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Research Article

Antibacterial Potential of Butterfly Pea Flower Kefir Water against *Salmonella typhi* and *Listeria monocytogenes*

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ABSTRACT

Foodborne diseases remain a major global health concern associated with the presence of pathogenic bacteria in food products. *Salmonella typhi* and *Listeria monocytogenes* are common causative agents frequently found in various foods, compromising food safety and quality. Developing functional foods with antimicrobial properties offers a potential strategy to improve both food safety and product value. Water kefir, a probiotic fermented beverage, contains beneficial microorganisms, while the butterfly pea flower (*Clitoria ternatea*) is rich in bioactive molecules, particularly anthocyanins, which may enhance the biological activity of the kefir. This study aimed to evaluate the impact of incorporating butterfly pea flower powder into water kefir on its ability to inhibit *S. typhi* and *L. monocytogenes*, as well as to assess its microbiological parameters. Butterfly pea powder was added at concentrations ranging from 1% to 5%. Antibacterial potential was evaluated using the Kirby -Bauer disk diffusion technique, while pH, lactic acid bacteria (LAB), total plate count (TPC), and yeast populations were statistically analyzed using Kruskal -Wallis and Mann-Whitney tests. The results revealed that water kefir supplemented with 2% butterfly pea powder exhibited the highest inhibitory effect against both tested bacteria. These findings suggest that butterfly pea water kefir has substantial potential as a functional food with enhanced antimicrobial activity.

Keywords: antibacterial, *Butterfly pea flower*, *Listeria monocytogenes*, *Salmonella typhi*, water kefir

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INTRODUCTION

Food becomes an esensial component for human survival, providing nutrients that are necessary and beneficial for the body (Setiarto, 2020). However, consumed food can also cause several health problems. Various microorganisms, such as bacteria, fungi, viruses and parasites can contaminate and muliply in food (World Health Organization, 2024). Contaiminated food can trigger various health problems known as foodborne diseases. Symptoms can vary, including nausea, diarrhea, and even more fatal conditiond. Globally, foodborne diseases cause approximately 600 million cases of illness and 40.000 mortality case each year (World Health Organization, 2024). Common bacterial agents that cause foodborne diseases include *Campylobacter*, *Salmonella*, *Shigella*, *Yersinia*, *Vibrio*, *Listeria monocytogenes* and Shiga toxin-producing *E. coli* (FoodNet Fast, 2023).

According to the Centers for Disease Control and Prevention, *Salmonella* and *Listeria monocytogenes* are to leading causes of death from foodborne diseases (FoodNet Fast, 2023). *Salmonella typhi,* the causative agent of typhoid fever, is a major global publik health concern. In 2019, this pathogen was responsible for approximately 9 million cases and 110.000 mortality deaths worldwide. Outbreaks have been reported globally, including in the Democratic Republic of the Congo in 2023 (World Health Organization, 2023), South Asian countries like Bangladesh (Garret et al. 2022), the Philippines in 2022 (Bhandari et al. 2024), and Indonesia, where 251 cases were documented at the Kampili Community Health Center in Gowa District between 2019 and 2020 (Mus et al. 2024). Contaminated food and beverages serve as key transmission routes. For instance, *S. typhi* contamination has been found in sugar and juice samples in Pontianak (Aufani et al. 2023), sushi in Malang (Pramida, 2023), and among students consuming contaminated food in Bandung Regency (Tampubolon et al. 2024).

Another significant concern in food safety is *Listeria monocytogenes*. Recent cases include outbreaks linked to frozen supplement products in the United States, resulting in 14 deaths. The World Health Organization (WHO) also reported that a significant portion of food safety incidents across its member countries were caused by this bacterium. In Indonesia, *L. monocytogenes* contamination has been identified in various food products, including fresh cow's milk in Enrekang and vaname shrimp (Yennie et al. 2021), green mussels in Bogor (Omura et al. 2024), and chicken meat (Panera-Martinez et al. 2024).

