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Research article

Comparative microbial analyses of hydroponic versus in-soil grown Romaine lettuce obtained at retail

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ABSTRACT

The overarching goal of this study was to assess the microbiological profile of hydroponically grown Romaine lettuce and in-soil Romaine lettuce (organic and conventional). Thirty-six samples of hydroponic lettuce, seventy-two samples organic lettuce (thirty-six bagged lettuce and thirty-six non-bagged lettuce), and thirty-six conventionally grown lettuce was purchased from retail stores. A portion of each sample was analyzed for aerobic bacteria (APC), coliforms and *E. coli*, and yeasts and molds (YM). Another portion of each sample was enriched for *Salmonella*, *E. coli* O157:H7, *Listeria monocytogenes*, and *Staphylococcus aureus*, and confirmed with RT-PCR. No statistical differences were found in the microbial profile ($P > 0.05$) between the different farming practices. The APC, coliforms, *E. coli*, and YM counts were similar across bagged samples. The results demonstrated that APC and *E. coli* were significantly higher ($P<0.05$) in organic non-bagged samples compared to other practices. *Salmonella* and *L. monocytogenes* were detected in some organically and conventionally grown lettuce samples but were only detected in 3 hydroponically grown lettuce samples. This study indicated that hydroponically grown lettuce obtained at retail may have food safety risks similar to organic and conventional systems. These findings highlight the need for food safety training and educational programs.

1. Introduction

Lettuce can be grown using different farming practices, including conventional or organic practices, using soil, or hydroponically, using a recirculated flowing film of nutrient solution (Coulombe et al., 2020). The hydroponic farming method is a system in which growers cultivate plants without soil in a controlled environment (Aires, 2018). In recent years, the controlled environment agriculture (CEA) industry, which includes hydroponics, has grown significantly as it has become an alternative production system for growers and popular product among consumers (Dankwa et al., 2020; Riggio et al., 2019).

Consumers value environmentally friendly and sustainably grown fresh produce (Aires, 2018), and leading to an increase in fresh produce consumption in the United States (Callejón et al., 2015; Carstens et al., 2019). Produce has been implicated in foodborne illness outbreaks leading to public health challenges (Callejón et al., 2015). Between 2010 and 2017, a total of 1797 foodborne outbreaks were recorded with a confirmed causative food product in the United States (U.S.) (Carstens et al., 2019), and 228 outbreaks were associated with fresh produce (CDC, 2017). Among fresh produce outbreaks, leafy greens are among the most common etiological sources of outbreaks (Murray et al., 2017; Turner et al., 2019). In most of these outbreaks, *Salmonella*, Shiga-toxin producing *E. coli* (STEC), and *L. monocytogenes* were the causative agents (Callejón et al., 2015; Carstens et al., 2019). In Canada, *E. coli* O157:H7 was the main pathogen causing foodborne outbreaks linked to leafy greens (Coulombe et al., 2020). Between 2008 and 2018, there were eleven *E. coli* O157:H7 outbreaks linked to leafy greens in Canada, including 7 associated with Romaine lettuce, 2 associated with iceberg lettuce, and 2 linked to other leafy greens (Coulombe et al., 2020). Studies also confirm the connection between outbreaks in the U.S. involving STEC found in leafy greens (Bottichio et al., 2020) and lettuce (Taylor et al., 2013). Another example is the 2018 multistate outbreak of *E. coli* O157:H7 in the U.S. that was associated with Romaine lettuce (CDC, 2018).

Maffei et al. (2016) stated that the sources of fresh produce contamination were varied, and the causative agents could be environmental, human, or animal at the pre- or post-harvest phase. The specific contaminations could involve soil, irrigation water, wild and/domestic animals, inadequately composted or raw animal manure, human handling, tools and equipment, containers, transportation, storage, and

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packaging. Greenhouse cultivation, which includes hydroponically grown produce, is reported as safer from microbiological issues compared to other farming practices due to reduction of risk factors including soil (Holvoet et al., 2015; Sirsat and Neal, 2013). However, hydroponic systems can become contaminated with pathogens from water, substrates, and human activity (Moriarty et al., 2019; Shaw et al., 2016). For instance, the Food and Drug Administration released a report of a multi-state salmonellosis outbreak associated with a hydroponic farm that led to 31 illnesses and 4 hospitalizations in 2021. The investigation showed that there was environmental *Salmonella* contamination that could have led to the outbreak due to cross contamination onto the produce (U.S. Food and Drug Administration, 2021).

