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Antimicrobial Activity of Nanoencapsulated Essential Oils of *Tasmannia lanceolata*, *Backhousia citriodora* and *Syzygium anisatum* against Weak-Acid Resistant *Zygosaccharomyces bailii* in Clear Apple Juice

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Abstract: The anti-yeast activity of oil-in-water encapsulated nanoemulsion containing individual or a combination of the three essential oils of Tasmanian pepper leaf (*Tasmannia lanceolata*), lemon myrtle (*Backhousia citriodora*), and anise myrtle (*Syzygium anisatum*) against weak-acid resistant *Zygosaccharomyces bailii* in clear apple juice was investigated. The effectiveness of the shelf-life extension of *Z. bailii*-spiked (1×10^3 CFU/mL) clear apple juice was evaluated and compared between natural (essential oils) and synthetic (sodium benzoate) antimicrobial agents. Essential oils showed an immediate reduction in the *Z. bailii* cell population at day-0 and exerted a fungicidal activity at day-4 of storage, with no further noticeable growth at the end of the experiment (day-28). At lower concentrations, Tasmanian pepper leaf oil of 0.0025% had >6 log CFU/mL at day-12 of storage. For lemon myrtle essential oils, the yeast population reached >6 log CFU/mL at day-24 and day-20 for concentrations of 0.02% and 0.01%, respectively. The fungicidal activity of Tasmanian pepper leaf oil reduced from 0.005% to 0.0025% *v*/*v* when mixed at a ratio of 1:1 with anise myrtle oil. The results of the present study suggest that these three native Australian herbs have the potential to be used in the beverage industry by controlling *Zygosaccharomyces bailii* in clear apple juice products.

Keywords: antimicrobial activity; essential oils; nanoemulsion; *Tasmannia lanceolata*; *Backhousia citriodora*; *Syzygium anisatum*; *Zygosaccharomyces bailii*; apple juice

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1. Introduction

Controlling the growth of food spoilage causing microorganisms is mainly achieved by the use of synthetic preservatives; however, they have been linked to negative health effects such as carcinogenicity, acute toxicity, teratogenicity, attention deficit hyperactivity disorder, asthma, and allergy, and to environmental pollution due to their slow degradation [1–5]. Even though synthetic preservatives are consumed regularly on a daily basis as effective food preservatives, their usage is limited by the U.S. Food and Drug Administration (FDA) due to safety issues [6]. The use of natural antimicrobial agents in food preservation is becoming an area of interest among researchers and food industries due to the great awareness and concern of consumers regarding what they perceive as harmful synthetic antimicrobial and preservative agents in food [7–12]. In addition, the emergence of weak-acid resistant microorganisms that are capable of growing in and spoiling beverage products even at the maximum permitted levels of synthetic preservatives; this turned the focus toward investigating new potential natural alternatives to synthetic preservatives to extend the shelf-life of beverage products [4,13–16]. The growth of the majority of spoilage, causing microorganisms in fruit juice products, is well controlled through the use