The pathogenicity of *S. typhi* and *L. monocytogenes* represents a major hazard to public health, prompting the widespread use of antibacterial agents as a therapeutic approach. However, controlling these pathogens with antibiotics faces serious challenges due to antimicrobial resistance. *S. typhi* has demonstrated resistance to fluoroquinolones, ciprofloxacin, chloramphenicol, nalidixic acid, and ampicillin (Yusof et al. 2022). Similarly, *L. monocytogenes* has shown notable resistance to several antibiotics, including ceftriaxone, trimethoprim, streptomycin, sulfamethoxazole, and vancomycin (Kayode and Okoh, 2022). These results underscore the escalating issue of antimicrobial resistance among foodborne pathogens and highlight the urgent need to explore alternative antimicrobial strategies to ensure food safety (Rippa et al. 2024).

Alternative antibacterial potential can be derived from natural ingredients, such as the butterfly pea flower (*Clitoria ternatea*). This flower contains active bioactive components, including flavonol glycosides, anthocyanins, flavones, phenolic acids, terpenoids, and alkaloids (Marpaung, 2020). Previous studies have shown that butterfly pea flower extract exhibits antibacterial activity against various bacterial species, including *S. typhi* and *S. aureus*. The potential of these natural ingredients can be combined to produce a stronger synergistic effect (Subianto et al. 2023).

A combination of kefir milk and butterfly pea flower has been reported to inhibit the growth of *E. coli* and *S. aureus*. However, this dairy-based combination cannot be consumed by individuals who are lactose intolerant. Therefore, water kefir is being developed as a non-dairy alternative that still contains probiotic microorganisms. Although the antibacterial potential of water kefir and butterfly pea flower has been demonstrated separately, studies on their combination remain limited, especially concerning their efficacy against *S. typhi* and *L. monocytogenes*. Consequently, evaluating the synergistic potential between water kefir and butterfly pea flower represents a relevant and promising topic for further research.

METHODS

Preparation of butterfly pea flower water kefir

Butterfly pea (*Clitoria ternatea*) flower solutions were prepared at concentrations of 1%, 2%, 3%, 4%, and 5%. The mixtures were heated at 60°C for 20 minutes and allowed to cool to room temperature before being filtered. A 6% sugar solution and a 5% water kefir starter were added to the filtrate and stirred until homogeneous. The suspension was fermented at 27°C for 48 hours. Following fermentation, the solution was filtered to separate the kefir grains from the water kefir (Setiawan et al. 2024).

Preparation of ciprofloxacin stock solution

A 500 mg Ciprofloxacin tablet was crushed using a blender and dissolved in 1000 mL of sterile water to produce a 0.5 mg/mL stock solution. The solution was filtered through a 0.22 µm filter. Subsequently, 10 µL of the 0.5 mg/mL solution was applied onto a sterile paper disc to yield a concentration of 5 µg per disc (Hamed and Abbo, 2023).

Bacterial culture preparation on nutrient agar medium

Pure bacterial cultures were streaked onto the surface of Nutrient Agar (NA) media using a sterile cotton swab. The cultures were then incubated at 30°C for 24 hours.

Hemolysis Test

A single bacterial colony from the NA culture was taken using an inoculating loop and streaked onto the surface of blood agar. The plates were incubated at 30°C for 24 hours (Amaria et al. 2023).

Biochemical tests

Biochemical tests for *Salmonella typhi* were conducted using urea, Triple Sugar Iron Agar (TSIA), and Lysine Decarboxylase (LDC) media. A single bacterial colony from the blood agar was inoculated into each biochemical medium and incubated at 30°C for 24 hours. For *Listeria monocytogenes*, biochemical tests were performed using D-xylose and L-rhamnose media. Similarly, a single colony from the blood agar was inoculated into the respective media and incubated at 30°C for 24 hours (Amaria et al. 2023).

Gram staining

Bacterial smears were prepared on glass slides and heat-fixed. Crystal violet was applied to cover the smear for one minute, then rinsed with water. Lugol's iodine solution was added for one minute, followed by rinsing with water. The smear was decolorized with 96% alcohol for 5–10 seconds and washed thoroughly. Safranin was then applied for 30 seconds as a counterstain. After a final rinse and blotting dry, the slide was examined under a microscope using a 100x objective lens with immersion oil (Amaria et al. 2023).

