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Antibiotic Resistance in Multi-Drug Resistant Pathogens

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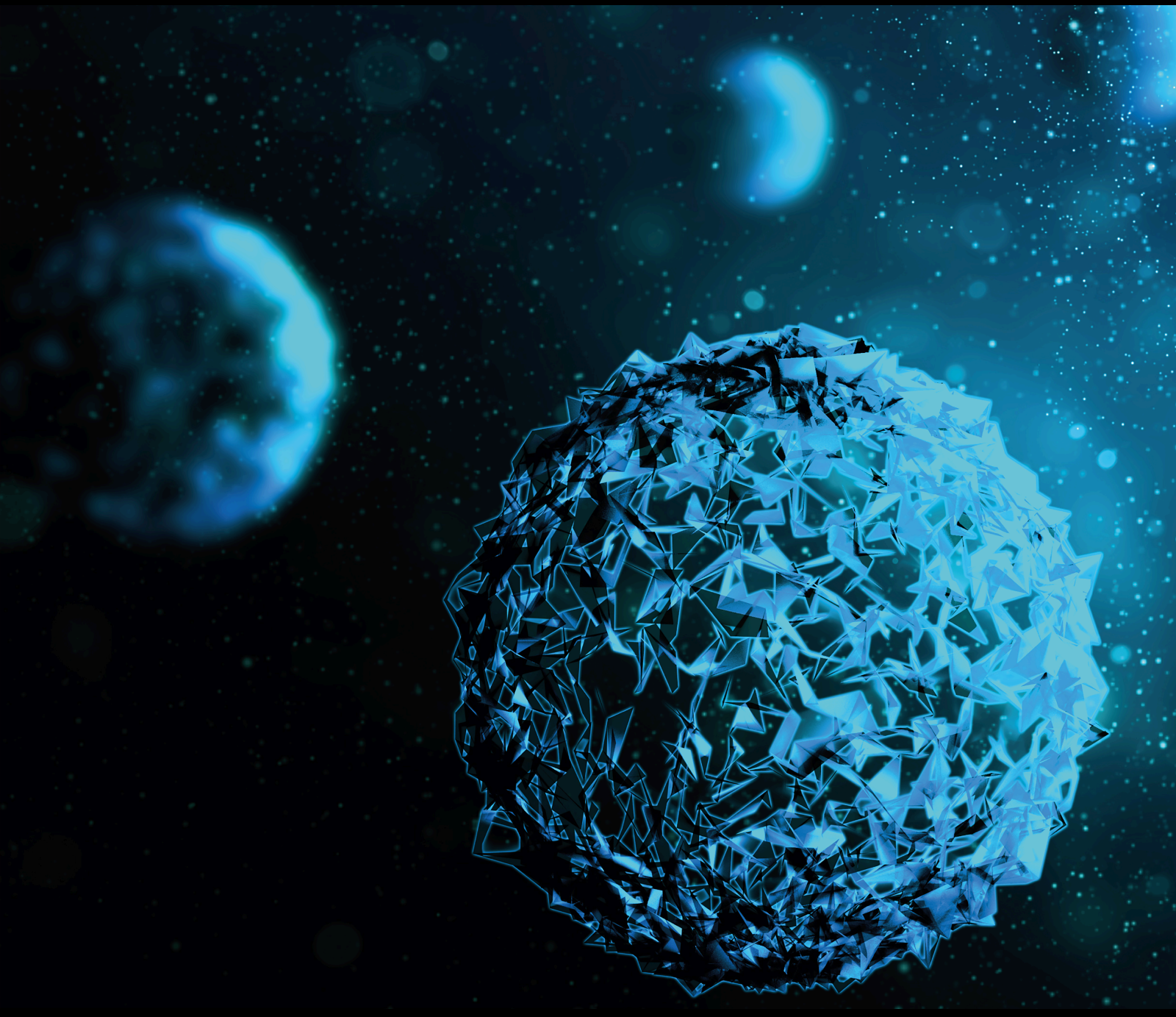

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Immunology

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Retraction

Retracted: Neuroglobin Is Involved in the Hypoxic Stress Response in the Brain

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This article has been retracted by Hindawi following an investigation undertaken by the publisher [1]. This investigation has uncovered evidence of one or more of the following indicators of systematic manipulation of the publication process:

(1) Discrepancies in scope

(2) Discrepancies in the description of the research reported

(3) Discrepancies between the availability of data and the research described

(4) Inappropriate citations

(5) Incoherent, meaningless and/or irrelevant content included in the article

(6) Manipulated or compromised peer review

The presence of these indicators undermines our confidence in the integrity of the article's content and we cannot, therefore, vouch for its reliability. Please note that this notice is intended solely to alert readers that the content of this article is unreliable. We have not investigated whether authors were aware of or involved in the systematic manipulation of the publication process.

Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

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[1] L. Shang, D. Mao, Z. Li, X. Gao, and J. Deng, "Neuroglobin Is Involved in the Hypoxic Stress Response in the Brain," *BioMed Research International*, vol. 2022, Article ID 8263373, 11 pages, 2022.

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Research Article

Examining the Extent of Contamination, Antibiotic Resistance, and Genetic Diversity of *Clostridioides* (*Clostridium*) *difficile* Strains in Meat and Feces of Some Native Birds of Iran

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Clostridioides (*Clostridium*) *difficile* (*C. difficile*) is one of the essential enteropathogens in humans and livestock and is a severe health threat, according to the Centre for Disease Control and Prevention. Also, antimicrobials are one of the most critical risk factors for *C. difficile* infection (CDI). The present study examined the infection, antibiotic resistance, and genetic diversity of the *C. difficile* strains in the meat and feces of some native birds (chicken, duck, quail, and partridge) in the Shahrekord region, Iran, from July 2018 to July 2019. Samples were grown on CDMN agar after an enrichment step. To determine the toxin profile, the *tcdA*, *tcdB*, *tcdC*, *cdtA*, and *cdtB* genes were detected via multiplex PCR. The antibiotic susceptibility of these isolates was examined using the disk diffusion method and followed based on MIC and epsilometric test. 300 meat samples of chicken, duck, partridge, and quail and 1100 samples of bird feces were collected from six traditional farms in Shahrekord, Iran. Thirty-five meat samples (11.6%) and 191 fecal samples (17.36%) contained *C. difficile*. Moreover, five toxigenic samples isolated had 5, 1, and 3 *tcdA*/*B*, *tcdC*, and *cdtA*/*B* genes. Out of the studied strains isolated from the 226 samples, two isolates belonging to ribotype RT027 and one isolated RT078 profile related to native chicken feces were observed from chicken sample. The antimicrobial susceptibility testing showed that all the strains are resistant to ampicillin, 28.57% are resistant to metronidazole, and 100% were susceptible to vancomycin. Based on the results, it can be concluded that the raw meat of birds might be a source of resistant *C. difficile* that poses a hygienic threat to the consumption of native bird meat. Nevertheless, further studies are essential to understand additional epidemiological features of *C. difficile* in bird meat.

1. Introduction

Clostridioides difficile (*Clostridium difficile*) is an anaerobic spore-forming bacterium that causes acute enteritis, colitis, and mortality particulary in susceptible people [1–3]. In 1978, this bacterium was the leading cause of antibiotic-induced diarrhea, called "antibiotic-associated diarrhea." It is also responsible for pseudomembranous colitis and patient mortality, especially in the elderly [4]. Two major toxins, A and B, are responsible for the disease. A third toxin (binary toxin) being of uncertain clinical signifiance might be encountered in several "hypervirulent" strains such as ribotype 027 (RT027) or RT078 [4].

C. difficile was an essential nosocomial pathogenic bacterium, with healthcare facility environments considered the most important sites of infection. Since 2003, the severity and mortality rate of "*C. difficile* nosocomial infection" has increased significantly in North America and many

European countries. Furthermore, several changes in bacterial epidemiology have been observed, including "community-acquired *C. difficile* infection," the occurrence of the disease in young people without risk factors, emergence of highly invasive strains, the emergence of fluoroquinolone-resistant strains, increase in disease incidence, mortality, and similarities between *C. difficile* isolated from humans and animal feces [5, 6].

Currently, *C. difficile* infection causes 250,000 hospitalizations and 14,000 deaths per year [7, 8]. The confirmation of the presence of bacteria from the feces of animals that humans consumed meat attracted many researchers to study *C. difficile* in animal meat. Based on the multiple studies on this bacteria, it was introduced as an emerging pathogen in animals used as human food [9]. Due to the use of antibiotics in animals, food can be one of the main tools for transmitting antibiotic resistance genes from animal meat to humans. Fluoroquinolone antibiotics (such as ciprofloxacin) and tetracycline are widely used in the livestock diet and are even used to treat diseases that can cause antibiotic residues in animal meat [10]. Therefore, when humans consume animal meat, this antibiotic enters the body and can indirectly predispose a person to "nosocomial infection of *C. difficile*" [11]. As a result, most studies on *C. difficile* in various food sources are dedicated to meat and meat products. For instance, a study by Heise et al. on *C. difficile* in meat indicated the presence of *C. difficile* [12]. In poultry feces, a high proportion of toxigenic *C. difficile* was described in two studies from Zimbabwe. However, the highest prevalence recorded was found in a layer farm in Slovenia (62.3%) with a high genotypic diversity of the isolates, most of them non-toxigenic [13]. High genetic diversity but low prevalence in poultry was observed in India, Austria, and the Netherlands [13]. Studies showed that *C. difficile* spores could survive at 71°C in the minimum recommended time for cooking meat [13]. It is noteworthy that food sources, especially poultry, are a critical means of transmitting pathogenic bacteria [13]. Therefore, poultry became the subject of focus in the present study. Available data from the Middle East and the Far East, including 12 studies, showed the prevalence rates of toxin genes carrying *C. difficile* in meat samples ranged from 0% to 10.8%. Still, none of these studies investigated meat from different kinds of native birds [13]. The frequent isolation of ribotypes which are also found in humans constitutes a substantial overlap and makes poultry meat a potential source for *C. difficile* infection in humans [14]. Also, an earlier report in Zimbabwe reported the incidence of *C. difficile* in poultry feces, with a prevalence of 29% in rural habitats and 17.4% in broilers [14]. Therefore, the present study is aimed at evaluating the extent of contamination, antibiotic resistance, and genetic diversity of the *C. difficile* strains in some native birds such as chicken, duck, quail, and partridge in Iran.

2. Materials and Methods

2.1. Study Procedures. This study was carried out in Shahrekord region, Iran. To do this, 300 samples of chicken, duck, quail, and partridge meat and 1100 samples of their feces were collected by the random sampling method from 6 traditional local farms in Shahrekord from July 2018 to July 2019. The samples were transported on ice to the Research Center for Nutrition and Organic Products, Islamic Azad University, Shahrekord branch, Iran.

2.2. Microbiological Analysis. To isolate *C. difficile*, 5 grams of meat and feces samples of native birds were enriched in 45 mL of *C. difficile* broth (CDB) and were anaerobically incubated at 37°C for 10-15 days. The samples were cultured on C. *diffiicle* Moxalactam–Norfloxacin (CDMN) Agar. The phenotypic experiments identified multiple colonies from each sample, including colony morphology, gram staining, colony odor, and L-proline aminopeptidase disk. E.Z.N.A.® Stool DNA Kit extracted the DNA of colonies identified by the classical method.

2.3. Molecular Analysis. Multiplex PCR was used to detect the *tcdA*, *tcdB*, *tcdC*, *cdtA*, and *cdtB* genes of toxigenic *C. difficile* isolates. Briefly, the PCR mixture consisted of 2.5 μL of PCR buffer, 2 μL of each deoxynucleotide triphosphates (dNTP) at a concentration of 10 mM, 1 unit of single DNA polymerase enzyme, 5 μL template DNA, and 0.1 μL of each primer including *tcdA*, *tcdB*, *tcdC*, *cdtA*, and *cdtB* and sterilized deionized water. The thermal cycle involved the following steps: "initial denaturation" at 94°C for one minute, annealing at 94°C for 45 seconds, annealing at 52°C for 60 seconds, and extension at 72°C for 80 seconds, based on the method introduced by Lemee et al. [15].

The PCR products were visualized by electrophoresis on 1.5% agarose gel for 1 hour at 80 V. The gel was stained with ethidium bromide solution and isolated bands were observed using UV-doc [15].

PCR ribotyping was performed using 200 μM of each dNTP mixture, 1.5 mM MgCl2, 2.5 U of Taq DNA polymerase, 50 μL of each primer, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, and 10 μL of DNA extract. The total reaction volume was 100 μL. The amplification was programmed for 30 cycles consisting of 95°C for 6 minutes in initial denaturation, 92°C for 60 seconds in denaturation, 55°C for 60 seconds in annealing, and 72°C for 6 minutes in extension steps. Amplicon product was loaded on 1.5% agarose gel for 6 hours at 80 V. Scanning by UV light was done after staining with ethidium bromide [16]. In the molecular tests, the strains of *C. difficile* ribotypes 027 and 078 were received from the Department of Pathobiology, University of Guelph, Canada, were used as positive controls.

2.4. Antibiotic Resistance Analysis. Antimicrobial susceptibility testing for different antibiotics was performed using gradient Etest (bioMérieux) and disc diffusion (Kirby Bauer). The minimum inhibitory concentration (MIC) was determined according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Interpretive breakpoints for vancomycin were based on the European Committee for Antimicrobial Susceptibility Testing (EUCAST). The inhibition zone's diameter was interpreted based on the CLSI guidelines for the disc diffusion method. This method was used for resistance testing towards amoxicillin, ampicillin,

ceftaroline, clindamycin, linezolid, meropenem, metronidazole, moxifloxacin, penicillin, pyracylene, tetracycline, and vancomycin on Mueller-Hinton agar medium according to the relevant protocols [17]. The diameter of the inhibition zone was read and interpreted after 48 hours of anaerobic incubation at 37°C. For antibiotic susceptibility testing, the innoculum was prepared at the 0.5 McFarland scale with a 24-hour young colony. These discs include amoxicillin (10 μg), ampicillin (25 μg), ceftaroline (64 μg), clindamycin (16 μg), linezolid (10 μg), meropenem (25 μg), metronidazole (8 gμ), moxifloxacin (10 μg), penicillin (10 μg), pyracylene (16 μg), tetracycline (30 μg), and vancomycin (4 μg). Based on the specification of the disks, the antibiogram test report for each antibiotic was characterized as susceptible, resistant, and intermediate.

Table 1: Contamination and genetic diversity of *C. difficile* strains in chicken, duck, quail, and partridge.

Type of native bird	Number of meat/ feces samples	Number of samples infected with *C. difficile* of meat/feces*	Toxin gene profile				
			tcdA	*tcdB*	*tcdC*	*cdtA*	*cdtB*
Chicken	90/300	22/95	+3	+3	+1	+2	+2
Duck	60/250	12/88	+1	+2	—	+1	+1
Quail	60/250	1/5	+1	—	—	—	—
Partridge	90/300	0/3	—	—	—	—	—
Total	300/ 1100	35/191	5	5	1	3	3

*Detection through L-proline aminopeptidase test for meat/feces.

3. Results

The present study observed that 35/300 bird meat samples (11.6%) and 191/1100 fecal samples (17.3%) contained *C. difficile* based on morphological examination of the obtained colonies. White-gray, opaque, circular, and slightly raised colonies indicated the presence of *C. difficile* according the morphological examination technique. Furthermore, multiplex PCR results revealed that 10 samples from chicken (06), duck (03), and quail (01) had tcdA/B gene, one sample from chicken had *tcdC* gene, and 6 samples from chiken (04) and duck (02) had *cdtA*/*B* genes. Among them, respectively, 2 and 1 ribotype profiles of 027 and 078 were observed relating to native chicken feces (Table 1).

The interpretation of antibiotic resistance was based on CLSI and EUCAST guideline. Based on the obtained antibiogram results (following EUCAST/CLSI breakpoints), the highest resistance was related to ampicillin, and the highest susceptibility was related to metronidazole and vancomycin (Table 2).