of synthetic preservatives (such as potassium sorbate and sodium benzoate) and through lowering the juice pH to 2 to 4 (by the addition of acidulants) [15,17–19]. A low pH of <4.5 prevents the spore germination and growth of the great majority of bacteria; however, there are some reported yeasts belonging to the *Zygosaccharomyces* genus capable of growing in a low pH and resisting the action of synthetic preservatives [16,20–22]. *Z. bailii* is a food and beverage spoilage yeast and is described as being notorious for its extreme resistance to weak-acid preservatives [22–24]. This yeast can grow above the legally permitted levels of preservatives, thus becoming a dangerous yeast in the food and beverage industries [22–24]. Sodium benzoate is a monocarboxylate weak-acid preservative (E211) commonly used in a low-pH (<4.5) beverage system; however, it has been found to react with ascorbic acid in drinks producing a carcinogen benzene, which occurs during storage due to the decarboxylation of benzoate [19,22,25]. The inhibition action of sodium benzoate against the growth of microorganisms is active only in a low-pH environment. Sodium benzoate is soluble in water and converted into benzoic acid once dissolved in water, which then becomes undissociated to its protonated acid form (–COOH) at a low pH; however, once it has entered into the microbial cells, reaching the neutral pH of the cellular cytosol (intracellular fluid), it then dissociates, producing an anion ($–COO^-$) and releasing a proton (H^+) inside the cell [26]. The accumulation of anions ($–COO^-$) inside a cell creates a turgor pressure, which causes oxidation stress by affecting the free radical production inside the cell through the mitochondrial respiratory chain, while the accumulation of protons (H^+) acidifies the cytosol, which delays or prevents essential metabolic functions of the cell [27–29]. *Z. bailii* cells are able to resist the action of sodium benzoate by pumping out anions ($–COO^-$) and protons (H^+), preventing their accumulation inside the cells. *Z. bailii* cells consume energy (ATP) to pump out anions ($–COO^-$) through membrane protein Pdr12 and to pump out protons (H^+) via membrane protein Pma1. Non-resistant yeasts lack the membrane protein Pdr12 and fail to eliminate or prevent the accumulation of anions ($–COO^-$) within cells, which eventually lead to their death. *Z. bailli* yeasts are considered to be preservative-resistant and ethanol-tolerant (spoiling of alcoholic beverages) species [17,21,30–32]. They are also considered to be a fermentative spoilage organism of fruits, fruit juices, and syrup concentrates (osmotolerant) [17,21,30–32], and they are known for spoiling acidic food sauces (acid-tolerant) and carbonated soft drinks [17,21,30–32]. *Z. bailli* cells have been reported to resist the action of benzoic acid at 1000 ppm [33,34]. With the current status of limiting the use of synthetic preservatives in food products, it has become a challenge for the beverage industry to increase the dosage of weak-acid preservatives to control the growth of resistant yeasts. This provides an opportunity for plant essential oils to be used as a natural source of antimicrobial agents in beverage products. Currently, plant essential oils are gaining much attention for their use as an alternative solution to control the growth of weak-acid resistant yeast and, at the same time, satisfying modern consumers by the application of natural antimicrobial agents in food products instead of synthetic preservatives [35–37]. The hydrophobicity of essential oils is their major limitation, which lowers their antimicrobial action and prevents their application into liquid-food or beverage products [38]. To overcome this hurdle, nanoemulsion technology could be utilized to reduce the size of essential oil particles to nanoscale and, with the aid of surfactant, to allow essential oils to be effectively dissolved in liquid while time maintaining their antimicrobial activity in liquid.

Tasmanian pepper (*Tasmannia lanceolata*) leaf, lemon myrtle (*Backhousia citriodora*), and anise myrtle (*Syzygium anisatum*) are three native Australian herbs reported to have antibacterial and antifungal activities [39–44]. Tasmanian pepper leaf belongs to the family Winteraceae, found in forested regions in Tasmania, Victoria, and New South Wales in Australia and it is characterized by a high content of sesquiterpene and monoterpene essential oils [45–48]. The essential oil of Tasmanian pepper leaf contains polygodial as its major bioactive compound responsible for its reported antimicrobial and antifungal activities [39,47,49,50]. Lemon myrtle is a member of the Myrtaceae family; its essential oil contains citral (82–91%) as its major bioactive component possessing strong antimicrobial

activity [51–53]. Anise myrtle belongs to the Myrtaceae family, where its essential oil is dominated by either anethole (94.97%) or methyl chavicol (77.54%) depending on the chemotype of anise myrtle trees [43,54,55]. The essential oil of Tasmanian pepper leaf and lemon myrtle has shown in vitro antimicrobial activity against many yeasts, including the most weak-acid resistant *Z. bailii*. Therefore, the inhibition action of encapsulated nanoemulsion of Tasmanian pepper leaf, lemon myrtle, and anise myrtle (individually or in synergy) were evaluated in an in vitro setting using a commercialized clear apple juice system free of chemical preservatives and microbial contamination. The clear apple juice was challenged with *Z. bailii* cells during the experiment and the inhibition action of oil-in-water encapsulated nanoemulsions and sodium benzoate at low, medium, and high concentrations were evaluated during 28 days of storage at 25 °C.

