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A Comparison of Laser Light-Scattering and Analytical Profile Index Systems for Foodborne Bacteria Identification

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ABSTRACT

Foodborne bacteria pose substantial risks to human health and food safety. Scientists worldwide have shown great interest in the development of rapid, reliable, and cost-effective methods for identifying foodborne bacteria. Among these methods, optical scattering technology (BARDOT) has emerged as the fastest and most efficient technique, offering a unique pattern of scattered light passing through the center of the bacterial colony for identification purposes. In this study, we examined 118 isolates of foodborne pathogenic bacteria, including *Escherichia coli*, *Enterobacter cloacae*, *Salmonella enterica*, *Hafnia alvei*, and *Proteus mirabilis*, derived from various food sources. To identify these isolates, we employed Analytical Profile Index (API) systems —specifically API 20E and ID 32E —which rely on biochemical tests, in addition to laser light-scattering technology. In this method, ideal colonies —which exhibited specific characteristics such as a suitable diameter, isolation from neighboring colonies, and a completely circular shape without any irregular edges —were selected to create scatter images. These scatter images revealed a distinct "fingerprint" that could be utilized to differentiate between the species. This "fingerprint" allowed for the successful identification of all isolates belonging to the five species in our current study, achieving 100% identification accuracy. Our findings demonstrated that laser light-scattering technology provided accurate identification in a cost-effective and safe manner. This method eliminated the need to open the plates containing the bacterial colonies, ensuring the colonies remained intact after identification. Furthermore, the laser light-scattering technique proved to be much more rapid compared to the API 20E and ID 32E systems, which were not only significantly more expensive but also time-consuming and labor-intensive.

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1. Introduction

Foodborne pathogens are a leading cause of diseases that significantly affect human safety and national economies. Therefore, the development of rapid and reliable techniques for detecting these pathogens is a crucial issue. The contamination of water and foodstuffs with pathogenic bacteria is considered a critical issue for human health (1). In the modern food industry, there is a great demand for rapid methods to detect foodborne bacteria because millions of individuals suffer from infections due to consuming foods contaminated with pathogens, leading to severe diseases or even death. It is estimated that there are about six hundred million cases of foodborne infections worldwide annually, with around four hundred and twenty thousand resulting in deaths (2). Some bacteria, such as *Salmonella enterica*, *Escherichia coli*, and *Staphylococcus aureus*, are considered highly virulent, as only a few cells can cause infections (3). Therefore, it is of utmost importance to develop rapid methods for the detection and identification of these organisms to prevent illnesses (4,5).

The traditional methods for bacterial detection involve several steps, including primary enrichment, growth on selective media, biochemical tests, and sometimes serological assays. These steps require a significant amount of time for results to be confirmed, because they rely on the organisms' ability to grow, divide, and produce visible colonies. Additionally, the preparation of culture media, streaking of plates, and other procedural steps make these methods labor-intensive. Modern methods for bacterial detection and identification, such as polymerase chain reaction and enzyme-linked immunosorbent assay (6,7), as well as modifications of traditional tests to expedite the process (8), have been developed. However, these techniques face obstacles such as high costs and the need for skilled operators (9). The initial use of light-scattering dates back many years and has been employed for an extended period in semiconductor inspection to detect defects on wafers. The differentiation of a sample through light-scattering relies on various characteristics, such as refractive value, shape, size, and chemical components.

When polarized homochromatic light is directed at an object (e.g., a bacterial colony), unique patterns form from scattered light, which can be utilized for identifying unknown bacteria. The system is based on the concept that variations in refractive indices and size, relative to the arrangement of cells in bacterial colonies, generate different scattering patterns (10). However, the reproducibility of this technique may be affected by colony age, culture media, growth temperature, oxygen concentration in the medium, and the concentration of bacteria suspended in the medium. Challenges arise when dealing with bacteria in suspension, including the purity of cultures and the arrangement of cells, which may appear in chains or clusters. The orientations and distances between cells change over time, necessitating an averaging method to account for relative orientation and movement. Conversely, a colony on a solid surface, such as agar, is more stable, and its optical response can be modeled using scalar diffraction theory. While optical back-scattering is widely used for wafer inspection and studying biological cells, it did not produce reproducible results when tested with bacterial colonies (11). In contrast, optical forward-scattering yielded reproducible scattering patterns (12).