4. Discussion

The present study assessed the degree of contamination, antibiotic resistance, and genetic diversity of Clostridioides (*C. difficile*) strains in meat and feces of some indigenous birds in Shahrekord region of Iran.

From this study, the overall prevalence of *C. difficile* in bird meat samples was 11.6% and 17.3% in feces samples. These results indicate that the meat and feces of some birds collected in this study are contaminated with *C. difficile*. Different studies have been conducted on the prevalence of *C. difficile* infection in poultry [12, 14, 18–21]. A study conducted in Isfahan and Khuzestan regions showed that the prevalence of *C. difficile* in beef, cow, sheep, goat, camel, and buffalo meat was 1.3% and 2.3%, respectively [22]. In relation to the present study, it can be inferred that the prevalence of *C. difficile* in meat and animal fecal samples in Iran is a real public health problem for the population. It could be considered a threat to public health because this bacterium is able to form very resistant spores that can persist in the environment for long periods of time, facilitating its transmission. It is therefore considered an opportunistic pathogen for humans and some animal species. Methods to remove the spores are therefore necessary. For example, Heise et al. [12] showed that a wet treatment of 85°C for 20 minutes can reduce the strain-dependent spore load by at least ~4 log units and that temperatures above 85°C are necessary to completely eliminate individual *C. difficile* spores in an aqueous environment.

In Zimbabwe in 2006, a study showed that 16.6% of indigenous chicken samples and 14.28% of fecal samples were contaminated with *C. difficile* [14]. In the work of Zamani et al. [21] on quail feces and meat in Iran, it was found toxinogenic *C. difficile* strains in feces samples. However, limited attention should be given to prevalence comparisons due to differences in study design. The portage of *C. difficile* in birds and wild animals would also be related to the fact that the bacterium is ubiquitous in the environment and several animal species can be colonized by this bacterium, e.g., pets and food animals and wild animals. Thus, contaminated meat, raw vegetables, and water may play an important role as sources of human infection since studies have linked strains of *C. difficile* isolated from animals, birds, and food to those identified in humans [23]. In addition, in Slovenia, a prevalence of 62.3% (highest prevalence) has been reported in laying hens [24]. In India, a high genetic diversity among *C. difficile* strains was noted with a prevalence of 14% in poultry [25]. The prevalence of *C. difficile* in poultry is lower in Austria (2.5%) and the Netherlands (2.3%) than in the USA (8.5%) and Canada (9.2%) [26, 27]. The prevalence of *C. difficile* in Sweden and Austria is 2.7% [28]. According to studies by Weese et al. [18] and Guran and Ilhak [29], *C. difficile* was isolated from 12% and 8% of broiler meats, respectively. *C. difficile* was isolated from fecal samples in 60% of broilers on the poultry farm in Slovenia [24]. Similarly, Harvey et al. reported that *C. difficile* was isolated from 2% of chicken fecal samples and 12% of broiler meat samples [27]. It should be noted that the results of the present study are consistent with previous reports of chicken meat contamination, except that the fecal contamination rate in our study was higher.

In the present study, the *tcdA*, *tcdB*, *cdtA*, and *cdtB* genes were studied in chicken, duck, and quail because they are considered the most common genes for *C. difficile* toxin

Table 2: Examining the resistance and susceptibility of *C. difficile* strains in chicken, duck, partridge, and quail samples of current study.

Antibiotics	Range	Concentration of antibiotics	Resistance	Susceptible (percentage)	Intermediate (percentage)	Resistant (percentage)
Amoxicillin	1-10	7	Intermediate	0	18 (51.4)	24 (5.68)
Ampicillin	5-25	5	Resistant	0	0	35 (100)
Ceftaroline	6-64	58	Intermediate	5 (14.28)	18 (51.4)	12 (34.28)
Clindamycin	1-16	1	Resistant	0	11 (31.4)	24 (68.57)
Linezolid	1-10	6	Intermediate	11 (31.4)	6 (17.1)	18 (51/42)
Meropenem	5-25	19	Intermediate	11 (31.4)	12 (34.3)	12 (34.28)
Metronidazole	0.125-80	80	Susceptible	35 (100)	0	0 (0)
Moxifloxacin	1-10	5	Intermediate	12 (34.2)	12 (34.3)	10 (28.57)
Penicillin	1-10	6	Intermediate	0	30 (85.7)	5 (14.28)
Pyracylene		13	Intermediate	12 (34.2)	12 (34/3)	11 (31.42)
Tetracycline		22	Intermediate	11 (31.4)	14 (42.9)	9 (25.71)
Vancomycin	0.25-4	4	Susceptible	35 (100)	0	0

typing. According to Weese et al. [18], all strains isolated from broiler meat (12% of total samples) had genes encoding toxins A and B. *C. difficile* was isolated from 26/203 (12.8%) chicken samples; 10/111 (9%) thighs, 13/72 (18%) wings, and 3/20 (15%) legs and all isolates were ribotypes 078 [18]. Three strains of *C. difficile* were noted to possess the *tcdA* and *tcdB* genes, 2 for ribotype 027, and one for the 078-profile linked to native chicken feces (Table 1). The results show that less than one percent of the birds carry these ribotypes, and these data corroborate other previous observations [26, 30–32]. It is worth remembering that ribotypes 027 and 078 are the most virulent and the diarrheal outbreaks due to *C. difficile*. Ribotypes 027 and 078 are often associated with infectious diarrhea in Iranian hospitals [33]. Three toxigenic strains were identified in this study. These results shows that measures must be taken against the dissemination of virulent and toxigenic strains in order to avoid diarrheal epidemics linked to these strains. In addition, the frequent isolation of ribotypes constituted a significant overlap, making poultry meat a potential source of *C. difficile* infection in humans [32–35]. From these data, it could be inferred that consumption of broilers and contaminated chicken meat could be a source of human disease. The *tcdC* gene was found in only one strain. According to the literature, this gene is present in all toxigenic strains. This result is probably due to the technique used or the working conditions. But it also opens other research perspectives with advanced techniques to detect this gene in identified isolates, such as whole-genome sequencing.

Antimicrobial resistance of *C. difficile* is highly variable in different birds and countries [6]. The results of the antibiotic susceptibility study of isolated bacteria showed that *C. difficile* strains exhibited a high rate of resistance to ampicillin and susceptibility to metronidazole and vancomycin. Although the susceptibility of *C. difficile* strains is studied, there is a lack of information on resistance to fidaxomicin, meropenem, and piperacillin/tazobactam [36]. According to Saha et al. [37], *C. difficile* resistance to vancomycin is increasing. Metronidazole and vancomycin are antibiotics to treat *C. difficile* infections. Although resistance to metronidazole and vancomycin is not yet a major problem, the reduced sensitivity to these antibiotics has progressively increased, which underlines the need for constant monitoring and regulation of the use of these molecules. Low resistance to tetracycline, clindamycin, and moxifloxacin was noted in this study. These results are contrary to those of Heidari et al. [38] who obtained high level resistance of tetracycline, clindamycin, and moxifloxacin.

The limitation of the present study is the lack of accessibility to reference profiles of *C. difficile* for a complete comparison of the isolates found with the frequently used international profiles for poultry such as RT001, RT002, RT014, and RT020. This study is also limited by the fact that it covers only Shahrekord region, Iran.

5. Conclusion

The current study concluded that feces and meat of poultry and native birds, including chicken, duck, quail, and partridge, can be sources of pathogenesis through *C. difficile* in Iran. The consumption of this group of animals is the favorite of Iranian people. Therefore, appropriate cooking of these animals is recommended. More studies are suggested to understand the different aspects of the epidemiology of *C. difficile* in Iran.

Data Availability

All data generated or analyzed during this study are included in this article.

Additional Points

Novelty Impact Statement. (i) *C. difficile* prevalence in meat samples was 11.6% and in fecal samples was 31.6%. (ii) The highest susceptibility for *C. difficile* isolates was related to metronidazole.

Conflicts of Interest

The author declares that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Authors' Contributions

Akbar Ansarian Barezi was involved in the formal analysis, methodology, investigation, writing—original draft, and writing—review and editing. Amir Shakerian was involved in the supervision, writing—original draft, and writing—review and editing. Ebrahm Rahimi was involved in the supervision and writing—review and editing. Zahra Esfandiari was involved in the supervision, resource acquisition, project administration, validation, visualization, methodology, and writing—review and editing.

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Research Article

Distribution of Virulence Genes in *Campylobacter* spp. Isolated from *Agaricus* Mushrooms in Iran

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The white button mushroom (*Agaricus*) is a significant nutritional and therapeutic species utilized in the human diet and could transmit various bacterial infections. *Campylobacter* species are the most common cause of foodborne illness across the world. The present study has been planned to determine the frequency of virulence genes and antibiotic susceptibility test in *Campylobacter* spp. recovered from *Agaricus* mushroom. In this study, 740 *Agaricus* mushroom samples were gathered randomly from various markets from June 2020 to December 2020. Confirmation of *Campylobacter* spp. using biochemical analyses and *23S rRNA*-based PCR was performed. The agar dilution technique was used to determine resistance to antibiotics using gentamicin (GM10μg), ciprofloxacin (CIP5μg), nalidixic acid (NA30μg), tetracycline (TE30μg), ampicillin (AM10μg), amoxicillin+ clavulanic acid (AMC30μg), erythromycine (E15μg), azithromycin (AZM15μg), clindamycin (CC2μg), and chloramphenicol (C30μg). Multiplex PCR was utilized to determine the prevalence of the *recR*, *dnaJ*, *wlaN*, *virBll*, *cdtC*, *cdtB*, *cdtA*, *flaA*, *cadF*, *pidA*, *ciaB*, *ceuE*, and *cgtB* genes. *Campylobacter* spp. were detected in 74 out of 740 *Agaricus* mushroom samples (10%). According to the data, *Agaricus* mushroom samples included 32 (4.32%) *C. jejuni*, 11 (1.48%) *C. coli*, and 31 (4.18%) other *Campylobacter* spp. Antimicrobial resistance was most common in *C. jejuni* isolates. *C. jejuni* isolates also had the lowest resistance rate to gentamycin, ciprofloxacin, and nalidixic acid. *C. coli* isolates were reported to have the highest antimicrobial resistance to ciprofloxacin, ampicillin, and erythromycine. Resistance to gentamycin and amoxicillin+ clavulanic acid was likewise lowest among *C. coli* strains. The *flaA* and *ciaB* genes were found in 100% of B-lactams-susceptible *C. jejuni* and *C. coli* strains. When examining the relationship between antibiotic resistance and the existence of virulence genes, it was observed that there is a statistically significant relationship ($p < 0.001$) between bacterial resistance and virulence genes. Our findings indicated that changes in resistance patterns in *Campylobacter* strains have emerged from multiple treatment approaches in *Agaricus* mushrooms.

1. Introduction

Mushrooms have been utilized in food and medical supplies for decades and have become a vital element of human nutrition. Due to their high-quality nutrients, carbohydrates, enzymes, essential fatty acids, dietary fibers, and low-calorie content, these substances have an appealing taste, fragrance, and nutritive quality [1]. Their production and sales have increased dramatically in recent years all over the globe. Mushrooms are regarded as a regular diet and a supplier of nutritional supplements because of their favorable impact on humans due to their presence of bioactive components and nutritional ingredients [2]. The white button (*Agaricus*) mushroom is among the essential edible mushrooms that have long been sought by people searching for food. The Chinese recognized the advantages of mushrooms, believing that mushrooms enhance the physical organs and prolong vitality and vigor [3]. This mushroom is being utilized as a

diet and medicinal plant in Iran and other nations. *Agaricus* mushrooms are particularly fragile because they lack a defensive cuticle, have a rapid breathing function, and contain much wetness. Consequently, they are subjected to mechanical degradation, pathogen assault, weight loss, and caramelization, resulting in a fast-postharvest reduction in quality. *Agaricus* mushrooms have a storage life of one to three days at room temperature (20–25°C) and five to seven days under a refrigerator (4°C) [4].

Mushrooms are ideal hosts for a wide range of pathogens. Various microorganisms grow on the surface of mushrooms that might infect humans. One of the most significant bacteria is *Campylobacter* [5]. *Campylobacter* spp., a primary cause of bacteremia gastroenteritis in mammals, are frequently found in animal digestive tracts and, through fecal pollution, in animal-derived products. Raw milk, chickens, and beef have all been related epidemiologically to epidemics of *Campylobacter* infection in humans [6, 7]. *Campylobacter* can be identified in 1 to 1.5% of agricultural packaged food. As a result, several items that are epidemiologically linked to *Campylobacter* outbreaks have been recognized as responsible for the transmission of the *Campylobacter* genera [8]. The Seattle-King Health Center researched to quantify the migration of *Salmonella* spp. and *Campylobacter* spp. from animals to humans through the food supply chain and discovered that people with *Campylobacter* enteritis ate mushrooms [9]. As a result, there seemed to be an increased relative incidence of *Campylobacter* infection in humans in people who consumed mushrooms. Our goal was to see if raw mushrooms are a reservoir of *Campylobacter* spp. [10]. Freshly *Agaricus* mushroom packets sold at local supermarket shops are high in *Campylobacter* [11].

Campylobacter can cause bladder infections, pneumonia, or neuropathies, including septic arthritis, Guillain-Barre syndrome (GBS), inflammatory bowel disease, and Miller-Fisher syndrome (MFS). The Agaricus mushroom population causes approximately 40% of people's problems. Recent genetic investigations have shed insight on the primary virulence components involved in the *Campylobacter* strain's virulence. Furthermore, the capacity of *Campylobacter* to attach via the *cadF*, *racR*, *virB11*, *pldA*, and dnaJ proteins infiltrates intestinal mucosa cells by the *ciaB* and *ceuE* genes and manufactures toxins via the *cdtA*, *cdtB*, and *cdtC* genes [12]. Even though *Campylobacter* diseases are typically self-limiting and do not require treatment, antimicrobial therapy is not necessary in most cases of protracted disease in humans and septicemia. Also, it is essential in some types of sepsis. The selection medications for treating human *campylobacter*iosis include macrolides (erythromycin and azithromycin), fluoroquinolones (ciprofloxacin), and tetracyclines. The primary reasons for rising susceptibility patterns are the overuse of antibiotics in human illnesses and abusing antibacterial medications in animal farming to treat animal diseases or boost growth by adding antibiotics to food within the *Campylobacter* genus [13]. Antimicrobial resistance is caused by several biological processes that have been well-documented. The chloroquine inhibition domain of the DNA gyrase gene, *gyrA*, is primarily responsible for fluoroquinolone resistance. The acquired *tet(O)* gene, which codes for a defensive ribosomal polypeptide, is usually linked to high tetracycline resistance. Mutations in the V motif of the *23S rRNA* gene and stimulation of the *CmeABC* multidrug efflux system are commonly linked to antibiotic resistance to macrolides. Antibiotic-resistant genes like *erm(B)*, *aadE*, or *sat4* (streptomycin/streptothricin resistance), *blaOXA-61* (b-lactams resistance), and *aphA-3* (aminoglycosides resistance) have also been linked to multidrug resistance in *Campylobacter* isolates [14]. Numerous studies have found a relationship between virulence and antibiotic susceptibility in bacterial infections, implying a correlation between antibiotic resistance and the bacteria's ability to colonize or invasion. This link has been investigated, and some studies have found that infection with antibiotic-resistant *Campylobacter* isolates in people is linked to a prolonged length of diarrhea [15, 16]. In this research, we focused on the incidence of *Campylobacter* infection in *Agaricus* mushrooms in Iran and the infection distribution and antimicrobial resistance in collected isolates. As a result, this analysis is aimed at looking into the genetics of antimicrobial resistance (AMR) and finding virulence indicators in *Campylobacter* spp. samples from *Agaricus* mushrooms.

