reports by Mir *et al.* (2021) and Niroula *et al.* (2019), who demonstrated that during sprout development, macronutrients were hydrolyzed into amino acids, simple sugars, and other nutritional components, while antinutritional compounds decreased and functional metabolites such as polyphenols and vitamin C increased. Furthermore, elevated levels of phenolics, anthocyanins, flavonoids, and antioxidant capacity were observed when sprouts were exposed to natural stimulants such as essential oils (Matan and Matan, 2025), which agreed with the present results obtained using limonene as a seed stimulant for bioactive compound production. Red amaranth sprouts were identified as a source of betalains, a natural antioxidant pigment composed of cyclic amine and phenolic structures that function as electron donors (Novais *et al.*, 2022). Among these, betacyanins, which contain betalamic acid as a key reactive group, have been shown to provide protective effects against oxidative stress-related disorders. In the present study, limonene, an aromatic compound, was found to enhance antioxidant activity and intensify red pigmentation, suggesting a synergistic effect on betalain biosynthesis. This observation was consistent with the findings of Schliemann *et al.* (2001), who reported that phenolic oxygen in betacyanins readily lost electrons to form stabilized radicals via aromatic ring delocalization, thereby enhancing the TEAC capacity of betalains. The results of this study aligned with those of Zhu *et al.* (2025), who reported that the application of nanotechnology, such as nanofertilizers and seed nanopriming, can enhance the absorption of compounds by plants and improve both cultivation and postharvest quality of sprouts. Sprouts, valued for their high phytochemical content and rapid growth, are increasingly recognized as functional and nutraceutical foods. The present research further indicates that these approaches have the potential to improve nutrient use efficiency, promote plant growth, and extend product shelf life. However, the possible impacts of nanotechnology on plant physiology, soil microbiota, and food safety warrant careful consideration.

Furthermore, anthocyanin content significantly increased following limonene treatment. Anthocyanins contribute to red pigmentation, enhance visual appeal, and have been extensively studied for their antioxidant and antimicrobial activities. Although anthocyanin color intensity varies with pH, sprouts treated with limonene exhibited a noticeably deeper red hue compared to untreated controls. Similar findings were reported by Chaidech *et al.* (2024), who observed intensified red pigmentation in rambutan peel cells treated with cardamom oil. Likewise, Phothisuwan *et al.* (2021) demonstrated that limonene vapor from orange oil preserved the red coloration of salacca fruit during storage and inhibited mold growth in a closed system. These studies further support the role of limonene in enhancing pigment stability and microbial resistance. The intensified red coloration observed in this study highlights the potential of red amaranth sprouts as a natural source of food pigments. Natural colorants such as anthocyanins (E163) have been approved by the Codex Alimentarius Commission (FAO/WHO) for use in a wide range of food and beverage products due to their established safety and health benefits (Novais *et al.*, 2022).

The nutritional composition of red amaranth sprouts was found to be consistent with the findings of Jahan *et al.* (2022), particularly regarding fiber and protein content. In the present study, high fiber and protein contents from a plant-based source were also confirmed. Fiber was detected in substantial amounts in the powdered whole plant, second to carbohydrates. This composition was consistent with the observed growth of probiotic bacteria. Additionally, dietary fiber, polysaccharides, phenolic compounds, flavonoids, and antioxidants were detected in red amaranth sprouts germinated with limonene. In this study, the presence of limonene in red amaranth sprouts was not found to inhibit the growth of the probiotic strains tested. Instead, these constituents were shown to support the survival and growth of three probiotic strains in vitro. These findings indicated a strong potential for the consumption of red amaranth sprouts containing limonene to provide additional prebiotic functionality. However, further investigations under simulated gut fermentation conditions were recommended before practical application. According to Gaur and Gänzle (2023), members of the *Lactobacillaceae* family produced enzymes capable of transforming bioactive dietary phenolic compounds present in plants and other food matrices, owing to their tolerance to phenolics during food fermentation. In the present study, phenolic compounds tolerated by probiotics were also associated with the inhibition of common pathogenic bacteria found in vegetables. Moreover, Mahmmodi *et al.* (2021) reported that the viability of *L. acidophilus* under stress conditions with essential oils was maintained, resulting in only slight changes in pH. In this study, the presence of limonene in the sprouts allowed the probiotic to grow. Antimicrobial potential was demonstrated by sprouts containing limonene, as a significant reduction in pathogenic bacterial populations was observed.