The Enterobacterales comprise gram-negative bacilli, encompassing over 100 bacterial species that typically reside in the intestines of humans and animals. When part of the normal intestinal flora, they are referred to as coliforms. Pathogenic species within the Enterobacterales can cause pneumonia, urinary tract infections, wound infections, and other nosocomial (hospital-acquired) infections. Under certain conditions, they may also lead to bacteremia and meningitis. Studies have demonstrated that Enterobacterales make up the majority of aerobic or facultatively anaerobic gram-negative bacilli isolated from intra-abdominal infections, with *Escherichia coli* being the most frequently isolated species (13). *Salmonella* species, particularly *Salmonella* Typhi, pose significant health risks. *S*. Typhi is a facultative intracellular pathogen responsible for both salmonellosis and human typhoid fever, which affects over 30 million people annually worldwide. Certain species of *Enterobacter* are considered pathogenic, with notable pathogenic species including *Enterobacter cloacae*, *Enterobacter aerogenes*, and *Enterobacter agglomerans* (14).

Proteus is known to cause urinary tract infections and serves as a secondary invader, leading to septic lesions in other parts of the body. It is present in the intestines of humans and a variety of animals, as well as in manure, soil, and polluted waters. *Hafnia alvei* is found in the feces of humans and other animals, including birds. It is also present in sewage, soil, water, and dairy products, and

some of its strains may cause diarrheal diseases (15). While traditional microbiological methods are currently the primary means of identifying enteric pathogens, they are cumbersome and time-consuming, often taking several days to yield results. Furthermore, DNA sequence-based methods, while accurate, are not accessible to all food microbiology labs. This study seeks to address these limitations by creating rapid and efficient tests for identifying foodborne bacteria using laser light-scattering technology. Implementing this technology would enable the food industry to promptly evaluate the microbiological safety of its products.

2. Materials and Methods

2.1. Food sources and bacteria isolates

A total of 118 bacterial strains (57 *E. coli*, 12 *E. cloacae*, 9 *S. enterica* subsp. enterica (*S.* enterica), 13 *H. alvei*, and 27 *P. mirabilis*) were isolated from various food sources (including poultry, meat, fruits, and vegetables) obtained from local markets in Damascus and its countryside in Syria.

2.2. Isolation of bacteria species on selective media

All bacterial species, except *S.* enterica, were isolated following the method outlined by Kilonzo-Nthenge (16): 25 g of the sample was added to a sterile bag containing 225 mL buffered peptone water (BPW). The bag was then placed in an incubator at 37°C for 20 h. Subsequently, 200 µL of the broth was streaked on MacConkey agar plates (Criterion, Hardy Diagnostics, Santa Maria, CA, USA), and the plates were incubated at 37°C for 24 h. *Salmonella* spp. were isolated according to the procedure described by Harrigan et al. (1998) (17).

2.3. Morphological, staining, and biochemical tests

Morphological and biochemical tests were performed to identify pure cultures. Gram staining was carried out following the method described by Benson et al. (2001) (18). Additionally, oxidase, catalase, motility, oxidation/fermentation of glucose, and the ability to grow on MacConkey agar were assessed following the protocol described by Harrigan et al. (1998) (17). Other biochemical tests were conducted using the API 20E and ID 32E systems in accordance with the manufacturer's instructions (bioMérieux, Marcy-l'Étoile, France). The identification of bacteria based on the results of biochemical tests was accomplished using AbiWeb v4.1 (bioMérieux, Marcy-l'Étoile, France).

2.4. Identification of isolates using LLS method

The preparation of bacterial plates for LLS (laser light-scattering), based on the method by Banada et al. (2007) (12), was carried out with some modifications. Pure cultures were inoculated into Luria-Bertani broth (LBB) and incubated at 37°C for 24 h. Dilutions ranging from 10^5 to 10^9 ($CFU.mL^{-1}$) in distilled water were prepared. These culture dilutions were then plated on the surface of Luria-Bertani agar (LBA) plates with a diameter of 90 mm. Sterile glass rods were used to spread the cultures, aiming to obtain 20-30 colonies per plate. The plates were incubated at 37°C until the colony diameter reached approximately 1-1.5 mm. Colony diameter measurements were taken using an optical microscope (Olympus CX41, Japan) equipped with ×4 and ×10 objective lenses, along with an ocular lens and a digital camera (Deltapix DP 450, Deltapix Insight software, The Netherlands). The LLS instrument used in the NCBT laboratory (Figure 1) consisted of a laser source with a wavelength of 635 nm and a Cable Charged Detector (CCD) Camera. Laser scattering images were captured and stored using PhotoImpression 5 software and further processed using IMatch software v3.6.