The Food and Drug Administration's (FDA) Food Safety Modernization Act (FSMA) has changed the strategy from responding to food safety risks to a more proactive prevention strategy (U.S. Food and Drug Administration, 2019a). The FSMA Produce Safety Rule was developed to provide science-based standards for growing, harvesting, packing, and holding produce for human consumption (U.S. Food and Drug Administration, 2019b). In 2016, when the final rule became effective, FDA stated that all covered fresh produce operations must perform certain practices based on produce safety requirements, including maintaining quality for agricultural water, microbial standards for biological soil amendments, standards for using domesticated animals, worker training, health and hygiene, and maintaining the sanitary standard for all agricultural tools, equipment, and facilities (U.S. Food and Drug Administration, 2019b). Specifically, the FDA announced that hydroponic systems are subject to the same potential routes of contamination as covered produce, including contamination from agricultural water (U.S. Food and Drug Administration, 2019b). Therefore, the Produce Safety Rule is applied to covered produce grown hydroponically (U.S. Food and Drug Administration, 2019b). Nevertheless, hydroponic growers are exempt from water testing requirements by the FSMA Produce Safety Rule if the water does not come in contact with the harvestable portion of covered produce (Stoeckel et al., 2017). Accordingly, hydroponic growers must understand how to assess the point of contaminations at every production stage, including planting, pre-harvest, and post-harvest practices (Francis et al., 2012). There is a paucity of research investigating the microbial profiles and food safety risks associated with hydroponic, organic, and/or conventionally grown produce addressing food safety risks of hydroponic systems. Hence, the objectives of this study were to investigate the baseline microbial profiles and microbiological food safety risks of Romaine lettuce that was grown hydroponically and compare those microbial profiles to the profiles of lettuces grown through in-soil farming practices, including organically and conventionally grown Romaine lettuce obtained at retail.

2. Materials and methods

2.1. Lettuce samples

For each biological replicate, 48 Romaine lettuce heads were purchased, including 12 hydroponically grown (plastic clam shell), 12 organically grown (bagged), 12 organically grown (not bagged), and 12 conventionally grown (not bagged) from a local supermarket in the Houston, TX area. The hydroponic lettuce with an intact root system was packaged in a plastic clam shell and marked as hydroponically grown. No wash steps were performed on the hydroponic and non-bagged lettuce obtained at retail. The bagged lettuce purchased was triple washed according to the label. The non-bagged Romaine lettuce did not go through sanitizer treatment when obtained at retail. The goal was to purchase fresh lettuce by visit the store right after the produce was restocked for the day, and the analysis was performed on the day of the purchase. Sell-by dates of at least 5-days in advance were ensured for hydroponic and bagged lettuce samples and same brands were purchased for each of the hydroponic and bagged lettuce, respectively, for each biological replicate. The same store was visited each time for sample collection to maintain consistency and brand availability. Three samples from each lettuce head were analyzed further. Thus, a total of 36 hydroponically grown, 36 organically grown lettuce (bagged), 36 organically grown (not bagged), and 36 conventionally grown lettuce samples were analyzed. The samples were transferred to the Food Microbiology Laboratory in an icebox for microbiological and molecular analyses on the same day. Three biological replicates were performed.