Preparation of bacterial suspensions

A sterile test tube containing 5 mL of physiological saline (NaCl) was prepared. Colonies of *S. typhi* ATCC 6579 and *L. monocytogenes* ATCC 13932 were taken from the NA medium using an inoculating loop, suspended in the saline solution, and homogenized. The turbidity of the suspension was adjusted to match the 0.5 McFarland standard (CLSI M02-A11, 2012).

Antibacterial testing: plate inoculation and disc placement

A sterile cotton swab was dipped into the standardized bacterial inoculum (0.5 McFarland). Excess liquid was removed by pressing the swab against the tube wall. The swab was streaked evenly across the entire surface of Plate Count Agar (PCA) media in three directions to ensure uniform distribution. The plates were allowed to dry for 3–5 minutes at room temperature. Sterile discs (6 mm in diameter) were impregnated with 10 µL of the respective test solutions and allowed to absorb for 5–10 minutes. The discs were then aseptically placed onto the inoculated PCA surface and gently pressed to ensure full contact. This step was completed within 15 minutes after plate inoculation (CLSI M02-A11, 2012).

Incubation and measurement of inhibition zones

The plates were inverted and incubated at 35°C for 16–18 hours for *S. typhi* ATCC 6539, and 20–24 hours for *L. monocytogenes* ATCC 13932 (CLSI M02-A11, 2012). Following incubation, the diameter of the clear inhibition zone around each disc was measured in millimeters (mm) using a scale. The results for each disc were systematically recorded.

pH measurement

Five samples of water kefir from each concentration were taken post-fermentation. A pH meter was standardized using reference buffers (pH 4 and 7). The electrode was rinsed with distilled water, dried with a tissue, and immersed into the sample. The pH value was recorded to two decimal places (Setiawan et al. 2024).

Enumeration of Lactic Acid Bacteria (LAB)

A one mL aliquot of water kefir from each concentration was serially diluted (10^-1 to 10^-6) in a 0.9% NaCl solution. One milliliter of the diluted sample was inoculated into de Man, Rogosa, and Sharpe (MRS) agar using the pour plate technique. The Petri dishes were incubated at 37°C for 48 hours under anaerobic conditions. The number of LAB colonies was counted using a colony counter and converted to log CFU/mL, accounting for the dilution factor. The procedure was performed in triplicate for each concentration.

Enumeration of total aerobic bacteria

A one mL aliquot of water kefir was serially diluted (10^-1 to 10^-6) in a 0.9% NaCl solution. One milliliter of the dilution was inoculated onto PCA media using the pour plate technique. The plates were incubated at 37°C for 48 to 72 hours. Total bacterial colonies were counted, converted to log CFU/mL, and analyzed in triplicate.

Enumeration of yeast

A one mL aliquot of water kefir was serially diluted (10^-1 to 10^-6) in a 0.9% NaCl solution. One milliliter of the dilution was inoculated into Potato Dextrose Agar (PDA) using the pour plate method. The plates were incubated at 25°C for 120 hours. Yeas t colonies were counted using a colony counter and expressed as log CFU/mL. This measurement was repeated three times per concentration.

Data analysis

The experimental data were processed using IBM SPSS version 25. Statistical analysis was performed using the non - parametric Kruskal–Wallis test to identify significant differences, followed by the Mann–Whitney test with a Bonferroni adjustment for post- hoc comparisons.

RESULTS AND DISCUSSION

Inhibition zone analysis

The agar diffusion test revealed that the control water kefir without butterfly pea powder (KBP0) did not produce an inhibition zone against *Salmonella typhi*, as its pH (3.56) was insufficient to inhibit the bacterium. Conversely, KBP0 exhibited a 5.3 mm inhibition zone against *Listeria monocytogenes*. This aligns with Sindi et al. (2020), who noted that kefir demonstrates antibacterial activity against various pathogens, including *L. monocytogenes*, via metabolites like lactic acid, acetic acid, and bacteriocins produced by lactic acid bacteria (LAB). The lack of activity against *S. typhi* may be attributed to its higher tolerance to these specific fermentation metabolites. The results of the inhibition zone of kefir flower water against the growth *of S. typhi* and *L. monocytogenes* bacteria, as shown in Figure 1.