2. Methods

2.1. Biochemical Identification of Campylobacter spp. In this investigation, 740 *Agaricus* mushroom samples were gathered randomly from various markets from June 2020 to December 2020 and transferred to the laboratory. The selection represented the two varieties of *Agaricus* mushroom brands produced in Iran. Samples were placed below 4°C during transportation, and testing was performed immediately after receiving the samples. When the *Agaricus* mushroom trends turned up in the lab, they were torn into pieces and blended in a sterile folder with a sterilized swab stick, and roughly 1 g of each template was homogenized in enrichment broth and incubated for 24 hours at 37°C. Homogenized solution was then scrubbed onto (predried) *Campylobacter* agar base (Sigma-Aldrich, Germany) plates supplemented with Karmali selective complement SR0167E (Sigma-Aldrich, Germany). Samples were incubated for 48 hours at 42°C in an anaerobic jar with a gas-generating sachet (Oxoid–CampyGen™) to establish a microaerophilic environment for the growth of *Campylobacter* spp.

On 24–48 h Karmali agar plates, colonies with a clean, flat, colorless, transparent to the grey appearance of diameters of 1 mm were chosen. Since colonies are frequently mixed with other microorganisms, the mobility of suspected bacteria was examined using a section contrast microscope. Colonies exhibiting exceptional mobility were isolated on 5% horse blood agar plates and incubated for 48 hours in an anaerobic jar with a fuel-producing sachet to generate microaerophilic conditions. Gram-negative, catalase-positive, and oxidase-positive samples were snap-frozen in glycerol broth at -70°C for further molecular characterization (PCR), antibiotic sensitivity testing, and resistance gene identification [17].

2.2. Confirmation of Campylobacter spp. Using 23S rRNA-Based PCR

2.2.1. DNA Extraction. In an anaerobic jar with a gas-generating pouch, frozen *Campylobacter* isolates were grown on 5% horse blood agar plates and incubated for 48 hours at 42°C. The bacterial cells were taken from the plates and put into Eppendorf vials containing 200 μL of sterile water when they had grown sufficiently. The suspensions were heated for 8 minutes at 98°C on a boiling tube. The supernatant was collected and was then placed into sterile microcentrifuge tubes and centrifuged at 17000 g for 5 minutes to serve as a genetic DNA material for the following polymerase chain reaction (PCR). The quality (A260/A280) and amount of the extracted DNA were next measured at an optical density of 260/280 nm using a spectrophotometer (NanoDrop, Thermo Scientific, Waltham, MA, USA). The DNA's validity was tested on a 1.5% agarose gel stained with ethidium bromide (0.5 g/mL) (Thermo Fisher Scientific, St. Leon-Rot, Germany). The polymerase chain reaction (PCR) was carried out using a PCR thermal cycler (Eppendorf Co., Hamburg, Germany) according to the Tohid and Shandiz technique [18].

2.2.2. Molecular Identification of Campylobacter Species. Corroborating the identity established using biochemical techniques, primers encoding the *Campylobacter* genus-specific *23S rRNA* gene and species-specific sequences of *Campylobacter jejuni*, *Campylobacter coli*, and other *Campylobacter* strains were utilized (Table 1). Five microliters of 5x PCR buffer, 4 mmol^{-1} MgCl2, 2 μL of 2 mmol^{-1} deoxynucleotide triphosphate (dNTPs), 0.5 μL of 25 pmol each of oligonucleotide primers, and 1 U of *Taq* polymerase (Promega) with 2 μL DNA template were included in the precursor solution. The volume was adjusted to 25 μL using deionized water. PCR conditions for *23S rRNA* gene detection were 30 cycles of denaturation at 95°C for 30 seconds; annealing at 46°C temperatures for 30 seconds, elongation at 72°C for 30 seconds, and final extension at 72°C for 7 minutes were used in the amplification. Electrophoresis with a 1.5% agarose gel including ethidium bromide was used to examine the PCR results [17, 18].

2.3. Antibiotic Resistance Analysis. The Kirby-Bauer procedure was used for antimicrobial disk susceptibility test. The Mueller-Hinton agar (Zist. Rouyesh, Tehran, Iran) plates containing 5% defibrinated sheep blood as a medium was used to evaluate antibacterial sensitivities to various antibacterial drugs in a twofold serial dilution tend to range from 0.063 to 128 μg/mL^{-1} depending on the Medical and Laboratory Standard rules (M100) [17]. The inocula were made by swabbing two to three colonies from 24 h culture into a sterile 0.85% sodium chloride (NaCl) solution to create a cell suspension that matched the 0.5 McFarland threshold. This seed contained about 2×10^8 CFU mL^{-1}, which was then adjusted 1 : 10 to achieve a ratio of 10^7 CFU mL^{-1}. This was injected onto multiple plates holding varied antimicrobial doses using a multipoint inoculator. To allow the *Campylobacter* spp. to proliferate, the cultures were kept at 42°C for 24 hours in an anaerobic jar with a gas-generating pouch (Oxoid–CampyGen™). The following antibiotics were used in this study: gentamicin (GM10μg), ciprofloxacin (CIP5μg), nalidixic acid (NA30μg), tetracycline (TE30μg), ampicillin (AM10μg), amoxicillin+ clavulanic acid (AMC30μg), erythromycine (E15μg), azithromycin (AZM15μg), clindamycin (CC2μg), and chloramphenicol (C30μg) [19].

2.4. Virulence Encoding Gene Detection. Multiplex PCR was used to determine the prevalence of the *recR*, *dnaJ*, *wlaN*, *virbll*, *cdtC*, *cdtB*, *cdtA*, *flaA*, *cadF*, *pidA*, *ciaB*, *ceuE*, and *cgtB* genes [20–23]. The primers and PCR conditions used to genotype the *recR*, *dnaJ*, *wlaN*, *virbll*, *cdtC*, *cdtB*, *cdtA*, *flaA*, *cadF*, *pidA*, *ciaB*, *ceuE*, and *cgtB* alleles are listed in Table 1. This amplified procedure was used in a multiplex PCR: 5 minutes of initial denaturation at 94°C, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 54°C for 30 seconds, and elongation at 72°C for 1 minute. In a final reaction volume of 25 μL, each solution contained 4 mmol^{-1} MgCl2, 1 μL of 25 pmol per primer, 2 μL of 2 mmol^{-1} dNTPs, and 4 μL of 5x PCR-buffer, as well as 1 U of *Taq* polymerase (Promega) and 2 μL DNA template. After that, the PCR electrophoresis was performed on a 1.5% agarose gel with ethidium bromide in a 1x TBE buffer. By measuring the sizes of the individual amplicons to a 100 bp ladder, the lengths of the different amplicons were established.

2.5. Statistical Analysis. The frequency of virulence genes was compared among species of bacteria (*C. jejuni* vs. *C. coli*) using a multivariate analysis of variance (ANOVA) with the number of genes as the dependent variable and the *Campylobacter* species as the factor analyzed with SPSS statistical software (version 24). When the p value was less than 0.05, the results appeared significant.

3. Results

3.1. Frequency of Campylobacter spp. The prevalence of *Campylobacter* spp. was investigated in 740 *Agaricus* mushroom specimens. To quickly identify *Campylobacter* spp., the Gram-staining, catalase, and oxidase analyses were utilized. After incubation, the 74 positive *Campylobacter* spp. were recognized by catalase and oxidase tests, which produced a purple hue, a blue/purple tint, and the formation of oxygen bubbles, respectively. *Campylobacter* spp. were detected in 74 out of 740 *Agaricus* mushroom samples (10%). According to the data, *Agaricus* mushroom samples included 32 (4.32%) *C. jejuni*, 11 (1.48%) *C. coli*, and 31 (4.18%) other *Campylobacter* spp.

All of the organisms were confirmed using PCR amplification of the *23S rRNA* gene. All 74 isolates were positive for *Campylobacter* spp. according to PCR data. *C. jejuni* (4.32%) and *C. coli* (1.48%) had the highest prevalence of *Campylobacter* spp. bacteria, whereas other *Campylobacter* spp. were detected in 31 samples (5.33%). There was a significant statistical difference ($p < 0.05$) between the samples and the prevalence of *Campylobacter* infections. In this study, the results demonstrated that biochemical test identification

Table 1: Primers of antibiotic resistance genes, annealing temperatures, and size of amplicons.

Gene	Primer sequences (5′-3′)	Annealing temperatures (°C)	Product size (bp)
23S rRNA	*23S rRNA*: TTAGCTAATGTTGCCCGTACCG ERY2075: TAGTAAAGGTCCACGGGGTCGC	46	485
recR	F: GATGATCCTGACTTTG R: TCTCCTATTTTTACCC	49	584
dnaJ	F: AAGGCTTTGGCTCATC R: CTTTTTGTTCATCGTT	53	720
wlaN	F: TTAAGAGCAAGATATGAAGGTG R: CCATTTGAATTGATATTTTTG	46	672
virbll	F: TCTTGTGAGTTGCCTTACCCCTTTT R: CCTGCGTGTCCTGTGTTATTTACCC	48	494
cdtC	F: CGATGAGTTAAAACAAAAAGATA R: TTGGCATTATAGAAAATACAGTT	47	182
cdtB	F: CAGAAAGCAAATGGAGTGTT R: AGCTAAAAGCGGTGGAGTAT	51	620
cdtA	F: CCTTGTGATGCAAGCAATC R: ACACTCCATTTGCTTTCTG	55	370
flaA	F: AATAAAAATGCTCATAAAAACAGGTG R: TACCGAACCAATGTCTGCTCTGATT	55	855
cadF	F: TTACTCCTACACCGTAGT R: AAACTATGCTAACGCTGGTT	45	283
pidA	F: AAGCTTATGCGTTTTT R: TATAAGGCTTTCTCCA	45	913
ciaB	F: TGGGCAGATGTGGATAGAGCTTGGA R: TAGTGCTGGTCGTCCCACATAAAG	44	284
ceuE	F: CCTGCTACGGTGAAAGTTTTGC R: GATCTTTTTGTTTTGTGCTGC	48	793
cgtB	F: TAAGAGCAAGATATGAAGGTG R: GCACATAGAGAACGCTACAA	50	561

accuracy was not significantly different from molecular PCR test accuracy ($p < 0.05$).

3.2. Antibiotic Susceptibility Test of Campylobacter Isolates. Antimicrobial resistance profiles of *Campylobacter* isolates were recovered from multiple specimens (Tables 2 and 3). *C. jejuni* isolates exhibited high resistance against tetracycline, ampicillin, amoxicillin+ clavulanic acid, and erythromycine. On the other hand, *C. jejuni* isolates had the lowest rate of resistance to gentamycin, ciprofloxacin, and nalidixic acid. Furthermore, a significant percentage of resistance to antibiotics azithromycin (40.62%), clindamycin (25%), and chloramphenicol (37.5%) was observed in different isolates. There was a statistical difference between the specimens and antimicrobial resistance incidence ($p < 0.05$).

C. coli isolates were reported to have the highest antimicrobial resistance against ciprofloxacin (72.72%), ampicillin (72.72%), and erythromycin (72.72%). However, the resistance to gentamycin (0%) and amoxicillin+ clavulanic acid (27.27%) was noticed to be lowest among *C. coli* strains. The results, on the other hand, revealed that both *C. coli* and *C. jejuni* isolates were completely sensitive to antibiotics gentamycin.

3.3. Prevalence of Virulence Factors. The rates of virulence genes among resistant isolates of *C. jejuni* were as follows for *recR*, *dnaJ*, *cdtC*, *cdtB*, *cdtA*, *flaA*, *cadF*, and *ciaB*, respectively. The *flaA* and *ciaB* genes were found in 100% (32/32) of *C. jejuni* strains when these genes were tested in susceptible isolates. The frequency of these genes in *C. jejuni* was noticed to be the lowest when the *wlaN*, *virbll*, and *ceuE* genes were examined (Table 3).

Table 3 shows the genotype distribution of *C. coli* isolates collected from various types of specimens. *FlaA* and *ciaB* were the most prevalent genotypes observed among *C. coli* recovered from mushroom (100%). *DnaJ*, *wlaN*, *virbll*, and *ceuE* were the *C. coli* strains found with the lowest frequency in samples (0%). The *recR*, *cdtC*, *cdtB*, *cdtA*, *cgtB*, *cadF*, and *pidA* genes were also discovered in a variety of *C. coli* isolates. These genes were found in between ten and forty percent of the population. There was a significant difference ($p < 0.05$) between the types of samples and the occurrence of genotypes.

3.4. Association of Virulence Genes with the Antibiotic Resistance Pattern. In terms of the association between the existence of virulence genes and antibiotic susceptibility/resistance profiles across strains, resistant *C. jejuni* isolates had more virulence-related genes than sensitive ones. Tetracycline-resistant isolates had more virulence genes than nalidixic acid and gentamycin-resistant strains isolated. The antibiotics GM10 and the *virBll* and *wlaN* genes, which

Table 2: Antibiotic resistance pattern of *C. jejuni* and *C. coli* isolates recovered from *Agaricus* mushroom.

	Antibiotics	*GM10*	*CIP5*	*NA30*	*TE30*	*AM10*	*AMC30*	*E15*	*AZM15*	*CC2*	*C30*
C. jejuni	Number and the percentages of the isolates	2 (6.25%)	6 (18.75%)	6 (18.75%)	15 (46.87%)	16 (50%)	13 (40.62%)	17 (53.12%)	13 (40.62%)	8 (25%)	12 (37.5%)
C. coli		0 (0%)	8 (72.72%)	5 (45.45%)	4 (36.36%)	8 (72.72%)	3 (27.27%)	8 (72.72%)	4 (36.36%)	5 (45.45%)	5 (45.45%)

Legend: gentamicin (GM10μg), ciprofloxacin (CIP5μg), nalidixic acid (NA30μg), tetracycline (TE30μg), ampicillin (AM10μg), amoxicillin+ clavulanic acid (AMC30μg), erythromycine (E15μg), azithromycin (AZM15μg), clindamycin (CC2μg), and chloramphenicol (C30μg).

Table 3: Prevalence of virulence factors in *C. jejuni and C. coli.*

	recR	*dnaJ*	*wlaN*	*virbll*	*cdtC*	*cdtB*	*cdtA*	*flaA*	*cadF*	*pidA*	*ciaB*	*ceuE*	*cgtB*
C. jejuni	27 (84.37%)	26 (81.25%)	3 (9.37%)	3 (9.37%)	26 (81.25)	26 (81.25%)	22 (68.75%)	31 (96.87%)	24 (75%)	18 (56.25%)	32 (100%)	13 (40.62%)	9 (28.12%)
C. coli	2 (18.18%)	0 (0%)	0 (0%)	0 (0%)	2 (18.18%)	2 (18.18%)	2 (18.18%)	11 (100%)	7 (63.63%)	7 (63.63%)	11 (100%)	0 (0%)	11 (45.45%)

Legend: virulence genes (*recR*, *dnaJ*, *wlaN*, *virbll*, *cdtC*, *cdtB*, *cdtA*, *flaA*, *cadF*, *pidA*, *ciaB*, *ceuE*, and *cgtB*).