2.2. Enumeration of microbial profile

Methods were designed based on a previously published study (Sirsat et al., 2021) with slight modifications. Upon arrival at the Food Microbiology Laboratory, representative samples of 10 g were collected from each lettuce sample for analyses using a sterile scissor. For each 10 g sample, a portion was cut from the top, botom, and heart of the lettuce to represent all areas of the lettuce sample. The scissor was sanitized between samples using 70% ethanol. The individual sample was placed in a stomacher bag containing 90 mL 0.1% buffered peptone water (PW) that was homogenized using stomacher lab (400 Seward Co. Ltd., London, UK) for 2 min. Serial 10-fold dilutions were made, and 1 mL from each dilution was plated on 3 M petrifilms for aerobic bacterial counts (APC, 3M Nelson-Jameson, Inc., WI, USA), *E. coli*/Coliforms (*E. coli*/Coliforms, 3M Nelson-Jameson, Inc., WI, USA), and Yeast/Mold (YM, 3M Nelson-Jameson, Inc., WI, USA) for each sample. All petrifilms were incubated at 35 °C for 24–48 h. Samples were also enriched in enrichment media for the presence of *Salmonella* entrica, *E. coli* O157:H7, *Listeria monocytogenes (L. monocytogenes)*, and *Staphylococcus aureus (S. aureus)*. For *Salmonella*, 5 mL of sample was added to 45 mL of Universal pre-enrichment broth (UPB, Hardy Diagnostic, Santa Maria, CA). For *E. coli*, 5 mL of sample was added to 45 mL of Modified Tryptone Soy Broth (mTSB, Neogen, Lansing, MI) and supplemented with novobiocin (Sigma Chemical Co., St. Louis, Mo.). For *L. monocytogenes*, 5 mL of sample was added to 45 mL of *Listeria* Enrichment Broth (LEB, Hardy Diagnostic, Santa Maria, CA). For *S. aureus*, 5 mL of each sample was added to the tube containing 45 mL Giolitti-Cantoni Broth (GCB, HiMedia™ Laboratories Pvt Ltd, Nashik, India). All enrichment media were incubated at 35 °C for 24 h. A loopful from each sample was streaked onto selective media and incubated at 35 °C for 24 h using a disposable sterile plastic inoculation loop (VWR International, Radner, PA, USA) after the incubation period. The selective media used was Eosin Methylene Blue agar (EMB, Hardy Diagnostic, Santa Maria, CA), Polymyxin Acriflavine Lithium Chloride Ceftazidime Aesculin Mannitol (PALCAM, Sigma-Aldrich Co. St. Louis, MO), and Baird Parker Agar (BPA, Neogen, Lansing, MI) used for *Salmonella*, *E. coli*, *L. monocytogenes*, and *S. aureus*, respectively. The individual growth colonies from each selective media was transferred to 9 mL TSB tubes, incubated at 35 °C for 24 h, and these cultures were used for RT-PCR testing to confirm whether the positive samples from enrichment procedures were true positives. We used RNA instead of DNA because were interested in detecting only viable microorganisms as DNA detects both viable and non-viable microorganisms.

2.3. RNA extraction and RT-PCR testing

Following microbial analysis and enrichment steps, RT-PCR assays were administered to detect specific pathogenic microorganisms for samples that were positive on selective media.

2.4. RNA extraction

RNA extraction was performed using ReliaPrep™ RNA Cell Miniprep System (Promega, Madison, WI, U.S), following the manufacturer protocol. Briefly, 5 mL of each sample was centrifuged at 1000 × g for 15 min using a centrifuge (Allegra X-22 Centrifuge, Beckman Coulter Inc., Palo Alto, CA) and the pellet was washed twice in PW. Following this, 1 mL of suspension was transferred to 2 mL elution tube (Promega, Madison, WI, U.S.). Next, 250 μL of the BL + TG solution (1-Thiolycerol + BL

Buffer) was added and vortexed for 1 min to lyse the cell. The lysate cell was transferred to minicolumn, centrifuged for 30 s, and the liquid in the collection tube was removed, followed by the addition of 85 μL of 100% Isopropanol (Sigma-Aldrich Co. St. Louis, MO). The minicolumn was inserted into the collection tube and 500 μL of RNA wash solution (RNA wash solution+ 95% ethanol) was added and centrifuged for 30 s. Following this, the liquid was discarded and DNase I solution (Yellow Core Buffer + MnCl2+DNase I enzyme) was prepared. Thirty μL of freshly prepared DNase I was added to the minicolumn and incubated for 15 min at 23 ± 2 °C. After the incubation, 200 μL of column wash solution (solution+ 95% ethanol) was added and centrifuged for 15 s. This was followed by adding 500 μL of RNA wash solution and centrifuging for 30 s. The liquid was removed and final wash was carried out by adding RNA wash solution (300 μL) and centrifuging for 2 min. The minicolumn was transferred to a 1.5 mL elution tube and 30 μL nuclease free water was added to and centrifuged for 1 min. The resulting purified RNA was stored at −80 °C until the time of RT-PCR testing. Total RNA quantity was determined using NanoVue Spectrophotometer (NanoVue Plus™, BioChrom, Holliston, MA).