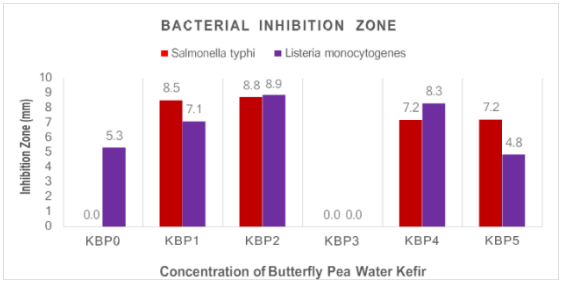

Figure 1. Result of inhibitor zone of butterfly pea flower kefir water

The incorporation of butterfly pea powder at 1% (KBP1) and 2% (KBP2) yielded the largest inhibition zones: 8.5 mm and 8.8 mm against *S. typhi*, and 7.1 mm and 8.9 mm against *L. monocytogenes*, respectively. These concentrations represent the optimal conditions for antibacterial activity. Interestingly, at a 3% concentration (KBP3), no inhibition was observed for either bacterium. However, antibacterial activity reappeared at 4% (KBP4) and 5% (KBP5), albeit with smaller inhibition zones compared to the optimal concentrations.

This non-linear phenomenon can be attributed to the behavior of secondary metabolites in the butterfly pea flower, such as f lavonoids, alkaloids, tannins, and saponins. At lower concentrations (KBP1 and KBP2), these bioactive compounds remain soluble and diffuse optimall y through the agar, creating clear inhibition zones. The absence of an inhibition zone at KBP3 is likely due to precipitation or self-association, where polyphenol molecules aggregate into larger complexes. This aggregation reduces their solubility and restricts their diffusion through the agar medium, thereby temporarily diminishing their observable antibacterial activity (Muntaha et al. 2025).

This dynamic aligns fundamentally with the findings of Rumidatul et al. (2021), who demonstrated that the antimicrobial efficacy of secondary metabolites—specifically phenolics and flavonoids derived from endophytic fungi of *Falcataria moluccana*—is closely tied to their production phases and stability. Both studies underscore a crucial concept in natural products chemistry and bioprospecting: the antibacterial effectiveness of plant-derived secondary metabolites is not exclusively concentration-dependent. Instead, it is heavily governed by the diffusion capacity and structural stability of the active compounds within the medium; when concentrations exceed the solubility threshold, aggregation occurs, limiting the compounds' ability to penetrate target bacterial cells.

The differing inhibition zones between *S. typhi* (Gram-negative) and *L. monocytogenes* (Gram-positive) are influenced by their respective cell wall architectures. Gram-positive bacteria possess a thick but relatively simple peptidoglycan layer without an outer lipid membrane, allowing antibacterial compounds to interact directly with vital cellular components. In contrast, Gram-negative bacteria feature a complex cell envelope with an outer lipopolysaccharide layer acting as a formidable barrier against antibacterial

penetration. Consequently, the synergistic combination of water kefir metabolites and butterfly pea bioactive compounds is generally more effective against Gram-positive bacteria (Nur'aini et al. 2024).

According to the CLSI M02-A11 (2012) interpretation guidelines, the positive control (Ciprofloxacin) produced a maximum antibacterial effect, with inhibition zones of 18.5 mm against *S. typhi* and 17.9 mm against *L. monocytogenes*. The antibacterial activity of KBP1, KBP2, KBP4, and KBP5 was categorized as moderate (ranging from 6.9 to 8.9 mm), while KBP3 was categorized as inactive. Overall, this combination exhibits a broad-spectrum antibacterial effect, targeting both Gram-positive and Gram-negative microbes through the synergy between kefir fermentation metabolites (organic acids, H_2O_2, antimicrobial peptides) and the flavonoids, anthocyanins, and phenolic compounds from the butterfly pea flower.

pH measurement analysis

The pH of the control water kefir without butterfly pea powder (KBP0) was measured at 3.56. Upon the addition of butterfly pea flower powder, the pH values varied slightly, ranging from a minimum of 3.19 in the 1% concentration (KBP1) to a maximum of 3.46 in the 5% concentration (KBP5). At lower concentrations, such as KBP1 and KBP2, the fermentation process remained highly optimal; lactic acid bacteria actively produced sufficient organic acids, which facilitated the initial decrease in pH. The results of this pH test through supplementation with butterfly pea flower powder are shown in Figure 2.