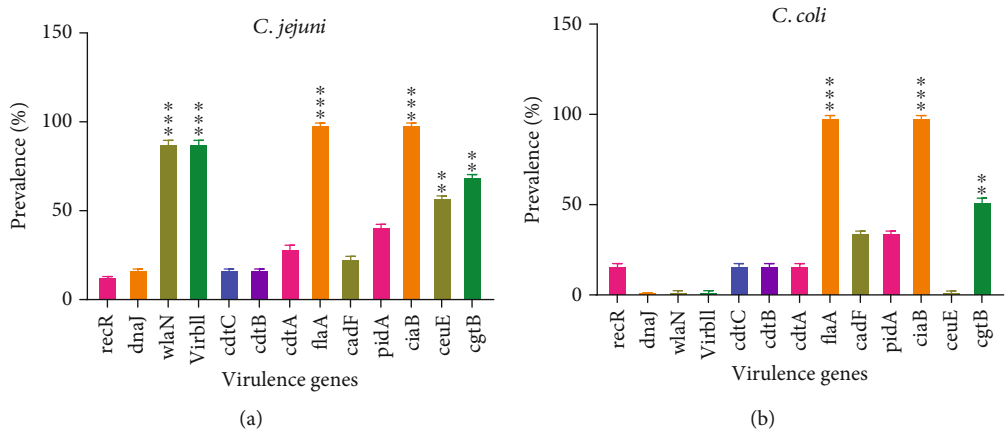

Figure 1: The prevalence of virulence genes in susceptible *C. jejuni* (a) and *C. coli* (b) isolates. Legend: virulence genes (*recR*, *dnaJ*, *wlaN*, *virbll*, *cdtC*, *cdtB*, *cdtA*, *flaA*, *cadF*, *pidA*, *ciaB*, *ceuE*, and *cgtB*). When researchers looked at the association between resistance to antibiotics and the presence of genetic variants, they discovered a positive significant association ($^{***}p < 0.001$) among bacterial resistance and the presence of virulence genes.

exhibit no variability (100% resistance), were omitted from the study. When examining the relationship between antibiotic resistance and the existence of virulence genes in *C. jejuni* isolates, it was shown that there is a statistically significant relationship ($p < 0.001$) between bacterial resistance and the existence of virulence genes (Figure 1(a)). The medicines GM10 were excluded out of the *C. coli* investigation, as were the *dnaJ*, *virbll*, *ceuE*, and *wlaN* genes, which had no variability (100% resistance). When researchers looked at the association between resistance to antibiotics and the presence of genetic variants in *C. coli* strains, they discovered a positive significant association ($p < 0.001$) among bacterial resistance and the presence of virulence genes (Figure 1(b)).

4. Discussion

Campylobacter bacteria cause foodborne infections, and MDR variants are a severe health problem. There has been no information on pathogenic molecular features of regional *Campylobacter* isolates because there have been few investigations on the bacteria in Iran [24]. As a result, the presence of virulence in *Campylobacter* genera isolated from *Agaricus* mushrooms was investigated in this work. Several researchers worldwide have found that gene expression related to mobility, colonization, epithelium invasion, and toxin generation is crucial in the development of *Campylobacter*-related illnesses [25–28]. Most of the strains in this investigation were found to have associated virulence genes linked to pathogenic adherence, colonization, and invasive features. This was in line with prior research, which had found *flaA*, *ciaB*, *racR*, *virB11*, and *pldA* to be often prevalent [23].

Furthermore, as described by various investigations [29, 30], the *cdtA*, *cdtB*, and *cdtC* alleles required to produce the CDT toxic substance were found in all *Campylobacter* strains. Concerning *C. jejuni*, 59% of the examined strains had the *ceuE* gene, which confers the ability to chelate iron. The *wlaN* gene was found in 90% of *Campylobacter* strains, consistent with research conducted in Iran [31], which found a high incidence of isolated *Campylobacter* strains (82.22%). Furthermore, the *cgtB* gene was found in 54% of *C. coli* and 71% of *C. jejuni*. As a result, we believe that the high prevalence of these alleles among the tested samples might imply their significant pathogenic capability and high danger to human health. Lipooligosaccharide of *Campylobacter* (LOS), similar to gangliosides in neurons, is considered a critical factor in the initiation of GBS neuropathies and Miller-Fisher syndrome after *C. jejuni* infection [31]. The higher prevalence of these genes may be associated with GBS in humans. Antibiotic resistance, a global concern for animal and human health, has received much attention. Because of the extensive utilization of antibiotics in the food sector, antibiotic resistance has become a significant problem [32]. Previous research has found that *Campylobacter* has a high antimicrobial resistance to various drugs [33–40]. Antimicrobial resistance patterns correspond well with the presence of genes expressing resistance to antibiotics, according to a study examining the genetic basis of antibiotic resistance in isolates tested [34, 35].

Our findings demonstrated that multiple virulence factors are related to resistant bacteria when examining the frequency of virulence genes and antibiotic susceptibility. Indeed, the detection of *cadF* and *ciaB* in amoxicillin/clavulanic acid-resistant bacteria, *ciaB* in ampicillin-resistant bacteria, *racR* in nalidixic acid-resistant isolates, and *cadF* and *ceuE* in chloramphenicol-resistant isolates was linked. Although the presence of positive or negative relationships among antibiotic resistance and virulence genes in microorganisms has been demonstrated, the *Campylobacter* species remains contentious [41–43]. *In vitro* experiments have shown that resistant bacteria invade more than susceptible strains, whereas others have highlighted the tendency of susceptible strains to produce more serious diseases than resistant organisms. In the current investigation, virulence genes and antibiotic resistance among *C. jejuni* samples were shown to have some favorable relationships ($p > 0.05$). A few virulence genes linked to antimicrobial-resistant *C. jejuni* isolates are implicated in bacterial attachment and invasion,

indicating that resistant strains have more adherence and attack potential than susceptible strains. Further research is needed to understand the association between virulent characteristics and antibiotic resistance in greater depth in *Campylobacter* isolates.

5. Conclusions

In the present study, a significant recovered rate of *Campylobacter* was found. Antibiotic resistance in *Campylobacter* strains isolated from *Agaricus* mushroom is a growing source of worry and might pose a severe public health danger. Resistance to multiple antibiotics with a significant association with virulent factors has been discovered. *Agaricus* mushroom *Campylobacter* strains from Iran have little resistance to antibiotics important to global health.

Data Availability

The datasets used and analyzed during the current study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

All authors participated in the review of the paper.

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Research Article

Bacterial Profile, Antimicrobial Susceptibility Pattern, and Associated Factors among Dental Caries-Suspected Patients Attending the Ayder Comprehensive Specialized Hospital and Private Dental Clinic in Mekelle, Northern Ethiopia

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Background. Dental caries is a major public oral infectious disease globally due to its high prevalence and significant social impact. Many studies have been conducted on dental caries in Ethiopia; however, they fail to convey the antimicrobial resistance in the oral environment. *Objective*. This study was conducted to determine the antimicrobial susceptibility patterns and biofilm formation in the bacteria isolated from dental caries and its associated factors of dental caries in THE Ayder Comprehensive Specialized Hospital and private dental clinics located at Mekelle, Ethiopia. *Methods*. A cross-sectional study was conducted from September 2019 to October 2020. Sociodemographic characteristic, behavioral, and clinical data were collected using structured questionnaires. A total of 422 dental caries-suspected patients were selected and coronal caries scraps were collected by the dentist aseptically; these samples were transported to a microbiological laboratory to identify the antibiotic sensitivity assay and biofilm formation by the isolated pathogens. The data was analyzed using SPSS version 22. The P value of ≤ 0.05 was considered statistically significant. *Results*. The overall prevalence of culture-positive samples was found to be 196 (46.4%). From the 196 culture-positive samples, 327 bacteria were isolated. Out of 327 bacterial isolates, 196 (46.4%) were identified as *Streptococcus mutans* and 69 (35.2%) were identified to be *Staphylococcus aureus*. From the isolated bacteria, 311 (95.1%) organisms were identified as positive for biofilm formation. From the AST assay, we have identified that penicillin has the highest resistance rate of 76.5%, followed by tetracycline at 64.8%. In contrast, the antibiotics such as cefoxitin and chloramphenicol have a sensitivity of 83.5% and 81.6% to all the bacterial isolates. The overall prevalence of multidrug resistance (MDR) in the isolates was found to be 40.4%. With respect to the associated risk factors, the white spot (AOR = 3.885, 95% CI 1.282-11.767, $P = 0.016$), gum bleeding (AOR = 2.820, 95% CI 1.006-7.907, $P = 0.049$), toothache (AOR = 2.27, 95% CI 0.58-0.885, $P = 0.033$), and chocolate consumption (AOR = 5.314, 95% CI 1.760-16.040, $P = 0.003$) were statically associated with dental caries bacterial infection. *Conclusion*. Based on our findings, we recommend the integration of routine culture and AST into clinical practice that might support the diagnosis and management of MDR in dental caries. The education on proper dietary habits might support the prevention and control of dental caries. It is important to provide health education on how to improve oral health in the study area. The education on proper dietary habits might support the prevention and control of dental caries. Further study is needed to find the other determinant factors of dental caries.

1. Introduction

Dental caries is the localized destruction of susceptible dental hard tissues by acidic by-products from bacterial fermentation of dietary carbohydrates [1, 2]. Dental caries is a major public oral infectious disease globally due to its high prevalence and significant social impact. Dental caries result from an ecological imbalance in the equilibrium between tooth minerals and oral biofilm [3, 4]. Dental caries is the biofilm-induced disease that can affect any age group and is highly related to and influenced by the patient's dietary habits, frequent sweet food intake. These factors, together with time, promote the microbial residence in the accumulated dental plaque to initiate dental caries infection [5]. Oral biofilm is a three-dimensional complex structure of different microorganisms inhabiting the oral cavity; if remained for a long time without treatment or interventions, biofilm can undergo maturation leading to dental caries development. It is believed that *Streptococcus mutans* plays an important role in forming multidimensional and complex structures on oral mucosa and tooth enamel [6]. Cells adhered to by biofilm are more resistant to conventional antibiotics compared to their planktonic ones. Bacterial extracellular polymeric substances (EPS) are strong barrier molecules that influence the rate of transport to the deep biofilm layer. In oral biofilm, bacteria are continuously challenged by changes in the environmental conditions. As a response to such challenges, *S. mutans* dominant in oral biofilms rely on the MDR transporter allowing withstanding toxic compounds produced by competing species or present in the plaque environment [7] [8]. According to the World Health Organization (WHO) reports, more than 90% of the population experiences caries, which is more prevalent in Asian and Latin American countries [9, 10]. In 2019, about 3.58 billion people have been assessed with dental caries infections [11]. Dental caries is the most chronic oral infectious disease in the world [12]. The policy of the WHO global oral health programme emphasizes that oral health is integral and essential for general health and the oral health is a determinant factor for the quality of life [13, 14].

The prevalence of dental caries was reported as 24.1% in Africa, 63.3% in Nigeria, 45.6% in China, and 65% in Kenya [15–19]. The main reason attributed for this high prevalence rate is because of a lack of knowledge, poor oral hygiene, and poor disease prevention methods [20, 21]. In the study conducted in Egypt, 74% prevalence of dental caries among children was identified [22]. In Ethiopia, 78.2% prevalence of dental caries was found in the patients attending the Debre Tabor General Hospital [23], 35.4% prevalence of dental caries was identified in the patients attending the Axum Primary School [24], and 68.7% prevalence of dental caries was found in the patients attending the Debre Berhan Referral Hospital [25]. Despite the fact that many studies have been conducted to study dental caries in Ethiopia, none assessed the bacterial profile of dental caries. Dental caries causes low to severe pain aggravated by chewing on any food substance, psychosocial disturbance, difficulty communicating due to missing or discolored teeth, and has the greatest impact on daily life activities [26]. However, if not treated early, it leads to severe intolerable pain, facial swellings, difficulty in chewing and swallowing, limited mouth opening, difficulty in breathing, and, in some cases, death [27]. Management strategies for tooth decay are adopted as surgical removal of the decayed tooth, and a more geometrically perfect cavity is created and filled with the most compatible and artificial materials. These materials might lead to secondary bacterial infection and bacteraemia [28, 29]. Nowadays, people spend huge amounts of money and time in treating dental caries. Therefore, it is essential to prevent and control dental caries [30].

The conventional methods to diagnose dental caries are based on the detection of visible color, texture change, tackling sensation using a dental explorer, and radiographs. However, radiographs are not useful for detecting early enamel caries. A microbiological approach of diagnosis would help in diagnosing early tooth decay. However, the implementation of this strategy is limited in resource-constrained settings. Therefore, generating evidence through research on the early screening for pathogenic bacteria and identifying their antimicrobial susceptibility test patterns are vital for infection prevention and implementers should consider the importance of this approach. In Ethiopia, there are few studies that show a higher prevalence of dental caries. However, the previous reports did not address the microbiological investigation and there is only one study done on the prevalence of *Streptococcus mutans* without assessing the AST assay to detect the AMR pathogens [25]. Moreover, there is no study on the bacterial profile in dental caries and the susceptibility pattern of the isolated bacteria [23–25]. Therefore, our study is aimed at determining the bacterial profile, antimicrobial susceptibility, biofilm formation in pathogens, and associated risk factors of dental caries in the study area. Our study will provide up-to-date information about the status of drug-resistant pathogens in the dental caries which could help the physicians select the best alternative drugs. Additionally, it could enroute the experts and policymakers to set the guidelines for treatments.

2. Materials and Methods

2.1. Study Area. The study was conducted at the dental clinic in ACSH and the union private dental clinics in Mekelle, northern Ethiopia. Mekelle town is the capital city of the Tigray regional state. Mekelle is located 787 km north of Addis Ababa, the capital city of Ethiopia, and covers an area of 109 square kilometers; its elevation is 2084 m above sea level. According to the 2007 census program, the total population of the town was reported to be about 258258. ACSH is the largest university hospital in the region and the second-largest hospital in Ethiopia. The ACSH dental clinic provides different services from dental care to different surgical procedures. According to the health management and information system of ACSH, an average of 5491 dental caries patients were treated in the year 2011. The inion special higher dental clinic, a pioneer of dentistry service in Tigray, was founded by Dr. Kidane Mamo Endale in 2002 at Kebelle along with 17 different branches. The clinic has a patient flow of about 1200/year.

2.2. Study Design: Inclusion and Exclusion Criteria. A cross-sectional study design was conducted from September 2019 to October 2020. All patients visiting the dental clinic of ACSH and the union private dental clinics were considered as a source of population. All patients suspected of dental caries who were attending a dental clinic in ACSH and the union private dental clinics were included in the study. All the age groups of people were included in this study. Patients who developed a complication and arrested dental caries were excluded from this study.

2.3. Sample Size Determination. The sample size was calcutated using single population proportion formula. The following parameters and assumptions were taken into consideration: Z = statistic for the level of confidence 95% (5%, $Z\alpha/2$, 1.9 and 6) and the margin of error $(d) = 5\%$. To our knowledge, there was no similar study previously done in Ethiopia; hence, we assume a 50% proportion ($P = 0.5$). Based on these assumptions, the actual sample size for the study is computed to achieve the following sample size.

$$n = \frac{(Z\alpha/2)^2 P(1-P)}{d^2}, \quad (1)$$

where n = sample size, $Z\alpha/2$ = critical value = 1.96, P = proportion = 0.50, d = precision(marginal error) = 0.05.

The sample size was $(1.96/.05)^2\, 0.5\,(1-0.5) = 384$. 10% contingency was $384\,(10/100) = 38.4, 384 + 38.4$, $n = 422$. Therefore, a total of *422* patients were included in the study.

2.4. Sampling Technique. A consecutive convenience sampling technique was employed as shown in Figure 1 to include the study participants. The total patient flow within six months in ACSH was 582 and 439 were in the union private clinic.

2.5. Study Variables. We have considered the following dependent and independent variables: the bacterial profile, biofilm formation, and antibiotic susceptibility patterns were considered dependent variables. The sociodemographic variables such as age, sex, residence, occupation, and education; clinical factors such as poor oral hygiene, dental trauma, sensitivity to cold and hot, history of chronic disease, and history of dental procedure; behavioral factors such as oral hygiene practice, smoking cigarettes, sweet intake, tooth brushing, and chat chewing were considered independent variables.

2.6. Data Collection Procedures. Structured questionnaires were prepared to collect patient's sociodemographic (including age, sex, residence, occupation, and education), clinical (including poor oral hygiene, dental trauma, sensitivity to cold and hot, history of chronic disease, and history of dental procedure), and behavioral data (including oral hygiene practice, smoking cigarettes, sweet intake, tooth brushing, and chat chewing). Complete questionnaires could be found in the supplementary document (available here). The questionnaires were translated into the local language (Tigrigna) to generate quality and reliable data. The coronal caries scraps were collected using a sterile dental elevator and inserted into a sterile tube containing 2 mL normal saline. The tubes were labeled and transported to the medical microbiology laboratory. The samples are refrigerated at 2–8°C until further use.