2.4.1. Reverse transcription real-time PCR procedure and testing

Two pairs of oligonucleotide primers were prepared based on previous studies (Mafu et al., 2009; Goto et al., 2007; Sharma et al., 1999) for each microorganism. The reactions contained 10 μL of 1x GoTaq® qPCR Master Mix (Promega, Madison, WI, U.S), 0.2 μL of Reference Dye, 2 μL of each forward and reverse primers, 4 μL of the RNA template, 0.4 μL RT enzyme Mix (RT-qPCR, Promega, Madison, WI, U.S), and 1.4 μL Nuclease-Free Water. The conditions were as follows: 37 °C for 15 m reverse transcription, 95 °C for 10 m RT inactivation, 40 cycles of 95 °C for 10 s denature, 60 °C for 30 s anneal, and 72 C for 30 s extend. For each cycle, negative control, and positive controls (with known pathogenic strains) were carried out.

2.5. Statistical analysis

Bacterial counts were enumerated and log10 units were calculated before the statistical analysis. For each of the APC, *E. coli*, coliforms, and yeast and molds counts the means and standard deviations were calculated and results were compared using one-way analysis of variance. Significant mean differences ($P < 0.05$) were compared with the Tukey test. All statistical analyses were performed using JMP statistical software v14 (SAS, Institute, Cary, NC). The means of positive samples from the enrichment media of sampling surfaces were calculated and recorded. The RT-PCR results were analyzed using AriaMx Software (data analysis software, Agilent Technologies, Santa Clara, CA).

3. Results and discussion

3.1. Romaine lettuce microflora

The overall microbiological profiles of lettuce samples (hydroponic, organic bagged and not bagged, and conventional) obtained from retail stores were compared using mesophilic aerobic counts (APC), generic *E. coli* and coliforms, YM analyses. The results showed that APC ranged from 3.2 to 3.7 log CFU/g, *E. coli* ranged from 1.3 to 2.0 log CFU/g, coliforms were found between 2.7–2.9 log CFU/g, and YM ranged from 2.5 to 2.6 log CFU/g across all lettuce samples, regardless of the farming method used. Since the Romaine lettuce was obtained at retail, there is a possibility of other factors such as time and temperature abuse and handing practices that can contribute to the microbial profile. No statistically significant differences in the number of APC, *E. coli*, coliforms, or YM counts were found between lettuce samples grown hydroponically, organically (bagged), or conventionally. This data is demonstrated in Table 1. The data suggests that the levels of natural background microorganisms are not affected by farming methods, and background microorganism levels are similar between soil-based systems and hydroponic systems.

Table 1. Baseline microbial profile test results for the presence of background microorganisms on hydroponic lettuce, organic lettuce, and conventionally grown lettuce. N = 36.

Microbial profile	[a]Mean Log (CFU/g) on Lettuce Samples			
	Hydroponic	Organic (Bagged)	Organic (Not bagged)	Conventional
APC	3.4 ± 0.1 A	3.3 ± 0.3 A	3.7 ± 0.2B	3.2 ± 0.1A
Generic *E. coli*	1.5 ± 0.6A	1.3 ± 0.6A	2.0 ± 0.3B	1.6 ± 0.5A
Coliforms	2.7 ± 0.8A	2.7 ± 0.1A	2.9 ± 0.3A	2.8 ± 0.3A
Y/M	2.6 ± 0.1A	2.6 ± 0.3A	2.5 ± 0.4A	2.5 ± 0.4A

[a] In the same row, mean with same letter A or B are not statistically different ($P > 0.05$).