3. Results

3.1. Identification by API 20E and ID 32E Systems

Pure cultures of bacteria, which were isolated on selective media, were reactivated by streaking on LBA plates. A single well isolated colony was removed and used to prepare the inoculum, which was then used to fill the microtubes containing dehydrated substrates. The results of the Gram stain revealed that all bacterial species were Gram-negative rods. The best-known biochemical test kits for identifying intestinal bacteria that can be used with complete identification rules are API 20E and ID 32E. The results showed that these databases are suitable for identifying the tested strains, since about 90-100% of the studied isolates were correctly diagnosed and did not require additional tests (19). The results of all other biochemical tests from the API 20E and ID 32E systems are shown in Tables 1 and 2, respectively. Table 1 presents the characteristics of different species. All species exhibited positive results for ornithine decarboxylase, NO_2 production, motility, and growth on MacConkey agar, glucose oxidation, and glucose fermentation.

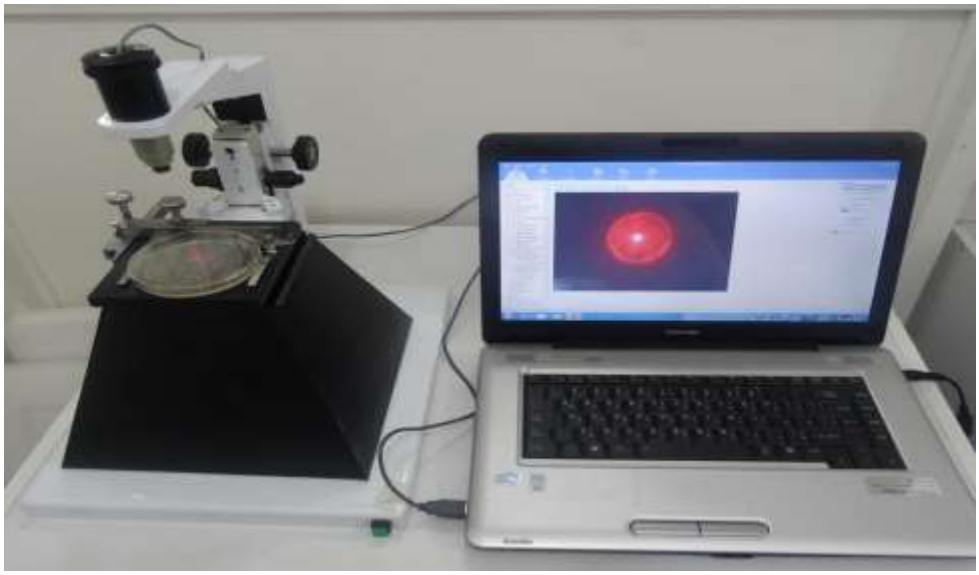

Figure 1. Laser Light Scattering instrument designed for current study.

Table 1. The results of biochemical test of API 20E.

Test	Bacterial species				
	E.coli	***E. cloacae***	***P.mirabilis***	***S. enterica***	***H.alvei***
β–Galacosidase	+	+	–	–	+
Arginine dihydrolase	–	+	–	+	–
Lysine decarboxylase	+	–	–	+	+
Ornithine decarboxylase	+	+	+	+	+
Citrate utilization	–	+	+	+	+
H_2S production	–	–	+	+	–
Urease	–	–	+	–	–
Trptophane deaminase	+	–	+	–	–
Indole production	+	–	–	–	–
Voges Proskauer	–	+	–	–	+
Gelatinase	–	–	+	–	–
Glucose fermentation/oxidation	+	+	–	+	–
Mannitol fermentation/oxidation	+	+	–	+	+
Inositol fermentation/oxidation	–	–	–	–	–
Sorbitol fermentation/oxidation	+	+	–	+	–
Rhamnose fermentation/oxidation	+	+	–	+	+
Saccharose fermentation/oxidation	+	+	–	–	–
Melibiose fermentation/oxidation	–	+	–	+	–
Amygdalin fermentation/oxidation	–	+	–	–	–
Arabinose fermentation/oxidation	+	+	–	+	+
Cytochrome oxidase	–	–	–	–	–
NO_2 production	+	+	+	+	+
N_2 production	–	–	–	–	–
Motion	+	+	+	+	+
Growth on McConkey agar	+	+	+	+	+
Glucose oxidation	+	+	+	+	+
Glucose fermentation	+	+	+	+	+
Identification accuracy	99.9%	95%	99.9%	89%	**99.9%**