2.7. Bacterial Isolation and Identification. The coronal caries scrap was vortexed for 30 seconds to become homogenized and then streaked on different agar plates such as Mitis Salivarius agar (HiMedia, India), blood agar, MacConkey agar, and Mannitol salt agar (Oxoid, Hampshire, UK) using a sterile cotton swab and incubated for 24 hours at 37°C. After incubation, colonies were observed for their morphology (opaque, spherical, and raised) and glistening bubbles on the surface due to excessive synthesis of glucan from sucrose on Mitis Salivarius agar media due to the growth of *S. mutans* [31][25, 32]. The isolated pure cultures were subcultured on a blood agar slant for further identification. Standard cultures including *E. faecalis* ATCC 29212, *E. coli* ATCC 25922, and *S. aureus* ATCC 25923 strains were obtained from the Tigray Health Research Institute.

2.8. Identifying Biofilm Forming Bacteria. The qualitative tube adherence method was used to detect the biofilm formation in the isolated bacteria [33]. Briefly, a loopful of the test organisms were inoculated in a test tube containing 10 mL of trypticase soy broth (TSB) medium (HiMedia, India) with 1 ml of 1% glucose and incubated for 24 hours at 37°C. After incubation, cultures were discarded and the tubes were left to dry. The dried tubes were stained with 0.1% crystal violet and subsequently washed with sterile deionized water to remove the excess stains. The positive biofilm-forming bacteria show the visible film on the side and bottom of the tube.

2.9. Antimicrobial Susceptibility Pattern of the Bacterial Isolates. Kirby-Bauer disk diffusion assay was employed to identify the AST. The turbidity of the isolate was set at 0.5 McFarland standards. The antibiotic disk such as tetracycline (TE) (30 μg), doxycycline (DOX) (30 μg), erythromycin (Ery) (15 μg), clindamycin (CL) (10 μg), penicillin (PE) (10 μg), gentamicin (GE) (10 μg), chloramphenicol (CAF) (30 μg), ciprofloxacin (CIP) (5 μg), cefoxitin (CEF) (30 μg), trimethoprim-sulfamethoxazole (SXT) (1.25/23.75 μg), and ceftriaxone (CEFT) (30 μg) were used. Briefly, the Muller Hinton agar plates were swabbed with the test cultures and the antibiotic disk was placed on the plates and incubated at 37°C for 24 hours. After incubation, the zone of inhibition was measured and the results were recorded as sensitive (S), intermediate (I), or resistance (R) [34]. The observed results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Multidrug resistance (MDR) was defined as acquired nonsusceptibility to at least one agent in three or more antimicrobial categories [35].

2.10. Data Analysis Procedures. The collected data were entered, cleaned, and analyzed using Statistical Package for Social Sciences (SPSS) software version 22.0 (IBM, USA). Descriptive statistical analyses were computed using frequency, percentage, crosstabs, and mean and standard

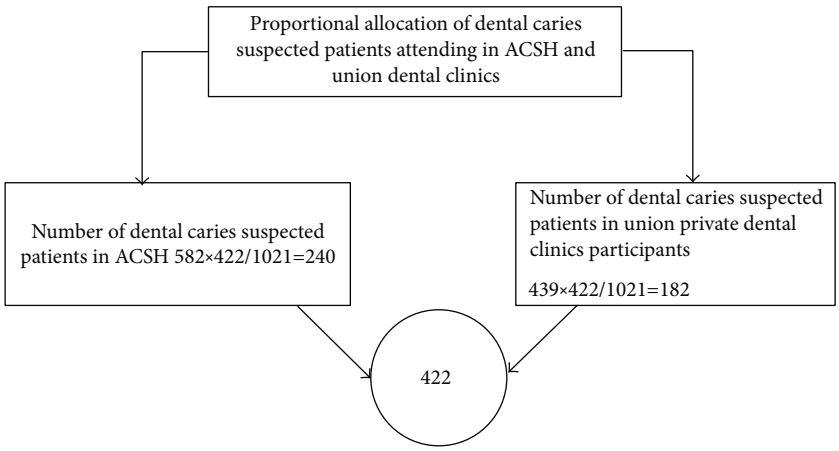

Figure 1: Schematic representation of the sampling procedure.

deviation. Binary logistic regression analysis was conducted to assess the presence and degree of association between dental caries and independent variables. The strength of association was presented by odds ratio, 95% confidence interval, and a P value of less than or equal to 0.05 on binary logistic regression; binary multiple logistic regression analyses were computed. The P value of less than or equal to 0.05 was considered a statistically significant association between risk factors and dental caries bacterial infection.

2.11. Ethical Clearance. The study was reviewed and approved by the Institutional Review Board (IRB) of CHS, MU. Support letters were obtained from respective concerned bodies. Informed consent or ascents were obtained after explaining the objective of the study to participants or guardians/parents, respectively. All the information collected through the study was kept confidential. The laboratory test results were communicated to the concerned clinicians.

3. Results

3.1. Characteristics of Study Participants. A total of 422 patients with dental caries were enrolled in the study, of which 227 (53.8%) patients were male. The majority 289 (68.5%) of the study participants were within the age group of 19–64 years while the lowest number 33 (7.8%) belonged to ≥65 years. Resident wise, 304 (72%) of the study participants were from urban areas and 118 (28%) of the study participants were from rural areas. Regarding the occupations, 186 (44.1%) of them were students and 65(15.4%) study participants were employed. From our study, majority of the study participants, 146 (34.6%), completed their primary education level and about 42 (9.9%) study participants have not taken formal education (Table 1).

3.2. Clinical Characteristics of Study Participants. From the 422 study participants, 224 (53.1%) had a toothache, 207 (49.1%) had a white spot, 177 (41.9%) had gum bleeding, 237 (56.2%) were sensitive to cold, and 229 (54.3%) were sensitive to hot (Figure 2). About 145 (34.4%) participants previously visited dental clinics for dental procedures. Study participants who had a history of dental trauma were 95 (22.5%), and those who had a history of chronic disease were 57 (13.5%) (Table 2).

3.3. Behavioral Characteristics of Study Participants. In Table 3, we conclude that the majority of the study participants 351 (83.2%) brushed their teeth as a daily habit, and from those, 114 (27%) used toothpaste and 42 (10%) used charcoal, respectively. Among the participants, 95 (22.5%) were brushing their teeth in a circular motion and 84 (19.9%) were top to bottom motion, respectively. 100 (23.7%) participants cleaned their teeth before and after a meal and 74 (17.5%) participants cleaned their teeth once a week. 53 (12.6%) study participants were having the habit of chewing chat, and 78 (18.5%) participants were having the habit of smoking cigarettes; 347 (82.2%) of the participants have the habit of taking soft drinks. 351 (83.2%) of the study participants have the habit of sweet food consumption, and out of 351 participants, the majority of 105 (24.9%) participants consumed chocolate.

3.4. Prevalence of Bacterial Isolates among Dental Caries-Suspected Patients. Streptococcus mutans are Gram-positive cocci, a nonmotile facultative anaerobic microorganism that can metabolize carbohydrates and is considered the principal etiological agent of dental caries. Other pathogens were not considered members of the usual oral microbiota, including *Staphylococcus spp*., coagulase negative Staphylococci, and *Acinetobacter*, where these pathogens were detected in high-frequency dental caries [36]. We have isolated about 327 pure bacterial isolates from the collected samples, the predominant isolates were identified to be *Streptococcus mutans* 196 (46.4%) followed by *Staphylococcus aureus* which was found to be 69 (35.2%), and coagulase-negative *Staphylococci* (CoNS) was found to be 62 (31.6%). Out of 196 *Streptococcus mutans* cultures, 160 (81.6%) cultures were found to be a mixed bacterial culture, whereas 36 (18.4%) cultures were found to be pure conies (Table 4). The high prevalence of *Streptococcus mutans* in the urban participants were found to be 46.4% (141) and

Table 1: Sociodemographic characteristics of patients attending dental clinics at ACSH and private dental clinic.

Variables	Frequency, *n* (%)
Sex	
Male	227(53.8)
Female	195 (46.2)
Age group (in years)	
≤18 (children)	100 (23.7)
19–64 (adult)	289 (68.5)
≥65 (elder)	33 (7.8)
Residence	
Urban	304 (72.0)
Rural	118 (28.0)
Occupation	
Employed	65 (15.4)
Unemployed	13 (3.1)
Merchant	52 (12.3)
Student	186 (44.1)
Farmers	52(12.3)
Housewife	54 (12.8)
Educational level	
No formal education	42 (9.9)
Primary education	146 (34.6)
Secondary education	113 (26.8)
Tertiary education	121 (28.7)

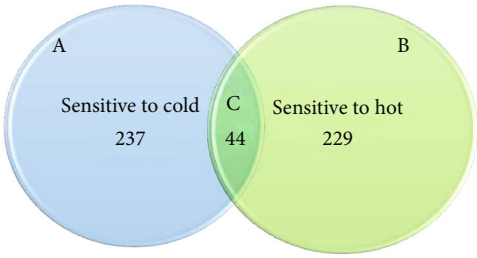

Figure 2: Venn diagram shows the study participant correlation between the (a) participants sensitive to cold, (b) participants sensitive to heat, and (c) participants sensitive to both hot and cold.

in the student participants of about 49.5% (92). This strain was mostly found in the age group below 18 years with 53% (53), and the participants who does not have any formal education was identified to be 72.7% (27). The prevalence of mixed isolates such as *Staphylococcus aureus* and *Streptococcus mutans* in urban participants were found to be 41.7% (62), this strain was mostly found in the age group between 19 and 64 with the 57.3% (55), the high prevalence of CoNS and *Streptococcus mutans* was found in the below 18 age category with 66% (35), these strains were mostly found in the female with 45.3% (29), the high prevalence of CoNS, *S. aureus*, and *S. mutans* was found in the rural population with 53.3% (24), and this strain is mostly found in the participants of the above 65 age group with 90.9% (10).

Table 2: Clinical characteristic frequency of dental caries-suspected patients attending ACSH and private clinics.

Variables	Frequency, *n* (%)
White spot	
Yes	207 (49.1)
No	215 (50.9)
Gum bleeding	
Yes	177 (41.9)
No	245 (58.1)
Sensation of tooth ache	
Yes	224 (53.1)
No	199 (47.2)
Dental trauma	
Yes	95 (22.5)
No	327 (77.5)
History of dental procedure	
Yes	145 (34.4)
No	277 (65.6)
Sensation to cold	
Yes	237 (56.2)
No	185 (43.8)
Sensation to hot	
Yes	229 (54.3)
No	193 (45.7)
History of chronic diseases	
Yes	57 (13.5)
No	365 (86.5)

3.5. Identifying Biofilm-Forming Bacteria. The result of the qualitative tube adhesion method revealed that out of a total of 327 pure bacteria isolates, 311 (95.1%) isolates were recognized as biofilm producers, while the rest of the 16 (4.9%) isolates exhibited no biofilm formation capabilities. The biofilm producers with strong tube adherence were found to be 53.7% (167) bacteria, moderate biofilm formers were found to be 34.4% (107) bacteria, and weak biofilm producers were found to be 11.9% (37) bacteria. Among the isolates, 52.6% (103) were found to be *S. mutans*-strong biofilm producers than *S. aureus* which accounts for 49.3% (34) and CoNS which accounts for 48.4% (30) (Figure 3).

3.6. Association of Risk Factors among Dental Caries Patients. Binary logistic regression analysis was performed to assess the degree of association between independent and dependent variables. Multivariate logistic regressions were also performed for variables that showed a significant association with the dependent variables in the binary logistic regression analysis. Of 207 study participants, participants with white spots were found to be about 116 (56.0%) and these participants were more likely to have dental caries (AOR = 3.885, 95% CI, 1.282-11.767, $P = 0.016$) than those who did not have a white spot. Similarly, the participants who had gum bleeding were found to be 99 (55.9%) and these participants were susceptible to two times at risk for

Table 3: Behavioral characteristics of dental caries-suspected patients attending ACSH and private clinics.

Variables	Frequency, *n* (%)
A habit of teeth cleaning	
Yes	351(83.2)
No	71 (16.8)
Kind of material used to clean teeth	
Toothpaste	114 (27.0)
Charcoal	42 (10.0)
Wooden tooth sticks	99 (23.5)
Others	96 (27.2)
Time & frequency of teeth clean	
Before & after a meal	100 (23.7)
Twice a day	89 (21.1)
Once a day	88 (20.9)
Once a week	74 (17.5)
Techniques of cleaning teeth	
Top to bottom	84 (19.9)
Sideway	94 (22.3)
Circular	95 (22.5)
Mixed	78 (18.5)
Mouth rising habit	
Yes	353(83.6)
No	69 (16.4)
A habit of sweet food eating/intake	
Yes	351 (83.2)
No	71 (16.8)
Kind of sweet intake	
Sugared coffee	34 (8.1)
Chewing gum containing sugar	42 (10.0)
Candy	61 (14.5)
Chocolate	105 (24.9)
Others (burger, biscuit)	64 (15.2)
Soft drink	
Yes	347 82.2)
No	75 (17.8
Smoking cigarette	
Yes	53 (12.6)
No	369 (87.4)
Chewing chat	
Yes	78 (18.5)
No	344 (81.5)

tooth decay (AOR = 2.820, 95% CI, 1.006-7.907, $P = 0.049$) than those without gum bleeding. Participants who had toothache were found to be 117 (52.2%), and these participants were three times at risk for developing dental caries (AOR = 2.27, 95% CI, 0.58-0.885, $P = 0.033$) than those who did not have a toothache. From our interpretation, 351 study participants consumed sweet foods; from the types of sweet foods, the participants consuming chocolates were identified as 65 (61.9%) and they are five times at risk for dental caries infection (AOR = 5.314, 95% CI, 1.760-16.040, $P = 0.003$) than those who did not consume chocolate (Table 5).

3.7. Antibiotic Susceptibility Patterns of the Bacterial Isolates. Table 6 summarizes the overall AMR profile of the Gram-positive bacteria. In this study, the sensitivity rates of the bacterial isolates ranged from 13.5% to 83.5% and the resistance rate of Gram-positive bacteria for antibiotics was between 3.4% and 76.5%. The highest resistance rate of 88.4% was observed in *S. aureus* against penicillin and 71% against ciprofloxacin. On the other hand, cefoxitin shows 81.9% sensitivity and chloramphenicol shows 73.9% sensitivity toward *S. aureus*. The isolated *S. mutans* (70.4% and 63.7.7%) show 70.4% resistance to penicillin and 63.7% resistance to tetracycline. On the other hand, 83.6% and 82.2% of *S. mutans* were 83.6% sensitive to cefoxitin and 82.2% sensitive to chloramphenicol, respectively. The isolated CoNS were 82.2% resistant to penicillin and 62.9% resistant to ciprofloxacin. However, they are 85.5% susceptible to cefoxitin and 87.1% sensitive to chloramphenicol (Table 6).

Multidrug resistance (MDR) was defined as acquired resistance to at least one agent in three or more antimicrobial categories according to the European CDC, 2015 [35]. All the bacterial species isolated from dental caries were resistant to one or more antimicrobial agents, and the overall MDR was found to be 40.4%. The multidrug resistance rate was high in *S. mutans* with 41.3% followed by *S. aureus* with 39.1% and CoNS with 38.7% (Table 7).