2. Materials and Methods

2.1. Oils, Reagents, and Apple Juice

Pure 100% essential oils of lemon myrtle and anise myrtle are supplied from Australian Rainforest Products Pty Ltd. (New South Wales, Australia), while the essential oil of Tasmanian pepper leaf was supplied by Essential oils of Tasmania Pty Ltd. (Devonport, TAS, Australia). The essential oils were kept at their original bottles protected from light exposure and stored at 4 °C until further use. Sunflower oil (100% pure, Crisco, Sydney, Australia) was purchased from a retail supermarket and stored at room temperature. Non-ionic surfactant Tween 80 was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Double distilled water purified in a Milli-Q system (Millipore Co., Bedford, MA, USA) was used in all experiments. Pasteurized clear apple juice (Golden circle, Coles, Melbourne, Australia) with no added preservatives, no added sugar, and a long shelf-life was used in the study.

2.2. Culture Preparation and Inoculation

The antimicrobial activity of oil-in-water nanoemulsion containing essential oils of Tasmanian pepper leaf, lemon myrtle, and anise myrtle was evaluated against a weak-acid resistant yeast, *Zygosaccharomyces bailii* (ATCC 38923). The *Z. bailii* culture was grown in tryptone soya yeast extract broth for 48 h at 25 °C prior to the day of experiment. The yeast culture suspension was measured at optical density (OD_{540}) = 0.5 McFarland to produce approximately 2.5×10^6 CFU/mL, where 420 µL of this suspension was added per 1050 mL of apple juice to provide a final concentration of 1×10^3 CFU/mL.

2.3. Addition of Essential Oil Nanoemulsion and Sodium Benzoate in Apple Juice

Apple juice treatment was divided into the following: Negative control (without yeast or treatment, sterility test); positive control (spiked with yeast and without treatment); sodium benzoate treatment at 500, 750, and 1000 ppm; Tasmanian pepper leaf oil nanoemulsion at 0.00125%, 0.0025%, and 0.005%; lemon myrtle oil nanoemulsion at 0.01%, 0.02%, and 0.04%. To evaluate the synergistic effects between oils, apple juice was also treated with a combination of Tasmanian pepper leaf oil and lemon myrtle oil (1:1) nanoemulsion at 0.01% and 0.02% and a combination of Tasmanian pepper leaf oil and anise myrtle oil (1:1) nanoemulsion at 0.00125%, 0.0025%, and 0.005%. After the application of a treatment, the apple juice samples were transferred into 50 mL sterile bottles and incubated at 25 °C. Microbial growth was monitored on the day of the experiment (day-0) and every fourth day afterward, lasting for 28 days, where two bottles were removed from each apple juice treatment on the measurement day and duplicate measurements were taken for each bottle using yeast mold agar (YMA) consisting of 3 g/L of yeast extract (Oxoid, LP0021), 3 g/L of malt extract (Oxoid, LP0039), 5 g/L of peptone (Oxoid, LP0037), 10 g/L of dextrose (Oxoid, LP0071), and 20 g/L of agar (Oxoid, LP0011). The pH of YMA was adjusted to 6.2 ± 0.2 using hydrochloric acid (1 M). Enumeration of inoculated *Z. bailii* growth in the apple juice samples was determined after 48 h of incubation at 25 °C.

2.4. Statistical Analysis

The experiment was repeated twice ($n = 2$), and measurement performed in duplicate ($n = 2$). Cell counts were converted into logarithm values to calculate significant differences between the treatments over time. Yeast cell counts between treatments were considered significant at the 95% confidence limit ($p \leq 0.05$). Statistical analysis of the results was performed using two-way ANOVAs, followed by Tukey's multiple comparison post-hoc tests, with significant differences observed at $p \leq 0.05$ using GraphPad Prism version 8.00 (GraphPad Software, San Diego, CA, USA), and figures were generated in Microsoft Excel (Office 2016).