Although the average level of *E. coli* on organic bagged lettuce samples had the lowest count when compared to other farming practices, no significant statistical differences were found between farming methods. The current study results are consistent with those found by Barnhart et al. (2015), who assessed the level of microorganisms on leafy greens produced in aquaponics, hydroponics, and soil-based agricultural systems at retail in the state of Minnesota (Minneapolis/Saint Paul Metropolitan area and the Duluth area). Similar to the current study, the researchers found no statistical differences in the level of microorganisms between different farming practices (Barnhart et al., 2015). Furthermore, significant differences ($P < 0.05$) in counts of APC and *E. coli*, and slightly higher counts of coliforms were found on organic non-bagged lettuce samples. The reason for lower aerobic counts in bagged lettuce samples could be because the lettuce was washed. However, the higher coliform counts in organic non-bagged lettuce samples may be due to contaminated water used for washing or irrigation, or poor handling practices.

3.2. Pathogenic microorganisms

Human pathogens such as *L. monocytogenes*, *Salmonella* enterica, *E. coli*, and human noroviruses are continually associated with fresh produce outbreaks (Berger et al., 2010). Therefore, evaluating the presence of pathogens on lettuce grown hydroponically and comparing it with soil-based farming practices, such as organic and conventional-soil practices, is important from a food safety and risk management standpoint. Table 2 demonstrates pathogen presence on lettuce samples. The results show that *Salmonella* was present in ten out of thirty-six samples of organic bagged lettuce samples and seven conventionally grown lettuce samples. While only three out of thirty-six hydroponic grown lettuce

Table 2. Results from enrichment and RT-PCR for the presence of microorganisms on the surface of hydroponic, organic, and conventionally grown lettuce.

Pathogenic microorganisms	Lettuce Samples[a]			
	Hydroponic	Organic (Bagged)	*Organic (Not bagged)	Conventional
Salmonella	3/36	10/36	5/36	7/36
[b]*E. coli O157:H7*	10/36	15/36	22/36	18/36
Listeria monocytogenes	3/36	9/36	6/36	7/36
Staphylococcus aureus	13/36	12/36	21/36	15/36

[a] This refers to the number of positive samples from enrichment methods and confirmed with reverse transcription PCR for lettuce samples. N = 36.

[*] The asterisk sign refers to the lettuce samples that were not bagged. All other lettuce samples were purchased in bags, except for one set of organic lettuce samples that were not bagged.

[b] The presumptive positive lettuce samples with *E. coli* O157:H7 were confirmed with RT-PCR as positive when samples tested positive for both *eae* and *stx*.

samples and five non-bagged lettuce samples grown organically were *Salmonella positive*. *Salmonella* occurrence on produce in this study (bagged and non-bagged organic lettuce samples, and conventionally grown lettuce samples) could be originated from soil or environmental contamination, since *Salmonella* was lower in hydroponically grown lettuce samples where no actual soil is involved in the hydroponic system. Another study analyzed food safety hazards in leafy greens in aquaponics, hydroponic, and soil-based systems and suggested the potential of pathogens, such as *E. coli* O157:H7 and *Salmonella,* in all of the farming methods studied, including hydroponics (Barnhart et al., 2015). However, lettuce samples were positive for *E. coli* O157:H7 regardless of the farming practice. Eighteen samples from conventionally and 22 samples from non-bagged organically grown lettuce samples were positive for *E. coli* O157: H7, while 10 out of thirty-six lettuce samples of hydroponic and fifteen organic bagged samples were positive for *E. coli* O157: H7. The presence of *E. coli* O157:H7 on lettuce samples indicates fecal contamination (Carstens et al., 2019). The source of contamination could be from the irrigation water and/or workers due to poor sanitation practices (Standing et al., 2013).

A previous study found that *E. coli* O157:H7 contaminated irrigation water can contaminate the edible portions of hydroponically grown lettuce through damaged plants or root injury (Moriarty et al., 2019). Therefore, hydroponic systems could pose a risk of pathogen harborage due to continuous reuse of nutrient solution (Moriarty et al., 2019). Hydroponic practices may reduce pathogen contamination risks because the growing media has little to no contact with the edible portion of the produce (Settanni et al., 2013). However, a comprehensive review of the research demonstrated that there could be a risk of pathogen internalization within the edible portion of leafy greens in lab-scaled hydroponic systems (Riggio et al., 2019). Despite the conflicting literature, *Salmonella, E. coli* O157:H7, human noroviruses, and *Listeria monocytogenes* have been identified in hydroponically grown produce (Lopez-Galvez et al., 2014) and therefore, good practices should be promoted to ensure the quality of water used in the system.