4. Discussion

There is limited data on dental caries, especially in the northern parts of Ethiopia. We attempted to assess the prevalence and associated factors of dental caries among patients attending the ACSH and union private dental clinics. This study revealed the overall prevalence of bacteria in dental caries as 196 (46.4%). The findings of our study were higher than those of the studies done in Nigeria (37.1%), Iraq, (36.2%), and Nepal, (40.3%) [15, 32, 37]. The difference might be due to increasing patterns of caries patients because dental caries is a chronic disease that progresses slowly in most people and every individual in the world is susceptible to dental caries once in their lifetime. However, this study was lower than studies conducted in Nepal by Yadav and Prakash which shows about 62.5% prevalence [38]. These differences might be due to different factors such as the study setting, sample size, sociocultural differences, and attitude. Supragingival plaque is dominated by Gram-positive bacteria including *Streptococcus mutans*, *Streptococcus sanguinis*, *Streptococcus mitis*, and *Streptococcus salivarius* [39–41]. *Streptococcus mutans* are the principal agent of enamel caries [38]. *Streptococcus mutans* are highly cariogenic, producing short-chain acids which soften hard tissues of the teeth and the ability to survive at low pH [42] and have three isozymes of glucosyltransferases catalyze and metabolize sucrose to synthesize insoluble extracellular polysaccharides, which increase their adherence to the tooth

Table 4: Prevalence of bacteria among dental caries-suspected patients attending at ACSH and union private dental clinics regarding sociodemographic 2020.

Variables	Bacterial isolates, *n* (%)			
	S. mutans (*n* = 196)	*S. aureus* & *S. mutans* (*n* = 69)	CoNS + *S. mutans* (*n* = 62)	CoNS, *S. aureus*, & *S. mutans* (*n* = 29)
Residence				
Urban	141 (46.4)	62 (41.7)	48 (41.7)	5 (4.3)
Rural	55 (46.6)	7 (53.9)	14 (31.1)	24 (53.3)
Sex				
Male	105 (46.3)	42 (43.8)	33 (34.4)	21 (21.9)
Female	91 (46.7)	27 (42.2)	29 (45.3)	8 (12.5)
Age categories				
≤18	53 (53.0)	13 (24.5)	35 (66.0)	5 (9.4)
19–64	127 (43.9)	55 (57.3)	27 (28.1)	14 (14.6)
≥65	16 (48.5)	1 (9.1)	0 (0)	10 (90.9)
Occupation				
Employed	28 (43.1)	28 (100)	0 (0)	0 (0)
Unemployed	4 (30.8)	4 (100)	0 (0)	0 (0)
Merchant	21 (40.4)	15 (83.3)	0 (0)	3 (16.7)
Student	92 (49.5)	22 (23.9)	62 (67.4)	8 (8.7)
Farmers	27 (51.9)	0 (0)	0 (0)	18 (100)
Housewife	24 (44.4)	0 (0)	0 (0)	0 (0)
Educational level				
No formal education	27 (72.7)	4 (16.7)	1 (8.3)	12 (83.3)
Primary education	74 (50.7)	23 (36.5)	28 (44.4)	12 (19.0)
Secondary education	50 (44.2)	21 (48.8)	20 (46.5)	2 (4.7)
Tertiary education	75 (62.0)	21 (58.3)	13 (36.1)	2 (5.6)

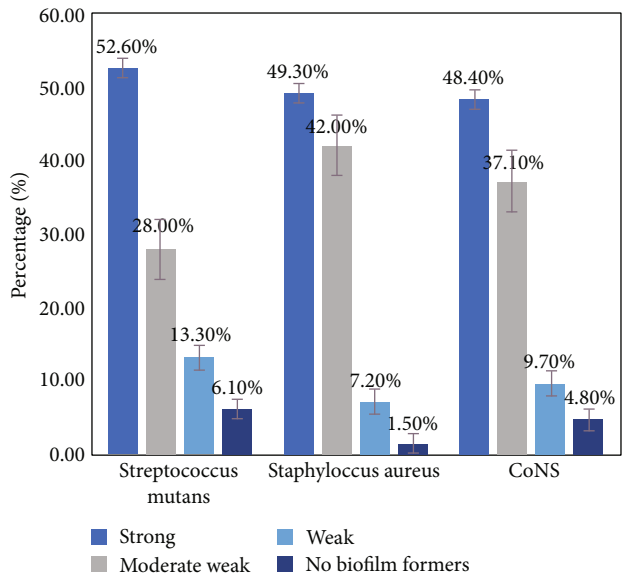

Figure 3: Biofilm detection by the collected bacterial isolates using the tube adhesion method.

surface and persuade biofilm formation [43]. In the current study, the predominant isolate bacteria were *Streptococcus mutans* with 46.4%. The findings of this study were consistent with other studies conducted in Nigeria (45.6%) [16] and Nepal (43.7%) [38]. And the current study shows more Streptococcus mutant isolate than the previous studies conducted in India (22.8%), Nepal (40%) [30, 37], and Nigeria (18.7%) [15]. In other contrast, this finding was lower than the study done in Debre Birhan (68.7%) [25]. Other than *S. mutans* isolate, our study shows 35.2% prevalence of *Staphylococcus aureus* which is higher than other studies conducted in Nepal (31.6%) and Nigeria (28.9%) [15, 16, 38]. The difference might be due to the methodology, socioeconomic backgrounds, dietary behaviors, and differences in knowledge and practice regarding the tooth brushing habit.

In our study, the prevalence of mixed bacterial growth was 81.6%, which was comparable with the previous study done in Nigeria (86%) [15]. In contrast to another study, this finding was lower than the study done in Nepal (90%) [37]. Biofilm formations are one of the major virulence factors and facilitate its adherence and colonization. The diversity of the oral microbiota and the ability of oral microorganisms to form dental biofilm on the teeth, implants, and oral mucosal surfaces in a sophisticated manner have been characterized [44]. In this study, the predominant strong biofilm

Table 5: Univariate and multivariate analysis of the associated risk factors among dental caries-suspected patients attending ACSH and the union private dental clinics.

Variables	Dental caries results		COR (CI 95%)	*P* value	AOR (95%)	*P* value < 0.05
	Positive	Negative				
Age						
≤18	53 (53.0)	47 (47.0)	0.695 (0.441-1.097)	0.118		
19–64	127 (43.9)	162 (56.1)	0.835 (0.380-1.835)	0.653		
≥65	16 (48.5)	17 (51.5)	1			
Sex						
Male	105 (46.3)	122 (53.7)	1		—	—
Female	91 (46.7)	104 (53.3)	1.017 (0.693-1.492)	0.933	—	—
Residences						
Urban	141 (46.4)	163 (53.6)	1			
Rural	55 (46.6)	63 (53.4)	1.009 (0.659-1.546)	0.966	—	—
Educational status						
No formal education	26 (61.9)	16 (38.1)	4.348 (1.097-17.226)	0.59	—	—
Primary education	74 (50.7)	72 (49.3)	1.676 (1.027-2.735)	0.39	—	—
Secondary education	50 (44.2)	63 (55.8)	1.294 (0.768-2.181)	0.333	—	—
Tertiary education	46 (38.0)	75 (62.0)	1			
Occupation						
Employed	28 (43.1)	37 (56.9)	1			
Unemployed	4 (30.8)	9 (69.2)	0.587 (0.164-2.104)	0.414	—	—
Merchant	21 (40.4)	31 (59.6)	0.895 (0.427-1.877)	0.769	—	—
Student	92 (49.5)	94 (50.5)	1.293 (0.732-2.284)	0.376	—	—
Farmers	27 (51.9)	25 (48.1)	1.427 (0.686-2.970)	0.341	—	—
Housewife	24 (44.4)	30 (55.6)	1.057 (0.511-2.188)	0.881	—	—
White spot						
Yes	116 (56.0)	91 (44.0)	2.151 (1.457-3.176)	0.000	3.885 (1.282-11.767)	0.016*
No	80 (37.2)	135 (62.8)	1			
Gum bleeding						
Yes	99 (55.9)	78 (44.1)	1.937 (1.309-2.866)	0.001	2.820 (1.006-7.907)	0.049*
No	97 (39.6)	148 (60.4)	1			
Toothache						
Yes	117 (52.2)	107 (47.8)	1.613 (1.096-2.373)	0.011	2.27 (0.58-0.885)	0.033*
No	79 (39.9)	119 (60.1)	1			
Dental trauma						
Yes	45 (47.4)	50 (52.6)	1.049 (0.664-1.658)	0.42	—	—
No	151 (46.2)	176 (53.8)	1			
Dental procedure						
Yes	70 (48.3)	75 (51.7)	1.119 (0.748-1.673)	0.585	—	—
No	126 (45.5)	151 (54.5)	1			
Sensation to cold						
Yes	114 (48.1)	123 (51.9)	0.859 (0.584-1.264)	0.440		
No	82 (44.3)	103 (55.7)	1			
Sensation to hot						
Yes	109 (47.6)	120 (52.4)	1.873 (1.265-2.773)	0.605		
No	87 (45.1)	106 (54.9)	1			
Do you clean your teeth?						
Yes	163 (46.4)	188 (53.6)	1			
No	3 3(46.5)	38 (53.5)	1.002 (0.601-1.670)	0.995	—	—

Table 5: Continued.

Variables	Dental caries results Positive	Negative	COR (CI 95%)	*P* value	AOR (95%)	*P* value < 0.05
If your answer is yes, how often?						
Before & after meal	48 (48.0)	52 (52.0)	1			
Twice a day	36 (40.4)	53 (59.6)	0.736 (0.413-1.311)	0.298	—	—
Once a day	36 (40.9)	52 (59.1)	0.750 (0.421-1.337)	0.330	—	—
Once a week	34 (45.9)	40 (54.1)	0921 (0.504-1.682)	0.788	—	—
Kind of material use						
Tooth paste	45 (39.5)	69 (60.5)	1			
Charcoal	12 (28.6)	30 (71.4)	0.613 (0285-1.321)	0.212	—	—
Wooden tooth sticks	46 (46.5)	53 (53.5)	1.331 (0.772-2.295)	0.304	—	—
Others	51 (53.1)	45 (46.9)	1.738 (1.003-3.010)	0.49	—	—
Type of techniques						
Top to bottom	38 (45.2)	46 (54.8)	1.354 (0.722-2.539)	0.344	—	—
Side way	39 (41.5)	55 (58.5)	1.219 (0.665-2.232)	0.522	—	—
Circular	41 (43.2)	54 (56.8)	1.234 (0.678-2.246)	0.492	—	—
Mixed	36 (46.2)	42 (53.8)	1			
Mouth rinsing habit						
Yes	163 (46.2)	190 (53.8)	1			
No	33 (47.8)	36 (52.2)	1.069 (0.637-1.791)	0.802	—	—
Sweet intake/food						
Yes	180 (51.3)	171 (48.7)	3.618 (1.996-6.559)	0.821		
No	16 (22.5)	55 (77.5)	1			
Kind of sweet intake						
Sugared coffee	12 (35.3)	22 (64.7)	1			
Chewing gum	20 (47.6)	22 (52.4)	1.667 (0.659-4.216)	0.281	1.238 (0.475-3.223)	0.662
Candy	32 (52.5)	29 (47.5)	2.023 (0.852-4.802)	0.110	1.471 (0.598-3.617)	0.400
Chocolate	65 (61.9)	40 (38.1)	2.979 (1.330-6.671)	0.008	5.314 (1.760-16.040)	0.003*
Others (burger, biscuit)	31 (48.4)	33 (51.6)	1.722 (0.731-4.059)	0.214	3.2565 (0.682-15.653)	0.139
Sugared tea	20 (44.4)	25 (55.6)	1.056 (0.640-1.743)	0.413	2.804 (0.568-13.852)	0.206
Soft drink						
Yes	162 (46.7)	185 (53.3)	1986 (1.131-3.486)	0.831		
No	34 (45.3)	41 (54.7)	1			
Smoking cigarette						
Yes	24 (45.3)	29 (34.7)	1.055 (0592-1.881)	0.856	—	—
No	172 (46.6)	197 (53.4)	1			
Chewing chat						
Yes	38 (48.7)	40 (51.3)	1.118(0.684-1.829)	0.656	—	—
No	158 (45.9)	186 (54.1)	1			
Hx of chronic disease						
Yes	29 (50.9)	28 (49.1)	1.228 (0.702-2.147)	0.471	—	—
No	167 (45.8)	198 (54.2)	1			

Hx: history; COR: crude odds ratio; AOR: adjusted odds ratio; CI: confidence interval.

producer bacteria were *S. mutans* (52.6%), followed by *S. aureus* (49.3%), and CoNS (48.4%) which is comparable with a study done in Egypt with *S. mutans* (48.5%), *S. aureus* (53.2%), and CoNS (46.8%) [45]. However, this study was lower than the study done in Kenya where *S. aureus* has 59.8% prevalence [46]. The difference might be due to the difference in detecting the biofilm detection methods. Patients developing white spots were significantly associated with the risk of acquiring dental caries infection than those who did not have white spots. Poor dental hygiene results due to irregular tooth brushing habits which result in the formation of plaque as a consequence caused tooth decay. The finding of this study was in line with studies done in Debre Birhan, Debre Tabor, Aksum, Ethiopia, and Egypt

Table 6: Antibacterial susceptibility patterns of the isolated bacterial at ACSH and the union private clinics 2020.

Isolated organisms (n)	Patterns	Antimicrobial drugs										
		PE (%)	CIP (%)	CL (%)	Ery (%)	GE (%)	TE (%)	DOX (%)	CAF (%)	SXT (%)	CEFT%	CEF%
S. mutans ($n = 196$)	S	32 (16.3)	113 (57.6)	112 (57.1)	103 (52.5)	96 (48.9)	45 (22.9)	103 (52.6)	162 (82.6)	91 (46.4)	121 (61.7)	164 (83.6)
	I	26 (13.3)	53 (27.0)	71 (36.2)	53 (27.0)	77 (39.3)	26 (13.3)	73 (37.2)	28 (14.3)	40 (20.4)	53 (27.0)	26 (13.3)
	R	138 (70.4)	30 (15.3)	13 (6.6)	40 (20.4)	23 (11.8)	125 (63.7)	20 (10.2)	6 (3.1)	65 (33.2)	22 (11.2)	6 (3.1)
CoNS ($n = 62$)	S	7 (11.2)	16 (25.8)	35 (56.5)	24 (38.7)	37 (59.7)	15 (24.2)	34 (54.8)	54 (87.1)	16 (25.8)	38 (61.3)	53 (85.5)
	I	4 (6.5)	7 (11.3)	15 (24.1)	20 (32.3)	14 (22.6)	16 (25.8)	20 (32.3)	5 (8.1)	15 (24.1)	18 (29.0)	6 (9.7)
	R	51 (82.2)	39 (62.9)	12 (19.4)	18 (29.0)	11 (17.7)	31 (50.0)	8 (12.9)	3 (4.8)	31 (50)	6 (9.7)	3 (4.8)
S. aureus ($n = 69$)	S	6 (8.7)	15 (21.7)	40 (64.5)	19 (27.6)	41 (59.4)	5 (7.2)	32 (46.4)	51 (73.9)	15 (21.8)	34 (49.3)	56 (81.9)
	I	2 (2.9)	5 (7.2)	15 (24.1)	21 (30.4)	21 (30.5)	8 (11.5)	25 (36.2)	12 (17.4)	18 (26.0)	26 (37.7)	4 (5.7)
	R	61 (88.4)	49 (71.0)	14 (20.3)	29 (42.0)	7 (10.1)	56 (81.1)	12 (17.4)	6 (8.7)	36 (52.2)	9 (13.0)	2 (2.9)
Total (327)	S	45 (13.7)	144 (44.0)	187 (57.2)	146 (44.6)	174 (53.2)	65 (19.8)	169 (51.7)	267 (81.6)	122 (37.3)	193 (59.6)	273 (83.5)
	I	32 (9.7)	65 (19.8)	101 (30.8)	94 (28.7)	112 (34.3)	50 (15.3)	118 (36.1)	45 (13.7)	73 (22.3)	97 (29.6)	36 (11.0)
	R	250 (76.5)	118 (36.1)	39 (11.9)	87 (26.6)	41 (12.5)	212 (64.8)	40 (12.2)	15 (4.6)	132 (40.4)	37 (11.3)	11 (3.4)

PE: penicillin; CIP: ciprofloxacin; CL: clindamycin; Ery: erythromycin; GE: gentamicin; TE: tetracycline; CAF: chloramphenicol; DOX: doxycycline; SXT: trimethoprim/sulfamethoxazole; CEFT: ceftriaxone; CEF: cefoxitin; S: sensitive; I: intermediate; R: resistant.