3. Results and Discussion

Effect of Sodium Benzoate and Essential Oils on Zygosaccharomyces bailii Cell Counts in Apple Juice during Storage

The clear apple juice samples from the negative-control group confirmed that the juices were free of microbes by showing no microbial growth initially (day-0). The apple juice had a pH average of 3.37. The treatment effect of nanoemulsion essential oils, either as a single oil or a combination of two oils, sodium benzoate on *Z. bailii* cell growth during storage (28 days) at 25 °C is graphically presented in Figures 1–5.

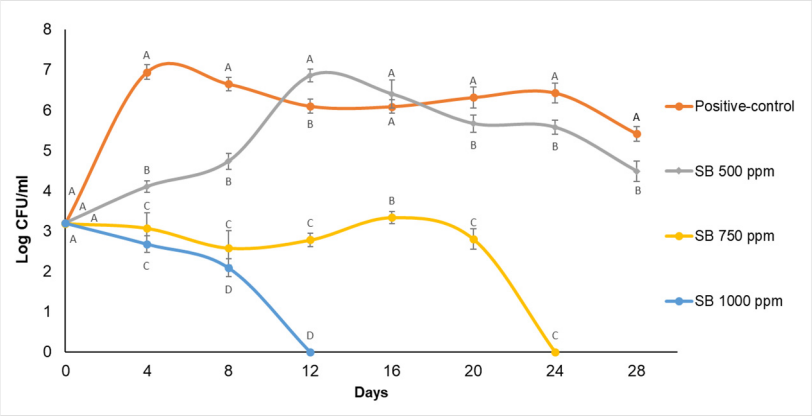

Figure 1. Effect of sodium benzoate (SB) on the growth rate of *Zygosaccharomyces bailii* in clear apple juice during 28 days of storage at 25 °C. Means with different letters on the same day are significantly different at $p < 0.05$.

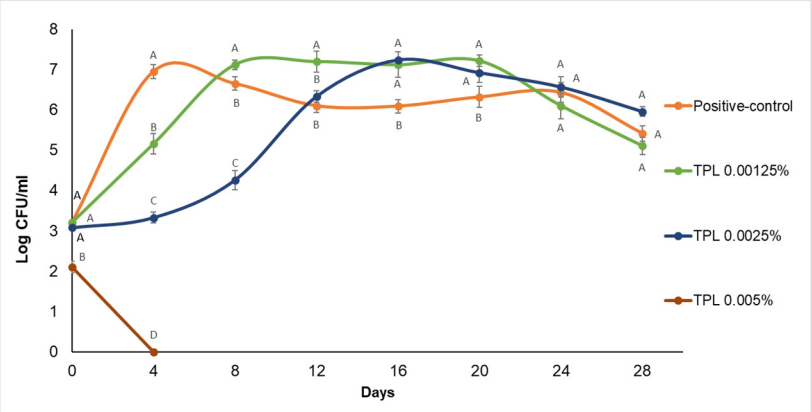

Figure 2. Effect of Tasmanian pepper leaf (TPL) nanoemulsion essential oil on the growth rate of *Zygosaccharomyces bailii* in clear apple juice during 28 days of storage at 25 °C. Means with different letters on the same day are significantly different at $p < 0.05$.

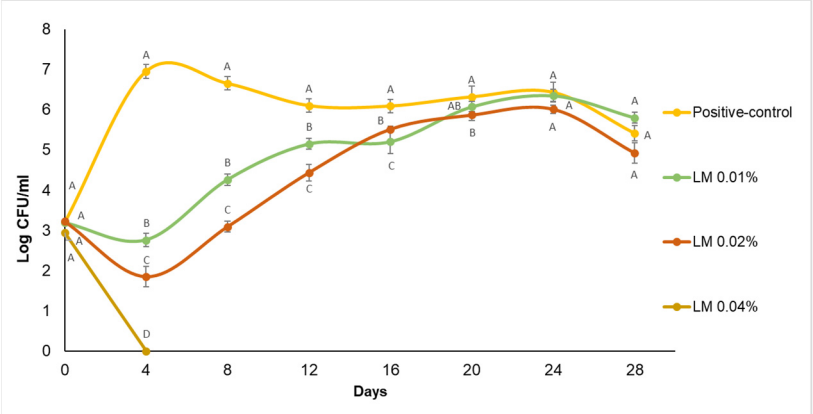

Figure 3. Effect of lemon myrtle (LM) nanoemulsion essential oil on the growth rate of *Zygosaccharomyces bailii* in clear apple juice during 28 days of storage at 25 °C. Means with different letters on the same day are significantly different at $p < 0.05$.