However, as the concentration of butterfly pea powder increased from KBP3 to KBP5, the pH value exhibited a slight upward tre nd. This specific pattern aligns with the findings reported by Putriana et al. (2025), who observed that an increase in the proportion of butterfly pea powder used in kefir formulation correspondingly elevated the pH levels. This phenomenon suggests that higher concentrations of bioactive compounds in the flower—namely anthocyanins, flavonoids, and phenolics—may partially inhibit the metabolic activity of acid-producing microorganisms during fermentation. Furthermore, Yuniati et al. (2024) highlighted that anthocyanins are not only highly sensitive to environmental pH changes but also possess a natural buffering capacity against hydrogen ions. Consequently, this intrinsic buffering effect acts to slow down the rapid decrease of pH in fermented products.

Despite these observable physical trends, the Kruskal-Wallis analysis, followed by the Mann-Whitney pairwise test utilizing a Bonferroni adjustment, confirmed that all comparisons between the treatment groups were not statistically significant ($p > 0.0033$). This statistical outcome implies that the supplementation of butterfly pea flower powder did not significantly alter the overall acidity of the water kefir. These results are highly consistent with a recent study conducted by Nur'aini et al. (2024), which similarly demonstrated that the inclusion of butterfly pea powder failed to exert a significant impact on the final pH of kefir products.

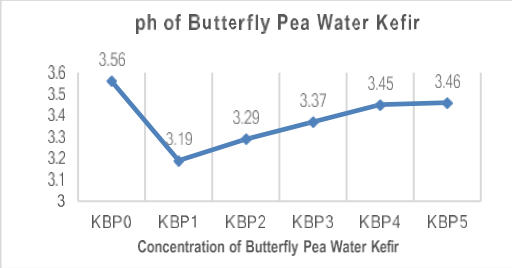

Figure 2. pH Measurement of butterfly pea flower kefir water

Total Lactic Acid Bacteria (LAB) examination

The enumeration of lactic acid bacteria (LAB) revealed robust populations across all treatments, with the average total LAB ranging from 7.77± 0.62 to 8.41 ± 0.53 log CFU/mL. Statistical evaluation using Kruskal–Wallis and Mann–Whitney post-hoc tests with a Bonferroni adjustment confirmed that the addition of butterfly pea flower powder did not significantly impact the total LAB count. This finding is strongly s upported by Setyawardani et al. (2025), who reported that the incorporation of butterfly pea powder into goat milk whey kefir did not inhibit LAB growth. Similarly, Nur'aini et al. (2024) observed that supplementing cow's milk kefir with butterfly pea powder had no detrimental effect on the v iability of lactic acid bacteria. Although Rifqi et al. (2021) noted that the anthocyanins and flavonoids present in the butterfly pea act as potent antibacterial agents, these bioa ctive compounds appear to be non-toxic to the intrinsic, beneficial microbiota of the kefir. The consistently high LAB population can be attributed to the favorable environmental conditions and abundant nutrients within the fermentation medium, which allow LAB to maintain high metabolic activity and regenerate rapidly, as suggested by Sionek et al. (2024).

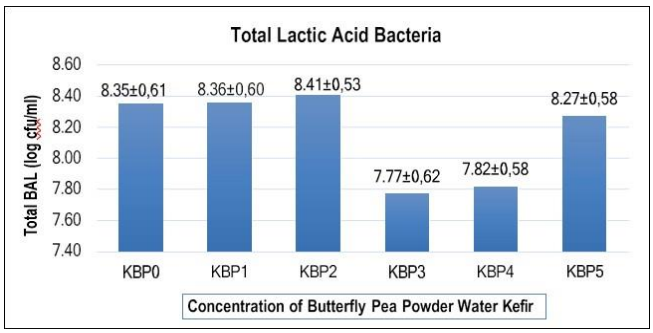

Figure 3. Total of Lactic Acid Bacteria examination

Total aerobic bacteria analysis

Total aerobic mesophilic bacteria were enumerated using Plate Count Agar (PCA) to account for the broader microbial community actively participating in the fermentation process, which includes both LAB and other bacteria such as acetic acid bacteria. After 48 hours of fermentation, the overall microbial load in the water kefir ranged from

7.4 ± 0.22 log CFU/mL in the control group (KBP0) to a peak of 8.1 ± 0.29 log CFU/mL in the 5% concentration group (KBP5). Statistical analysis indicated no significant differences across the varied treatments ($p > 0.05$). These results are highly consistent with the findings of Gülhan (2023), who demonstrated that the number of mesophilic bacteria in water kefir typically varies between 7.9 and 8.7 log CFU/mL and undergoes no significant changes during the fermentation and storage phases. This suggests that the supplementation of butterfly pea flower powder safely maintains the stability of the active mesophilic bacterial population that is essential for proper kefir fermentation.