Table 7: Multidrug resistance patterns of the isolated Gram-positive bacteria ($n = 327$) at the Ayder Comprehensive Specialized Hospital and union private dental clinic.

Bacterial isolates	Total (%)	Antimicrobial-resistant pattern						
		Ro	R1	R2	R3	R4	≥R5	MDR
S. mutans, $n = 196$	196 (59.9)	10 (5.1)	15 (7.6)	30 (15.3)	23 (11.7)	28 (14.3)	10 (10.2)	81 (41.3%)
S. aureus, $n = 69$	69 (21.2)	4 (5.8)	9 (13.)	13 (18.8)	6 (8.7)	14 (20.3)	11 (15.9)	27 (39.1)
CoNS, $n = 62$	62 (18.9)	12 (19.3)	5 (8.1)	0 (0.0)	4 (6.4)	9 (13.1)	4 (6.5)	24 (38.7)
Total	*327 (100.0)*	*26 (7.9)*	*29 (8.9)*	*33 (10.1)*	*33 (10.1)*	*51 (15.6)*	*25 (7.6)*	*132 (40.4)*

Ro: not resistant; R1: resistant to one; R2: resistant to two; R3: resistant to three, R4: resistant to four; R2: resistant to five antibiotics; MDR: multidrug resistant.

[22–25, 47]. This indicates underutilization of dental health facilities; unhealthy dietary habits and gaps in the knowledge, attitude, and practice of dental hygiene were the major causes of poor oral hygiene to develop dental caries infection [48]. Gum bleeding was significantly associated with dental caries. Patients with gum bleeding were more likely to have dental caries than others who did not have gum bleeding. The gum bleeding increased the colonization of bacteria, and in severe cases, it causes the loss of a tooth. This might be an indicator of poor oral hygiene. These findings correlate with the previous studies [23–25].

The higher prevalence of dental caries in those who did not attend formal education than their counterparts could possibly be due to the indirect effect of education on dental caries. Those who attended formal education might have awareness of dental caries and take regular appropriate measures to prevent dental caries. An educated person can read and obtain information about oral health while those who are not educated may not know the cause of dental caries and the measures to be taken for its prevention. Moreover, information related to oral health might be given during formal education. Toothache was one of the major indicators of dental caries infection. The experience of tooth pain; the problem with eating, smiling, and communication due to missing; and discoloration have a foremost impact on people's everyday life [38]. In this study, patients who have

toothache were 2.27 times more likely to have dental caries bacterial infection. This finding was supported by other studies done in Bahir Dar, Ethiopia, and Kenya [47, 49]. Consumption of sweet food like chocolate has been significantly associated with dental caries. The finding was in agreement with studies done in Bahir Dar, Finote Selam, Ethiopia, Egypt, and Zimbabwe [21, 22, 47, 50]. This might be associated with plentiful acid production by cariogenic bacteria that are adherent to the tooth as a consequence of the fermentation of sweet foods. Later on, the enamel of the hard teeth went into tooth decay [1, 2]. Treatment of dental infections depends on whether it is a low-level local infection or a severe infection of the fascial spaces. If possible, removal of the source of infection is the most important step in treating dental infection [51, 52]. Furthermore, it depends on the extent of dental caries and can range from the insertion of restorative material (filling) to tooth extraction. Irreversible pulpitis treatment includes root canal and extraction, and there is insufficient evidence to recommend antibiotics. A periapical abscess can complicate pulpitis. An uncomplicated periapical abscess is treatable with incision and drainage only. Periapical abscess complicated by systemic symptoms, cellulitis, or immunocompromised patients should receive antibiotics in addition to drainage [53]. Antibiotic therapy for dental infections is necessary for systemic symptoms, fascial space infections, and infections that spread to the bony cortex and surrounding soft tissue. Antibiotics such as amoxicillin, clindamycin, tetracycline, and erythromycin are the most common medication prescribed for dental infections [54–56].

In our study, we used the antibiotics such as tetracycline, erythromycin, and penicillin antibiotics to detect the antibiotic susceptibility test and found that the isolated bacteria were resistant to most of the used antibiotics. The overall prevalence of multidrug-resistant pathogens was identified to be 40.4%. This study's finding was comparable with other studies [37, 54]. The main reasons for multidrug resistance were because of the misuse of drugs. Caries can be prevented through perfect oral hygiene [45]. Many mechanical methods can be employed for this purpose. Among those, tooth brushing is the most frequently advocated and most widely exercised. The horizontal vibrating method also known as the bass method stresses the removal of plaque in the gingival sulcus and the interproximal spaces [34]. Minimizing the frequent consumption of more carbohydrates during night time is greatly helpful to prevent the formation of dental caries [57]. The burden of dental caries can be reduced by providing proper health education on how to keep oral hygiene and regular visit to a dental clinic.

5. Limitation

The major limitation of the present study is that the strictly anaerobic bacteria from the collected coronal caries scraping has not been isolated. Sweet food items and drinks were assessed by the usual patterns of intake, but the amount and the duration of intake were not assessed. The difficulty of radiological examination due to the lack of instruments and laboratory setup might reduce the actual magnitude of the problem. The monthly income of participants was not assessed. The determinant factors were not exhaustive. There could be other determinant factors of dental caries that our study did not address.

6. Conclusion

The overall prevalence of dental caries infection and biofilm detection for the present study was high in the study area. The predominantly isolated bacterium was *Streptococcus mutans*. *S. mutans* and *S. aureus* showed high multidrug resistance toward commonly used antibiotics and most of the bacteria isolates were found to be sensitive to cefoxitin and chloramphenicol. Based on our findings, we recommend the integration of routine culture and AST into clinical practice might support the management of multidrug-resistant pathogens among the study population. The educational status, tooth brushing habits, consumption of sugary food, chat chewing, smoking, mouth rinsing habits, kind of material used for brushing, white spots, gum bleeding, sensation to hot and cold, toothache, chronic diseases, and oral hygiene status were significantly associated with the prevalence of dental caries. It is important to provide health education on how to improve oral health in the study area. The education on proper dietary habits might support the prevention and control of dental caries. Further study is needed to find the other determinant factors of dental caries.

Data Availability

All the data are available within the manuscript and its supporting information.

Conflicts of Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary Materials

Structured questionnaires to collect patient's information. A) Sociodemographic (including age, sex, residence, occupation, and education, B) clinical (including poor oral hygiene, dental trauma, sensitivity to cold, hot, history of chronic disease, and history of dental procedure), and C) behavioral data (including oral hygiene practice, smoking cigarettes, sweet intake, tooth brushing, and chat chewing). *(Supplementary Materials)*

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Review Article

Molecular Mechanisms of Antifungal Resistance in Mucormycosis

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Mucormycosis is one among the life-threatening fungal infections with high morbidity and mortality. It is an uncommon and rare infection targeting people with altered immunity. This lethal infection induced by fungi belonging to the Mucorales family is very progressive in nature. The incidence has increased in recent decades owing to the rise in immunocompromised patients. Disease management involves a multimodal strategy including early administration of drugs and surgical removal of infected tissues. Among the antifungals, azoles and amphotericin B remain the gold standard drugs of choice for initial treatment. The order Mucorales are developing a high level of resistance to the available systemic antifungal drugs, and the efficacy still remains below par. Deciphering the molecular mechanisms behind the antifungal resistance in Mucormycosis would add vital information to our available antifungal armamentarium and design novel therapies. Therefore, in this review, we have discussed the mechanisms behind Mucormycosis antifungal resistance. Moreover, this review also highlights the basic mechanisms of action of antifungal drugs and the resistance landscape which is expected to augment future treatment strategies.

1. Introduction

Emerging number of fungi linked to illnesses in humans, animals, and plants is increasing, thanks to developments in molecular methods and phylogenetic analyses, which have led to a better understanding of fungal taxonomy and the creation of new dangerous fungi [1]. Aggressive fungal infections are rare since the body's immune system is capable of destroying large organisms, but they can be difficult for clinicians to treat, especially in severely ill individuals and patients who are immunocompromised as a result of disease or immunosuppressive medications. Infections produced by fungi can cause high mortality rates in some patients, particularly those who are immunocompromised or critically unwell. Invasive fungal infections are difficult to treat because eukaryotic pathogens' medication target sites are very similar to those of the human host, limiting therapy options [1].

Antifungals belong to many pharmacological classes that target various biological processes, either fungistatic or fungicidal the pathogenic yeast's growth. The creation of the cell wall, cytoplasmic membrane, and RNA biogenesis are among these biological activities, biosynthetic routes involving a series of enzymes. The pharmacokinetics of medications is a significant element that influences drug efficacy [2]. Factors such as drug metabolism, intake, and distribution can all have an impact on a medicine's effectiveness. Furthermore, treatment efficacy is affected by the severity of the illness as well as the size of the infecting organisms' population. Antifungal drugs' efficacy in the treatment of fungal infections may be influenced by differences in drug bioavailability between various tissues. Azole medications' bioavailability is much lower in low-pH vaginal tissues than in the blood. Importantly, the tissue distribution and toxicity of different lipid formulations differ [3].

Table 1: Antifungal drugs used in mucormycosis.

Commonly used antifungal drugs in mucormycosis		
Target mechanism of action in the pathogen	Antifungal drugs	Reference
Ergosterol biosynthesis	Azoles Polyenes Allylamines Morphine	[10, 11]
Cell wall biosynthesis	Echinocandins	[12]
Nucleic acid synthesis	5-Flucytosine	[13]

Biofilms can be formed on the surfaces of medical equipment such as catheters by fungi. These biofilms appear to be resistant to antifungal penetration and may have fewer therapeutic targets. Successful therapies rely heavily on the human immune system. Antifungal medications, azoles, in particular, rely on the immune system of the host to treat invasive fungal infections [3, 4]. Antifungal medication resistance of the infected species is another key aspect that can lead to therapeutic failure.

In long-term usage of azole medications such as fluconazole, for example, it is possible that pathogenic yeasts will develop resistance to it, rendering the therapy ineffective. Fluconazole is the most commonly prescribed azole for fungal infection prevention and treatment. However, azole resistance has emerged in a number of species, which is a new concern that is generating therapeutic failures [5].

2. Mucormycosis

Mucormycosis is a fatal infection caused by fungi of the Mucoromycotina subfamily and Mucorales order, which belong to a group of lower fungi previously known as zygomycetes and are a phylogenetically archaic group of microorganisms. The groups Rhizopus, Mucor, Lichtheimia, Cunninghamella, Rhizomucor, and Apophysomyces are among the causative agents of mucormycosis [6]. The group contains a variety of widely dispersed ancestral lineages in the fungal tree of life. Mutations are thought to have accumulated over time. The opportunistic Mucorales are divided into the families Cunninghamellaceae, Lichtheimiaceae, Mucoraceae, Saksenaceae, and Syncephalastraceae, with Mucoraceae and Lichtheimiaceae causing the vast majority of human infections. Rhizomucor was shown to be outside the Mucoraceae family in the molecular phylogenetic analysis [7].

Mucormycosis outbreaks have been found in wards, among victims of disasters like the Joplin disaster, and among soldiers recovering from combat-related injuries, circinelloides-related foodborne outbreaks in healthy people, illustrate Mucorales' propensity to cause serious illnesses.

It has become more common in hematologic malignancies and organ transplantation as the condition is the second most prevalent mould infection, and it is also becoming more common in people with uncontrolled diabetes or ketoacidosis. Despite rigorous antifungal treatment, in certain, significant surgical debridement, mucormycosis-related mortality remains unacceptably high [8].

A recent report shows an increase in a number of activity of mucormycosis in COVID-19 patients. It can damage the sinuses, brain, or lungs and is thus fairly prevalent in patients who have COVID-19 or are recovering from it [9] Swelling on one side of the face, fever, headache, nasal or sinus congestion, and black lesions on the nasal bridge or upper inside of the mouth are all common signs of mucormycosis (Table 1).

3. Antifungal Drugs

Antifungals belong to many pharmacological classes that target various biological processes, either fungistatic or fungicidal pathogenic yeast's growth. The production of the cell wall, cytoplasmic membrane, and RNA biogenesis are among these biological activities, biosynthetic routes involving a series of enzymes [14] (Figure 1).

4. Mechanism of Action of Antifungal Drugs

4.1. Targeting Ergosterol Biosynthesis. Ergosterol is the most abundant sterol in fungal cell membranes, especially plasma and mitochondria. In the cell membrane, sterols and sphingolipids combine to generate lipid rafts. Lipid rafts contain bioactive proteins including those involved in signaling, stress response, breeding, and nutrition transport. The structure of these membranes is critical for fungi to survive. A cascade of 25 enzymes catalyzes the manufacture of ergosterol. Because ergosterol is an essential lipid for fungus and plants, but not for humans, this metabolic pathway is a good target for medicines [15]. There are various groups of drugs that effects the biosynthesis of ergosterol mechanisms such as the azoles, polyenes, allamines, and morphines. The most prevalent antifungal medicine class used to treat fungal infections is azoles. Azoles inhibit the enzyme 14–demethylase (Erg11p), which is involved in the production of ergosterol. Azoles attach to Erg11p, thereby lowering ergosterol levels in cells. When Erg11p is blocked, subsequent enzymes in the pathway (Erg6p, Erg25p, Erg26p, Erg27p, and Erg3p) produce a fungistatic poisonous sterol; azoles are also responsible for increasing reactive oxygen species levels (ROS). The infecting fungus' development is inhibited by both high ROS levels and harmful sterol synthesis [16].

Whereas the polyene antifungal drugs attack the ergosterol within the cell membrane. It creates pores when they bind to ergosterol. Monovalent ions (K+, Na+, H+, and Cl) seep quickly through pores, resulting in fungal cell death. Amphotericin B and nystatin are polyene medicines; however, amphotericin B is still utilized for systemic treatment [17]. In the allylamine group of drugs, squalene epoxidase in the ergosterol production is the target of these antifungals. Terbinafine (Lamisil), flunarizine, and naftifine are examples of these medications. Terbinafine (Lamisil) is a drug that is frequently used to treat dermatophyte infections [18]. Fenpropimorph, tridemorph, and amorpholine are all examples of morphines. Ergosterol biosynthesis C-14 sterol reductase is the target (Erg24p). Morpholines are widely employed in

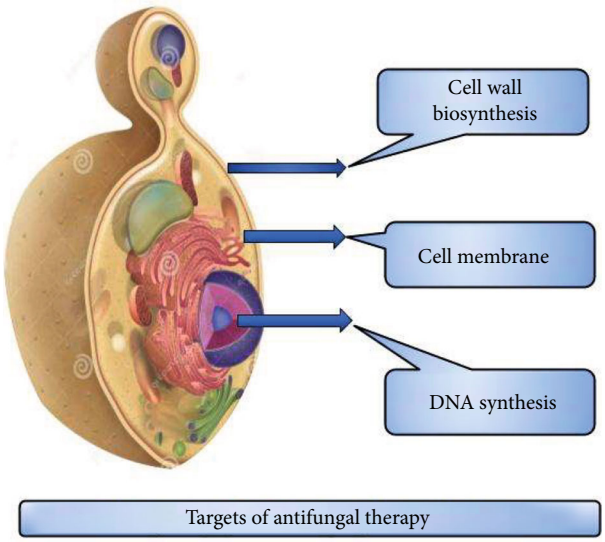

Figure 1: Targets of antifungal drugs.

agriculture, but they are extremely harmful to people. Nail dermatophyte infections are treated with a 5% amorolfine hydrochloride-containing nail lacquer solution [19]. In addition to conventional antifungal agents, medicinal plants are also widely explored against fungal pathogens. The aqueous extract of Tulbaghia violacea acquired by maceration exhibits antifungal activity by decreasing the production of ergosterol which negatively impacted lipid production in Aspergillus flavus [20]. Deciphering the mechanism behind this action, it was found that the enzyme oxidosqualene cyclase was mainly targeted by the extract which led to the accumulation of 2,3-oxidosqualene.