The current study results showed that lettuce samples also tested positive for *L. monocytogenes*. Nine out of thirty-six organic bagged lettuce samples, six non-bagged organic lettuce samples and seven conventionally grown lettuce sample were *L. monocytogenes positive*, while only three hydroponic lettuce samples were positive for *L. monocytogenes*. Packaging can affect the levels of oxygen and carbon dioxide and impact the types and growth rates of microorganisms present on the produce (Oliveira et al., 2010), which may explain the reason for the fewer *L. monocytogenes* on hydroponic and bagged organic lettuce samples. The presence of *L. monocytogenes* on lettuce could be from the soil or other environmental sources, such as water, animals, and food contact surfaces (Smith et al., 2018). An outbreak of *L. monocytogenes* in nine states was linked to contaminated leafy greens, possibly including Romaine lettuce, between 2015 and 2016 (Self et al., 2016). Although fewer lettuce samples from the hydroponic system were positive for *L. monocytogenes,* one study found no differences in *L. monocytogenes* attachment between hydroponic and soil-grown lettuce samples (Kyere et al., 2019).

In the current study, lettuce samples were contaminated with *S. aureus* for all farming practices. For example, 13 out of thirty-six hydroponic lettuce samples, 12 out of thirty-six samples bagged organic lettuce, and 21 non-bagged organic and 15 conventional lettuce samples were positive for *S. aureus*. Since *S. aureus* is a human pathogen, the sources of contamination could be due to poor personal hygiene of workers (Bennett et al., 2013) or to improper handling (Standing et al., 2013). A previous study suggests that *S. aureus* has similar internalization mechanisms to other human pathogens that are also associated to leafy greens outbreaks such as, *E. coli* O157:H7, *S. Typhimurium* and *L. monocytogenes*; and this study found the potential internalization of *S. aureus* and other pathogens in lettuce grown both in soil and hydroponically (Standing et al., 2013).

These results indicate that human pathogens can contaminate fresh lettuce regardless of the farming practice selected, including hydroponic, organic, and conventional practices. Contamination could occur at any time during growing, harvesting and packaging through irrigation water, contaminated equipment, improper handling, and substrate solution (Dankwa et al., 2020). In soil-based farming, contamination could occur through soil, irrigation water, animals, environment, contaminated equipment, poor personal hygiene, and improper handling (Carstens et al., 2019).

In hydroponic farming, water is the fundamental pillar (Dankwa et al., 2020) and may become a source of pathogens. Wang, Deering, & Kim (Wang et al., 2020) investigated the presence of foodborne pathogens in aquaponics and hydroponic systems and found positive samples of Shiga toxin-producing *E. coli* (STEC) in water in both systems. Water is used in irrigation and for nutrient solution formula. In the closed hydroponic farming system, the solution is constantly recirculated (Dankwa et al., 2020). Therefore, the nutrient solution and water quality should be monitored and changed frequently to ensure optimum safety and avoid food safety hazards. Good handling practices (GHP) and good agricultural practices (GAP) are essential to minimize risks of microbial food safety hazards in hydroponic systems since any contamination could spread pathogens into the entire system. Furthermore, water quality should be monitored to ensure optimum quality and safety.

Food safety training and educational resources specific for hydroponic systems can enhance food safety knowledge. Regardless of the farming system used, education and outreach is important to address appropriate post-harvest handling practices (Kuan et al., 2017).

4. Conclusion

A key limitation of the study is that the results do not capture the microbial profile of produce immediately after harvesting. Further research is needed to investigate different environmental factors, such as closed versus open systems, and pre-harvest and post-harvest handling practices in hydroponic, organic, and conventional systems. In addition, more research is needed to investigate the prevalence and differences of microbial profile in different farming practices with larger sample size and at farm level. Furthermore, this study provides useful recommendations to serve as a basic guideline for developing food safety outreach programs for hydroponic growers and packers.

Declarations

Author contribution statement

Zahra H. Mohammad: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Isabelle do Prado: Analyzed and interpreted the data; Wrote the paper.

Sujata A. Sirsat: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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Data availability statement

Data will be made available on request.

Declaration of interest's statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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