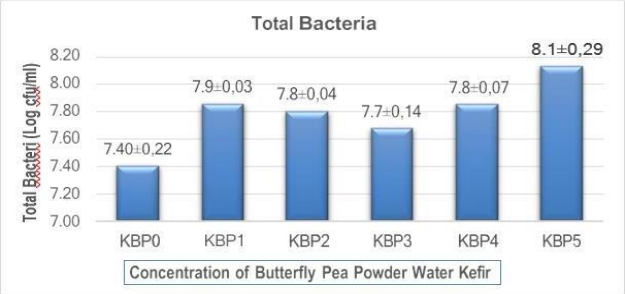

Figure 4. Total aerobic bacteria

Total yeast analysis

Yeast population dynamics were evaluated, yielding counts that ranged from a high of 7.64 ± 0.05 log CFU/mL in KBP1 to a low of 6.20 ± 0.11 log CFU/mL in KBP3. Statistical assessments confirmed that there were no significant differences between the treatme nt groups ($p > 0.0033$), indicating that butterfly pea powder supplementation did not considerably alter the overall yeast population. This aligns perfectly with Putriana et al. (2025), who found that adding butterfly pea extract to cow's milk kefir resulted in no significant quantitative difference in yeast counts.

The initial stability and slight increase in the yeast count observed at lower concentrations (KBP1 and KBP2) suggest that th e bioactive compounds are not toxic to yeast at foundational levels. However, the modest reduction observed at higher concentrations (KBP3) mirrors the concentration - dependent effects described by Setiawan et al. (2024), who reported that while low levels of butterfly pea extract (up to 0.9 %) can stimulate yeast, elevated concentrations introduce higher levels of bioactive compounds that can inhibit yeast growth. As noted by Islamy et al. (2022), butterfly pea flowers are rich in flavonoids and alkaloids. The cellular mechanisms of these secondary metabolites have been well -documented; Aboody & Mickymaray (2020) explained that flavonoids can disrupt fungal cell membrane integrity and inhibit essential metabolic enzymes. Furthermore, Rezaldi et a l. (2023) elaborated that flavonoids induce protein denaturation in yeast, while alkaloids can penetrate the cell wall and suppress DNA replication mechanisms. Despite these potential inhibitory mechanisms at higher concentrations, the overall yeast population in this study remained statistically s table and viable for effective fermentation.

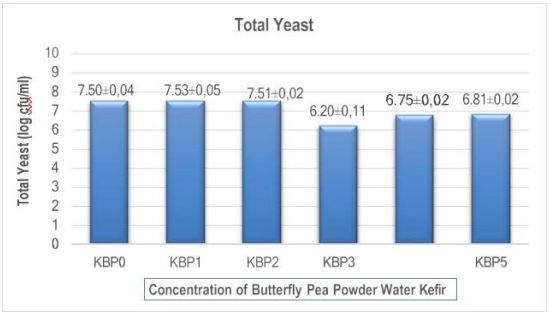

Figure 5. Yeast test result

CONCLUSION

Based on the study outcomes, the combination of water kefir and *Clitoria ternatea* flower powder demonstrates significant antibacterial activity. This is evidenced by the formation of inhibition zones against *Salmonella typhi* (Gram-negative) and *Listeria monocytogenes* (Gram- positive). These results suggest that the combination exhibits broad-spectrum antibacterial potential, capable of inhibiting the growth of both bacterial groups. The synergistic interaction between the bioactive metabolites produced during kefir fermentation and the phenolic constituents in the butterfly pea flower emphasizes its potential utility for further development as a natural antibacterial agent in the food and health industries.

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AUTHOR CONTRIBUTIONS

All authors have reviewed and approved the final manuscript for publication.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

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