Table 2. The results of biochemical tests of ID 32E system.

Test	Bacterial species				
	E.coli	***E. cloacae***	***P.mirabilis***	***S. enterica***	***H.alvei***
Ornithine decarboxylase	–	+	+	+	+
Arginine dihydrolase	–	+	–	+	–
Lysine decarboxylase	+	–	–	+	+
Urease	–	–	+	–	–
L–Arabitol acidification	–	–	–	–	–
Galacturonate acidification	+	+	–	–	+
5 Ketogluconate acidification	–	–	–	+	–
Lipase	+	–	–	+	–
Phenol Red acidification	+	–	+	+	+
ß–Glucosidase	–	+	–	–	–
Mannitol acidification	+	+	–	+	+
Maltose acidification	+	+	–	+	+
Adonitol acidification	–	–	–	–	–
Palatinose acidification	–	+	–	–	–
ß–Glucuronidase	+	–	–	–	–
Malonate	–	+	–	–	+
Indole Production	+	–	–	–	–
N–Acetyl–ß–Glucosaminidase	+	–	–	–	–
ß–Galactosidase	+	+	–	–	+
Glucose acidification	+	+	+	+	+
Saccharose acidification	–	+	–	–	–
L–Arabinose acidification	+	+	–	+	+
D–Arabitol acidification	–	+	–	–	–
α–Glucosidase	–	–	–	–	–
α–Galactosidase	+	–	–	+	–
Trehalose acidification	+	+	+	+	+
Rhamnose acidification	+	+	–	+	–
Inositol acidification	–	+	–	+	–
Cellobiose acidification	–	–	–	–	–
Sorbitol acidification	+	+	–	+	–
α–Maltosidase	–	+	–	–	–
L–Aspartic acid arylamidase	–	–	+	–	+
Identification accuracy	99.9%	99.7%	99.4%	99.9%	**99.9%**

They all showed negative results for inositol fermentation/oxidation, cytochrome oxidase, and N_2 production. *E. coli* is characterized by its negative citrate utilization and positive indole production. On the other hand, *P. mirabilis* demonstrated positive urease and gelatinase production, but negative results for mannitol, rhamnose, and arabinose fermentation/oxidation. *E. cloacae* was distinguished from other species by its ability to ferment/oxidize amygdalin. Table 2 provides additional information on the biochemical tests conducted (including ornithine decarboxylase, urease, mannitol, maltose, L-arabitol, glucose acidification, and indole production). It was observed that all species tested negative for L-arabitol, adonitol, and cellobiose acidification, while displaying positive trehalose acidification. *E. coli* exhibited positive ß-glucuronidase and N-acetyl-ß-glucosaminidase. *E. cloacae* demonstrated positive ß-glucosidase and α-maltosidase production, as well as acidification of palatinose, saccharose, and D-arabitol. *S.* Enterica showcased positive 5-ketogluconate acidification.

3.2. Identification using LLS technology

Dilutions of 10^5 to 10^9 were prepared from overnight cultures grown on LBB. Each dilution (200 µL) was spread on LBA plates and incubated at 37°C. The diameter of the resulting colonies was measured at intervals of 2 h using an optical microscope until they

reached a diameter of 1-1.5 mm. Plates with 20-30 colonies were selected, and colonies exhibiting ideal characteristics (e.g., *S.* Enterica colony, as shown in Figure 2) were tested using an LLS instrument.