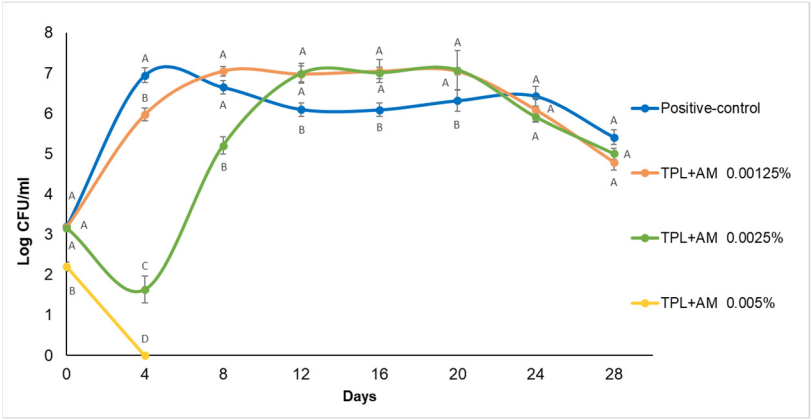

Figure 4. Effect of combining anise myrtle (AM) with Tasmanian pepper leaf (TPL) nanoemulsion essential oil on the growth rate of *Zygosaccharomyces bailii* in clear apple juice during 28 days of storage at 25 °C. Means with different letters on the same day are significantly different at $p < 0.05$.

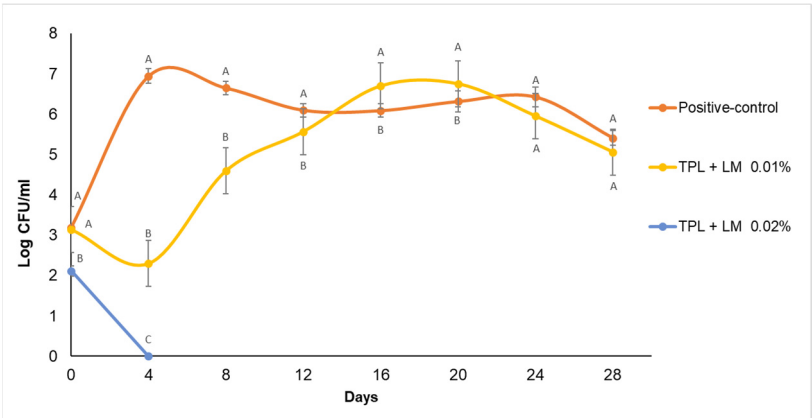

Figure 5. Effect of combining lemon myrtle (LM) with Tasmanian pepper leaf (TPL) nanoemulsion essential oil on the growth rate of *Zygosaccharomyces bailii* in clear apple juice during 28 days of storage at 25 °C. Means with different letters on the same day are significantly different at $p < 0.05$.

The positive-control group of clear apple juice samples spiked with yeast but without treatment had 3.2 log CFU/mL initially at day-0, but experienced a significant ($p < 0.05$) yeast growth at day-4 of 6.8 log CFU/mL. There were no significant differences within the

results of the sodium benzoate treatment groups and the positive-control group. After the addition of yeast cells at day-0, microbial counts from sodium benzoate treatment groups of 500, 750, and 1000 ppm were observed to be 3.2, 3.1, and 3.2 log CFU/mL, respectively, which is similar to the microbial counts of the positive-control group (Figure 1).