Furthermore, these enhancements were attributed to the functional properties of limonene, which has been recognized for its antimicrobial activity (Dos Santos *et al.*, 2024). The antimicrobial properties of plant fiber in leafy amaranths, used as a vegetable for health promotion, have been reported by Sarker and Oba (2019). In this study, the growth of foodborne pathogens was suppressed by the application of limonene pretreatment at a low concentration (5 μL/mL) before germination. This was consistent with previous studies in which nanoemulsion was employed to enhance the antimicrobial activity of limonene (Zhang *et al.*, 2014). The conversion of limonene into nanoemulsion form avoided the use of higher concentrations typically needed to inhibit foodborne pathogens. In earlier work, limonene was encapsulated within other substances to improve its stability and efficacy (Zahi *et al.*, 2015). In the present study, once the nanoemulsion of limonene was absorbed by the sprouts, the internal tissues acted as natural carriers, effectively encapsulating the active compound and enhancing its antimicrobial action against foodborne pathogens. These results are also consistent with those reported by Dos Santos *et al.* (2024), who demonstrated that the antibacterial activity of limonene is greater against Gram-positive bacteria than against Gram-negative bacteria. The structural characteristics of Gram-negative bacteria, particularly the presence of an outer lipopolysaccharide layer that acts as a permeability barrier and enhances resistance to antimicrobial agents, have been attributed to this discrepancy (Sperandeo *et al.*, 2019). In the present study, higher numbers of *E. coli* and *Salmonella* Enteritidis were detected compared to the Gram-positive bacteria *S. aureus* and *L. monocytogenes*, further supporting the selective antimicrobial effect of limonene.

Fresh red amaranth sprouts containing limonene were found to retain their quality for at least 5 days or longer under cold storage, while remaining within the accepted safety threshold of 5 $\log_{10}$ CFU/g for fresh produce. No foodborne pathogens were detected during this period. These sprouts could therefore be utilized in the production of safe, fresh salad vegetables. Typically, sprouts derived from seed germination (Matan and Matan, 2025) and fresh vegetables that have not undergone minimal processing have been reported to be highly susceptible to bacterial contamination (Zhang *et al.*, 2020). This approach is promising for the development of functional, health-promoting vegetables with prebiotic properties and improved microbial safety. It can be applied at an industrial scale to produce functional foods, with limonene-treated sprouts offering potential for diverse formulations. Moreover, limonene can be derived from citrus by-products, underscoring its relevance to sustainable agriculture and the circular economy. Incorporation of such sprouts into salads and other vegetable-based products may further support gut health and the development of microbiome-targeted foods. Further studies should investigate the use of sprouts containing these bioactive compounds to promote the growth of gut probiotics to enhance probiotic performance in the gastrointestinal tract. Additionally, sensory evaluations should be conducted to optimize flavor and aroma for improved consumer acceptance. Future research could also explore the application of these sprouts in a variety of products and assess the stability and degradation of bioactive compounds during storage under different conditions.

Conclusions

Red amaranth sprouts treated with 5 μL/mL limonene germinated effectively without compromising quality.

The nanoemulsion was absorbed during an 8-hour seed-soaking period, allowing gradual internalization without causing embryo damage. Morphological changes were observed, including smoother leaf texture, swelling of vascular tissues, and the formation of a thin surface film. Limonene treatment significantly enhanced the accumulation of bioactive compounds such as phenolics, flavonoids, and anthocyanins. These compounds contributed to deeper red pigmentation and improved antioxidant capacity. The sprouts also supported the growth of probiotic bacteria and exhibited antimicrobial activity, particularly against Gram-positive pathogens. These functional properties were attributed to the antimicrobial effects of limonene along with the sprouts' prebiotic fiber and phytochemical content. The internal tissues of the sprouts were found to adsorb limonene, which maintained the antimicrobial activity for at least 5 days during cold storage. This method was considered suitable for developing safe and functional vegetables for the food industry and was regarded as a simple approach for practical implementation.

Data Availability Statement

Data available on request due to privacy or ethical restrictions.

Ethical Approval

This study did not involve human or animal subjects. All microbiological experiments were conducted in accordance with the Institutional Biosafety Committee of Walailak University (Thailand) under protocol number WU-IBC-67-034. This study was also reviewed and approved by the Ethics Committee for Human Research, Walailak University, based on the Declaration of Helsinki (Approval number WUEC-24-144-01).

Authors Contribution

Narumol Matan: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Resources, Software, Writing - original draft, Writing – review & editing, Visualization, Supervision, Project administration, Funding Acquisition. Katthayawan Khunjan: Data curation, Formal analysis, Investigation Methodology, Validation, Resources, Writing – review & editing.

Conflicts of Interest

The authors have no conflicts of interest.

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