The ideal colonies, which exhibited specific characteristics such as a suitable diameter, isolation from neighboring colonies, and a completely circular shape without any irregular edges, were selected to create scatter images. These scatter images, as depicted in Figure 3, revealed a distinct "fingerprint" that can be utilized to differentiate between the species. This "fingerprint" allowed for the successful identification of all isolates belonging to the five species in our current study, achieving 100% identification accuracy. The laser scattering images of all studied bacterial species exhibited regular concentric star polygons, with central wide star polygons, due to their affiliation with the same family (*Enterobacterales*), when compared, for example, to bacterial species belonging to the genus *Staphylococcus*, which displayed concentric regular circles in their laser scattering images, as shown in the following Figure 4 (20).

However, the central wide polygon varied across different species, making it a useful tool for species differentiation. In Figure 3, the laser scatter image of *E. coli* depicted the relative location of the central wide star polygon, representing one-fourth of the total scattering image (Figure 3A). On the other hand, the scattering image of *S.* Enterica was characterized by a wide central star polygon, occupying one-third of the total scattering image (Figure 3B). Additionally, the central wide star polygons in *E. cloacae* and *P. mirabilis* represented one-half and two-thirds of the total scattering images, respectively (Figure 3C and 3D). Lastly, the laser scattering image of *H. alvei* featured a central shining disc, covering one-half of the total scattering image (Figure 3E).

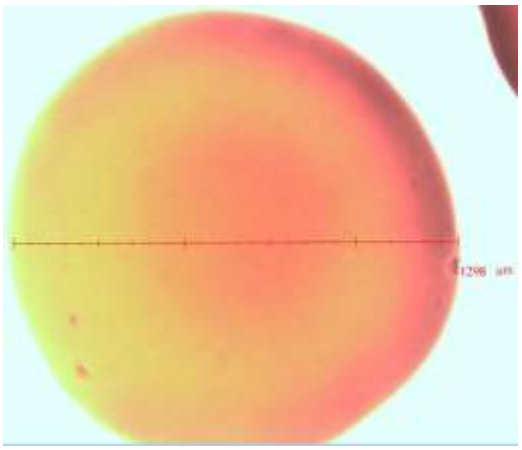

Figure 2. Ideal *S. enterica* colony (magnification ×40) with a diameter of ≈1.3 mm.

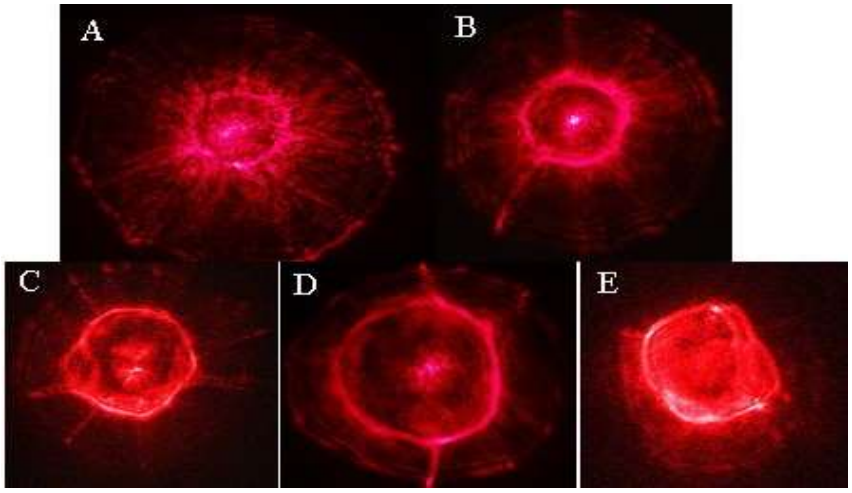

Figure 3. Scatter images of foodborne bacteria: colony diameter 1–1.5 mm, laser light wavelength 625 nm; A) *E. coli*, B) *S. enterica*, C) *E. cloacae*, D) *P. mirabilis* and E) *H. alvei*.