Sodium benzoate did not show a fungicidal effect against *Z. bailii* inocula, but instead exerted a fungistatic effect. However, for treatment of Tasmanian pepper leaf oil at 0.005%, lemon myrtle oil at 0.04%, a combination of Tasmanian pepper leaf oil and lemon myrtle oil (1:1) at 0.02%, and a combination of Tasmanian pepper leaf oil and anise myrtle oil (1:1) at 0.005% caused a significant ($p < 0.05$) reduction in microbial counts at day-0 to 2.1 log CFU/mL, 2.9 log CFU/mL, 2.1 log CFU/mL, and 2.2 log CFU/mL respectively. Unlike sodium benzoate, essential oils, either alone or in synergy, caused an immediate inhibition of yeast cells at day-0. In addition, the essential oil nanoemulsion concentrations mentioned above not only caused an immediate reduction in yeast cell counts at day-0, but also caused a total elimination (inactivation) of yeast cells at day-4 and continued to show no signs of growth until the last day of the experiment (day-28). These concentrations exhibited biocidal activity against weak-acid resistant *Z. bailii*, which reduced yeast cell counts from 3.1 log CFU/mL to 0 log CFU/mL (in comparison to the positive-control group) by day-4 of storage at 25 °C. In our experiment, the time between apple juice inoculation with *Z. bailii* cells and the addition of nanoemulsion essential oils was determined to be around 30 min. During this period of exposure (30 min), there was a noticeable decrease in yeast cells ranging from 0.2 log CFU/mL to 1.1 log CFU/mL from the treatments of Tasmanian pepper leaf oil at 0.005%, lemon myrtle oil at 0.04%, a combination of Tasmanian pepper leaf oil and lemon myrtle oil (1:1) at 0.02%, and a combination of Tasmanian pepper leaf oil and anise myrtle oil (1:1) at 0.005%. Other reports found a complete inactivation of yeast cells within 24 h of exposure to essential oils. For instance, Souza et al. [56] reported on the anti-yeast property of *Origanum vulgare* essential oil and found it to cause a prominent biocidal effect against *Candida albicans* and *C. krusei* cells at 24 h of exposure, as well as a biostatic effect against *C. tropicalis* at 12 h of exposure. Furthermore, Loeffler, Beiser, Suriyarak, Gibis, and Weiss [13] evaluated oil-in-water emulsions containing a combination of essential oil components, cinnamaldehyde, perillaldehyde, and citral against *Z. bailii* in diluted 2% apple juice and found these compounds to have biocidal activity, causing an inactivation of *Z. bailii* cells in apple juice within 24 h of storage at 25 °C. In addition, Friedman et al. [57] evaluated the antibacterial activity of 17 plant essential oils and nine essential oil compounds in a clear apple juice system (pH = 3.7) and found that citral, geraniol, eugenol, carvacrol, oregano oil, cinnamon leaf oil, clove bud oil, lemongrass oil, cinnamon bark oil, and lemon oil (in the range of 0.018–0.093%) caused inhibition against 50% of the tested *Escherichia coli* inocula within 1 h of exposure. In addition, the essential oils Melissa, oregano, lemon, lemongrass, and cinnamon leaf and the essential oil compounds citral, terpeineol, geraniol, carvacrol, and linalool (in the range of 0.004–0.011%) caused inhibition against 50% of the tested *Salmonella enterica* inocula within 1 h. Moreover, Friedman, Henika, Levin, and Mandrell [57] observed that the antimicrobial activity of essential oils could be categorized as slow- or fast-acting, where a biocidal effect could occur within 1 h of treatment. The major bioactive compounds found in Tasmanian pepper leaf oil, polygodial, lemon myrtle oil, and citral were reported to possess biocidal activity not only toward yeasts, but also against bacteria [49,50,58–68]. Citral was among the essential oil components that Souza, Stamford, Lima, and Trajano [56] and Friedman, Henika, Levin, and Mandrell [57] reported to be effective against *Z. bailii*, *Z. bisporus*, *C. albicans*, *E. coli*, and *S. enterica* in beverage model systems. Lemon myrtle essential oil has been reported to contain approximately 82–91% citral as its main bioactive compound, responsible for its strong antimicrobial activity [51–53]. In a previous study, the yeast cells of *Saccharomyces cerevisiae* exposed to lemon myrtle extract for a period of 30 min showed morphological changes, where the cells became swollen with a round-shaped structure; however, the cells showed membrane rupture when treated for 1 h. In addition, in our previous study [69], we found that the essential oils of lemon myrtle and Tasmanian pepper leaf have a fungicidal

effect through targeting yeast cell membranes, causing membrane lysis, membrane pore formation, and cell content leakage. This could explain the phenomenon where the *Z. bailii* cell count was significantly lower than the positive-control group at day-0 shortly after being exposed (30 min) to a nanoemulsion of Tasmanian pepper leaf and lemon myrtle essential oils.

Among all of the tested concentrations (0.00125%, 0.0025%, and 0.005%) of Tasmanian pepper leaf nanoemulsion (Figure 2), only the 0.005% concentration was able to reduce the yeast growth to 0 log CFU/mL (cell inactivation) at day-4, which is a 7-log reduction in comparison to the positive-control group, and no further yeast growth was observed at any day throughout the storage period of 28 days. Tasmanian pepper leaf nanoemulsion oil of 0.005% is considered the minimum fungicidal concentration (MFC) needed to kill all inocula yeast cells in clear apple juice. When the concentration of Tasmanian pepper leaf nanoemulsion oil lowered to 0.0025%, it did not kill the yeast cells, but instead delayed their growth and reduced their population significantly to 3.3 log CFU/mL at (<0.05) day-4 (3.7 reduction compared to the positive-control group), to 4 log CFU/mL at day-8, and to 6 log CFU/mL at day-12 of storage. A further decrease in the Tasmanian pepper leaf nanoemulsion oil concentration to 0.00125% reduced the yeast population to 5 log CFU/mL at day-4 of storage and to 7 log CFU/mL at day-8.

The effect of different concentrations of lemon myrtle nanoemulsion essential oil (0.01%, 0.02%, and 0.04%) on the growth rate of *Z. bailii* cells during storage is presented in Figure 3. The MFC of lemon myrtle nanoemulsion essential oil against *Z. bailii* in clear apple juice was 0.04%, which resulted in complete inactivation, where the yeast population reached 0 log CFU/mL at day-4 of storage. They did not show any further growth afterward. As the concentration of lemon myrtle nanoemulsion essential oil decreased, its antimicrobial activity decreased as well. When its concentration lowered to 0.02%, the yeast population reached 1.8 log CFU/mL at day-4 of storage, which was a significant (<0.05) decrease in yeast growth by 5.2 log CFU/mL compared to the positive-control group; however, the yeast growth reached 3.1 log CFU/mL, 4.4 log CFU/mL, and 5.5 log CFU/mL at day-8, day-12, and day-16 of storage. Moreover, the yeast growth from lemon myrtle nanoemulsion essential oil of 0.02% was significantly (<0.05) lower than that of the positive-control group from day-4 to day-28. Lemon myrtle nanoemulsion essential oil of 0.01% had a similar growth pattern of 0.02%, but was higher in yeast growth between log CFU/mL of 0.29, 1.17, and 0.72 at day-4, -8, and -12, respectively. Still, lemon myrtle nanoemulsion essential oil of 0.01% was able to maintain a yeast population below 5 log CFU/mL until day-8 of storage and was significantly (<0.05) lower than that of the positive-group at day-4, -8, -12, and -16 of storage.

In the case of anise myrtle nanoemulsion essential oil, it did not show antimicrobial activity against *Z. bailii* when tested up to a 2% concentration ($p = 0.9391$). Anise myrtle has been found to consist of two chemotypes that vary in essential oil compositions [43,55]. The first chemotype contains E-anethole (94.97%), methyl chavicol (4.43%), α-pinene (0.09%), 1, 8-Cineole (0.02%), and α-farnesene (0.07%), while the other chemotype consists of methyl chavicol (77.54%), E-anethol (19.95%), 1,8-Cineole (0.80%), α-pinene (0.40%), and α-farnesene (0.11%) [55]. Mainly, both chemotypes of anise myrtle contain the same components but at different amounts. The differences in the amount of E-anethol ($C_{10}H_{12}O$) and its isomer methyl chavicol, known as estragole, between the two chemotypes could influence the antimicrobial property of anise myrtle essential oil [70]. For instant, Wilkinson and Cavanagh [71] evaluated the antimicrobial activity of two chemotypes of anise myrtle essential oil, where the first sample exhibited no activity against *E. coli* and *C. albicans*, while the second sample showed inhibition against *E. coli*, *S. aureus*, and *C. albicans.* In addition, a study conducted by Hood et al. [72] reported antimicrobial activity of anise myrtle essential oil against *E. coli* and *S. aureus*; however, another report by Nirmal et al. [73] found no activity against *E. coli* and *S. aureus*. In addition, anise myrtle essential oil did not work in synergy with lemon myrtle essential oil, but it enhanced the antimicrobial activity of Tasmanian pepper leaf essential oil. Thus, anise myrtle was included in this