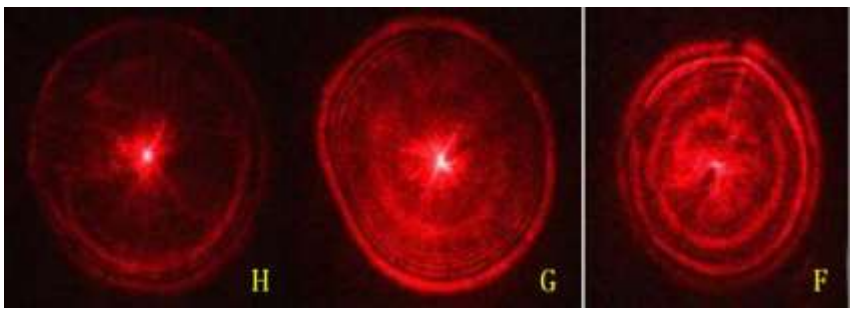

Figure 4. LLS images of *Staphylococcus* species; F) *S. aureus*, G) *S. haemolyticus* and H) *S. hominis*.

4. Discussion

Foodborne bacteria have significant effects on human health and food safety. Various methods have been developed for their detection and identification. While traditional laboratory methods are accurate, they tend to be time-consuming and labor-intensive. The API 20E and ID 32E systems have helped reduce the time required for preparing media and reagents, thus expediting the identification process by combining biochemical tests.

However, these methods pose health hazards, as they involve handling viable foodborne bacteria. Tamber et al. (2020) (21) utilized the API 20E system to identify and count *S.* Enterica in live oyster shellstock harvested from Canadian waters. Meanwhile, Budiarso et al. (2021) (22) opted for the commercial API 20E system to conduct biochemical tests on enteric bacteria (such as *E. coli*, *K. pneumoniae*, *Y. enterocolitica*, *E. cloacae*, *P. mirabilis*, among other enteric bacteria) isolated from snakes.

This decision was made after initial identification using CCA, SSA, SMAC, and DFI media, as the API 20E system offers high accuracy. Lastly, Xiong et al. (2023) (23) employed the API 20E system to biochemically characterize mutant strains of *Salmonella enteritidis*. Interestingly, no differences were observed between the mutant strains using this system. On the other hand, the LLS technique offers several advantages. It is not only a rapid, cost-effective, and accurate method for identifying foodborne bacteria, but also ensures safety by eliminating the need for direct handling of pathogenic bacteria. Identification can be achieved without direct contact, as it utilizes closed plates. Furthermore, the LLS method is non-destructive, allowing colonies to remain intact after the identification process.

Therefore, we recommend the use of the LLS methods for the identification of foodborne bacteria over traditional methods such as the API 20E and ID 32E systems. A laser light scattering system was used to identify various pathogens, but our study is the first to identify *E. cloacae*, *P. mirabilis*, and H. alvei using this system. In Hussain et al. (2020) (24) study, a laser light scattering system was developed for the identification of certain pathogens. The system comprises three main components: a laser source, a photodetector, and a data processing system. The researchers utilized this system to identify three specific species, namely: *E. faecalis*, *E. coli*, and *S. aureus*. The accuracy of the system in identifying these species was found to be 99%, 87%, and 94%, respectively.

However, it should be noted that this system was more complex in terms of design compared to the current system. Additionally, the sample preparation process was more challenging, as it required the bacteria to be mixed with 10mL of distilled water and placed inside the system's chamber. These findings are consistent with the research conducted by Banada et al. (2007) (12), who were able to differentiate between *Listeria monocytogenes* and *L. innocua* using a similar technique. Furthermore, Bhunia et al. (2009) (25) successfully discriminated between different *Salmonella serovars* using the same approach.

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Authors' Contribution

Study concept and design: B. AO, R. BAD, M. HM.
Acquisition of data: R. BAD.
Analysis and interpretation of data: R. BAD, E. I.
Drafting of the manuscript: B. AO, R. BAD.
Critical revision of the manuscript for important intellectual content: M. HM, B. AO.
Statistical analysis: R. BAD, E. I. B. AO.
Administrative, technical, and material support: R. BAD, E. I. B. AO.

Ethics

We hereby affirm that all ethical standards have been upheld in the preparation of the submitted article, in accordance with the guidelines set by the Ethics Committee of the University of of Damascus and Isfahan University of Technology, Syria and Iran.

Conflict of Interest

The authors declare that they have no conflict of interest.

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Data Availability

The data supporting this study's findings are available on request from the corresponding author.

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