experiment to evaluate if it enhances the antimicrobial activity of Tasmanian pepper leaf in clear apple juice during the storage period. The growth rate pattern of *Z. bailii* cells when treated with anise myrtle and Tasmanian pepper leaf oils (1:1) at 0.00125%, 0.0025%, and 0.005% is shown in Figure 4. Anise myrtle and Tasmanian pepper leaf of 0.005% was the MFC that inactivated the *Z. bailii* cells at day-4 of storage. Since the two oils were mixed, this means that half of the concentration of Tasmanian pepper leaf oil of 0.0025 in the mixture was able to inactivate *Z. bailii* cells at day-4 of storage. The anise myrtle oil worked in synergy by reducing the MFC of Tasmanian pepper leaf oil from 0.005% (when tested alone) to 0.0025%. When the concentration of anise myrtle and Tasmanian pepper leaf nanoemulsion oils (1:1) lowered to 0.0025%, it significantly (<0.05) reduced the yeast population to 1.64 log CFU/mL at day-4 and 5.21 log CFU/mL at day-8 of storage compared to the positive-control group. Anise myrtle oil enhanced the Tasmanian pepper leaf oil inhibition action and significantly (<0.05) reduced the yeast growth compared to Tasmanian pepper oil when tested alone at 0.00125% at day-4 by 3.55 log CFU/mL and day-8 by 2.0 log CFU/mL. Anise myrtle and Tasmanian pepper leaf oils at a concentration of 0.00125% was not enough to cause a major reduction is yeast growth, except at day-4, where the yeast population reached 5.97 log CFU/mL, which is significantly (<0.05) lower than the positive-control group.

There was no synergistic effect found between lemon myrtle and Tasmanian pepper leaf essential oils. They performed better when tested as individual oils over their mixture in reducing the growth of *Z. bailii* cells during storage. The combination of lemon myrtle and Tasmanian pepper leaf nanoemulsion essential oils at 0.02% was the MFC that caused cell inactivation at day-4 of storage, and no further yeast growth was observed at any time until the end of the storage period (Figure 5). However, their combination at 0.01% did significantly (<0.05) lower yeast growth compared to the positive-control group at day-4, -8, and -12 to log CFU/mL of 2.3, 4.6, and 5.6, respectively.

4. Conclusions

The action of natural antimicrobial agents, essential oils, is dose- and time-dependent, which showed biocidal activity at fungicidal concentrations against the weak-acid resistant *Z. bailii* in a clear apple juice. A reduction in *Z. bailii* cells was observed to be immediate (at day-0) after a short period (30 min) of exposure to essential oils at biocidal concentrations. In addition, essential oils exerted biostatic activity instead of biocidal activity when added to clear apple juice at lower concentrations, which caused a delay in the lag phase in the growth of *Z. bailii* cells. Sodium benzoate did not exhibit biocidal activity, but only showed biostatic activity during storage. The results of this study clearly showed that essential oils of lemon myrtle and Tasmanian pepper leaf as individual oils and the combination of anise myrtle and Tasmanian pepper leaf were effective in controlling the growth of *Z. bailii* in clear apple juice within the studied storage period (28 days) and temperature (25 °C). These results indicate the great potential of native Australian herbs in controlling one of the most resistant yeasts to the action of weak-acid preservatives in clear apple juice.

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