4.2. Targeting Cell Wall Biosynthesis. The fungal outer wall has a stiff exterior covering, and it serves as the initial line of protection against osmotic stress. Because mammalian cells lack cell walls, enzymes in cell wall synthesis are the main targets. Echinocandin antifungal drugs such as caspofungin, micafungin, and anidulafungin target the cell wall [21]. They act on the enzyme 1-3 glucan synthase, which is encoded by three genes: FKS1, FKS2, and FKS3. The 1-3 glucan synthase enzyme is a three-protein complex (Fks1p, Fks2p, and Fks3p) that uses UDP-glucose to synthesize 1-3-glucan, a key component of the fungal cell wall. These drugs are often fungicidal and are generally chosen due to their low human toxicity [22]. To date, antifungal agents have been developed for inhibiting the cell wall component biosynthesis. Poacic acid inhibits β-1,3-glucan synthesis, breaking its integrity by inhibiting the activity of Gas and Crh enzymes involved in cell wall remodeling [23]. It was found to modulate and affect the regulatory mechanisms involved in rescue responses.

Owing to the selectively targeting of fungal cell wall, echinocandins do not exert their activity on mammalian cells. High specificity reduced the off targeted effects and adverse events with echinocandin use in comparison to other antifungal drugs such as azoles and amphotericin B [24]. In a randomized double-blinded study, caspofungin (50 mg) has no adverse drug reactions in HIV-infected patients treated for esophageal candidiasis [25]. In contrast, its use in invasive candidiasis and aspergillosis was associated with infrequent reports of hepatotoxicity and nephrotoxicity [26, 27]. The safety of caspofungin in 1205 patients with daily doses ranging from 35 to 100 mg exhibited no dose-related adverse effects and toxicity [28]. Surprisingly, even at higher doses (150 mg/day), no adverse effects were observed in patients with invasive candidiasis [29]. In treatment for invasive candidiasis, caspofungin showed excellent safety profile without any drug-related side effects [30]. Interestingly, a clinical trial evidenced high safety and efficacy in immunocompromised paediatric patients [31]. Similar to caspofungin, micafungin also was found to be safe in both adult and pediatric population. In pediatric patients, it was well tolerated with only 4.7% patients experiencing serious side effects [32]. A multicentrer, randomized, open-label study (Phase III) investigating the prophylactic use of micafungin (50 mg/day) along with itraconazole in neutropenic patients with haemotopoietic stem cell transplantation demonstrated no drug-related adverse effects. In addition, it was found to be safer in prevention of fungal infections invasion compared to intraconazole [33]. In allogenic haematopoietic stem cell transplantation procedure, micafungin was found to be safe with only 1.4% discontinuation [34]. In *Candida* infections, patients with daily dose (50 mg, 75 mg, and 100 mg) of anidulafungin and follow-up study indicated no dose-dependent adverse effects and only 9.0% deaths were reported in highly complicated and comorbid patients [35]. A prospective, multicenter study in critically ill patients following various clinical conditions and candidaemia, invasive candidiasis, anidulafungin IV therapy accounted to only 1.9% of severe adverse effects. In the pediatric population, 0.75 mg/kg and 1.5 mg/kg per day dose of anidulafungin was associated with no adverse effects and well tolerated by children. Overall, these reports indicate that echinocandins is safe and well-tolerated profiles were observed in adult and pediatric patients [36].

4.3. Targeting the Synthesis of RNA and DNA. 5-Flucytosine (5FC) prevents the formation of nucleic acids. The cytosine permease enzyme is used by sensitive cells to import 5FC. 5FC is metabolized to 5FU, which is then transformed to 5-fluorouridine triphosphate. Rather than uridine triphosphate, 5FUTP is integrated into fungal RNA, altering protein translation. 5FU can also be transformed to 5-fluorodeoxyuridine monophosphate (5FdUMP), which inhibits thymidylate synthase, a key enzyme in DNA biosynthesis [37]. Figure 2 shows the mechanism of actions.

5. Mechanism Antifungal Drug Resistance

5.1. Azole Drug Resistance. Azoles inhibit ergosterol biosynthesis by actively targeting cytochrome P450-dependent enzyme, lanosterol 14-α-demethylase. Ergosterol biosynthesis inhibition leads to the intracellular accumulation of toxic intermediates which perturbs the membrane stability and arrests fungal growth [38]. Resistance to azole antifungals has also been linked to the overexpression of 14-α-

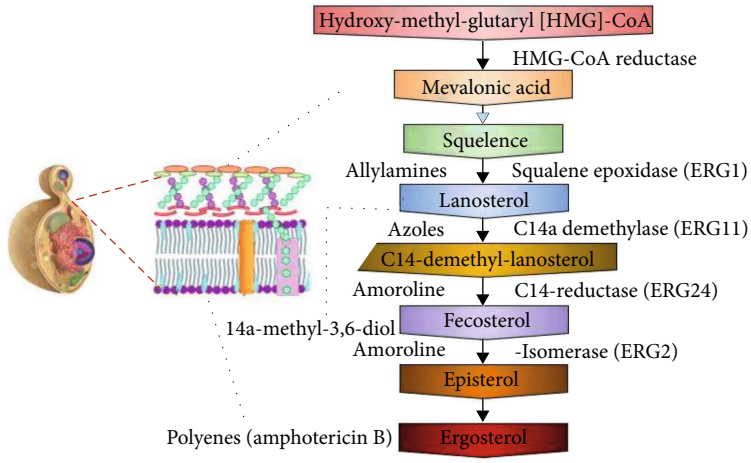

Figure 2: Mechanism of actions of antifungal drugs.

demethylase. In Aspergillus and Candida resistance strains, overexpression/alteration in *ERG11*/*cyp51A*/*cyp51B* was observed with substitutions in amino acid residues proximal to the heme-binding site of 14-α-demethylase [39]. Constitute expression of *ERG11* owing to a gain-of-function mutation in the Upc2, a transcriptional activator confers azole resistance to C. *albicans [40]*. C. glabrata transcription factor (TF) CgRpn4 was found to be responsible for azole resistance by reducing fluconazole accumulation and regulating the membrane permeability [41]. Candida also develops azole resistance by mutating *ERG11*. In a recent report, Set1 mediates the H3K4 histone methylation and loss of SET1 increases susceptibility to azoles by affecting *ERG11* expression [42]. In addition to this, *ERG3* mutations results in ergosterol depletion or alternative sterols accumulation and this depends on three stress response regulatory proteins including molecular chaperone Hsp90, protein phosphatase calcineurin, and protein kinase C1 [43].

Overexpression of cytochrome P450 enzymes is another route adopted to confer resistance to azoles. Researchers investigated the azole-resistant C. glabrata isolate and discovered that it had a higher ergosterol content. In-depth investigations portrayed a higher microsomal P450 levels leading to increased ergosterol synthesis in the resistance strain and was responsible for both azole and amphotericin B resistance [44]. These findings indicate that the cross-resistance to such two triazoles was caused by elevated P450 levels [45]. In the small number of clinical isolates with 14 α-demethylase overexpression, this phenomenon was only seen in C. glabrata, and the possibility that other resistance mechanisms are active in the same strain all suggests that increased expression of the target enzyme ends up playing just a minor role in clinical azole resistance [46].

Resistance to antifungal agents is also conferred by overexpression of multidrug transporters, alteration of drug target, and the initiation of stress responses. Pathogenic yeast contains a considerable number of membrane proteins which are located in the cell membrane, vacuolar surface, and mitochondrial membrane. [47]. They are involved in environmental sensing, nutrient transport, signal transduction, drug efflux, drug alteration, and drug detoxification processes. For instance, membrane protein found in the mitochondrial membrane Atm1p, an ABC transporter, is involved in iron homeostasis, whereas Mlt1p, a membrane transporter found at the vacuolar membrane, transports phosphatidylcholine [48]. A solitary membrane carrier can perform a variety of physiological tasks. Azole resistance has been linked to two forms of membrane transporters found in fungi [49]. ABC-Ts (adenosine triphosphate binding cassette transporters) are ATP-dependent active transporters. Each ABC-T is made up of two lattice domains (MSD) with six transmembrane sections and each two nucleic acid binding domains. Each NBD has an ABC (ATP-binding cassette), which binds ATP [50]. Major facilitator transporters (MFS-T) require a gradient of protons in the cytoplasm as an energy source in order to transfer xenobiotics. MFS-Ts have 12 to 14 transmembrane segments and lack the NBDs that define ABC-Ts [51]. The pleiotropic drug resistance transporter (PDR) belonging to the ABC transporters was found to be tightly associated with drug resistance. In *Mucor circinelloides*, out of the eight *pdr* genes, pdr1 and pdr2 was found to be involved in the resistance towards isavuconzaole, ravuconazole, and posaconazole [52] According to a comparison of fluconazole accumulation by C. albicans and C. krusei [53], all research strains accumulated the same amount of fluconazole in the first 60 minutes. C. krusei, on the other hand, collected 60% less fluconazole after 90 minutes of incubation than C. albicans, demonstrating that active efflux is implicated in the fluconazole sensitivity of these C. krusei strains.

The rise of resistance to azoles by various fungal species has worsened the landscape of treating several fungal diseases. Azole derivatives such as azole-triphenylphosphonium conjugates have shown to alleviate drug resistance in *Candida* strains by interfering with mitochondrial functions and while retaining the ability to inhibit ergosterol biosynthesis [54]. As most azole drugs exhibit their activity by targeting Erg11, by mining the existing repository of chemical compounds, new compounds targeting Erg 11 are explored to combat antifungal resistance. A

2,5-disubstituted pyridine compound CpdLC-6888 was found to inhibit Erg 11 similar to conventional azole drugs [55].

5.2. Polyene Drug Resistance. Resistance to polyene drugs like amphotericin B, and nystatin is unusual, but resistant isolates have been found. Polyene resistance is linked to changes in ERG3 and ERG6. In fungal strains, disrupting ERG3 and ERG6 causes decreased ergosterol concentrations and amphotericin B sensitivity in vitro [56]. One of the major mechanisms behind resistance is the polyene-induced reduction in oxidative stress [57]. Apart from this, altered sterol composition of the membrane was also found to induce resistance to AmB in *A. terrus* [57]. A catalase-dependent mechanism is more likely to be involved in the resistance of counteracting oxidative stress induced by AmB [58].

Candida auris exhibits resistance to amphotericin B and the underlying mechanism depicted alterations in membrane lipids and in chromatin modifications. More importantly, increased phosphorylated MKc1 cell integrity MAP kinase in response to AmB treatment was found to be a major resistance pathway behind the resistance [59]. Another mechanism behind the AmB resistance was found to be the involvement of heat shock proteins Hsp70 and Hsp90, key players in governing cellular stress. Three plausible mechanisms were proposed behind the acquisition of AmB resistance: (1) Hsp90 disruption would lead to the generation and endurance of new genetic variations, (2) Hsp90 may chaperone various cell signaling regulators to augment the development of new adaptive phenotypes, and (3) active Hsp90 would regulate the stabilization of several mutated cell regulators which have the tendency to induce AmB resistance [60]. Other mechanisms behind the AmB resistance are reviewed extensively [61]. Fryberg proposed that resistance occurs as a result of the selection of sources of resilient cells, which are present in small proportions throughout the population. These naturally resistant cells create sterols with a decreased affinity for nystatin. The usual growth rate as well as the rate at which nystatin destroys the cell membrane dictate the rate of growth in the presence of nystatin. The affinity of nystatin for membrane sterols is hypothesized to influence the rate of membrane damage: the greater the nystatin-sterol affinity, the greater the rate of membrane damage [62]. The biochemical notion that resistance develops as a result of changes in the sterol contents of the cells, either quantitatively or qualitatively, resistant cells with lower sterol content bind fewer polyene than sensitive cells. This reduced polyene binding in C. albicans mutants could be attributable to a drop in the cell's total ergosterol concentration without corresponding changes in sterol composition, or the replacement of some or all of the polyene-binding sterols with ones that bind polyene less tightly [63]. Other studies show a relationship between the polyene employed to isolate mutants and cross-resistance, as well as the selection of mutants with specific pol gene mutations. The wild type had the most ergosterol and dehydroergosterol, according to sterol analysis of the parent and mutations. The latter sterol, on the other hand, was absent in the pol2 mutant and only present in trace amounts in the pol3 mutant. Though the relationship between pol genes is unknown, evidence from UV spectroscopic analysis revealed that these mutants act in sequence rather than parallel, showing that they are epistatically linked [64] (Figure 3).

6. Possible Approaches to Tackle Antifungal Resistance in Mucormycoses

Mucorales are very destructive and cause lethal infections (Mucormycosis) in patients with altered immunity and predisposing conditions. The international treatment guidelines for mucormycosis involve amphotericin B as a first-line treatment strategy and posaconazole is followed as salvage therapy [65]. However, fungi are now intrinsically resistant to these routinely used drugs which narrow down the choice of drugs. For instance, a very unique mechanism through which *Mucor circinelloides* confers resistance to rapamycin and antifungal agents FK506 is by epimutation [66]. RNAi-based silencing of the genes targeted by the drug for short period and reexpression following drug passage is the causative mechanism behind the resistance [52]. This transient epimutation observed requires in-depth elucidations to unravel the mechanisms and develop novel approaches to combat drug resistance. Besides this, monotherapies using a single drug would be surpassed with a combinatorial therapeutic strategy involving multiple antifungal drugs in a phased manner. Mouse models of diabetes and neutropenic infected with *R. arrhizus* showed improved survival upon combined treatment with an echinocandin and a polyene rather than monotherapy [67]. Synergistic interactions between L-micafungin, AmB, and an iron chelator deferasirox showed higher efficiency against Mucorales fungi in diabetic rats [68] Micafungin inhibits fungal efflux pumps and upon combined administration with azole which enhances the intracellular uptake and retention [69]. Furthermore, in a study in patients with rhinocerebral mucormycosis, a combination of caspofungin and a polyene improved survival [70]. Thus, it is clear that combinatorial therapy is superior to the use of a single antifungal agent which results in the development of drug resistance.

Another strategy to negate antifungal drug resistance is therapeutic drug monitoring in patients. The development of drug resistance at high doses and off-targeted effects can also be minimized by maximizing the therapeutic potential through the aforementioned approach. Futuristic investigations are needed to delineate the mechanisms of tolerance and resistance. In vitro simulations and model predictions by utilizing the available pharmacokinetic data will aid in developing drugs with safer therapeutic doses. Integrated pharmacodynamics and pharmacokinetic approaches will aid in the identification of drug concentrations with maximum kill kinetics and reduction in antifungal resistance [71]. Truncated knowledge of Mucorales physiology and its molecular mechanisms of pathogenesis also dampens the development of new antifungals. The critical question of how to stem the rise of antifungal resistance can be addressed in the forthcoming years by involving next-

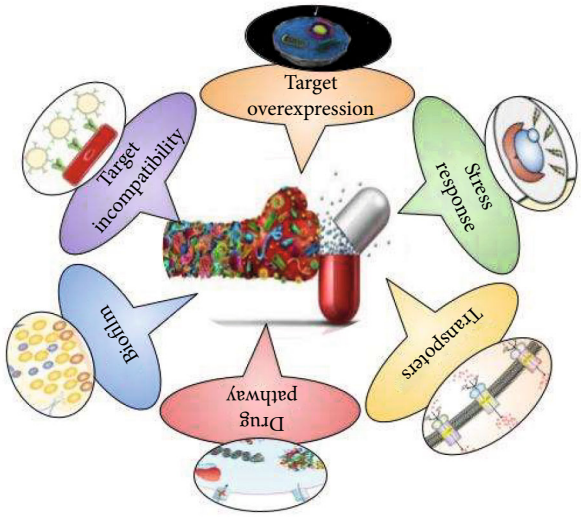

Figure 3: Mechanism of antifungal drug resistance.

generation sequencing and functional genomics to better identify the virulence determinants.

An extensive understanding of mucormycosis necessitates the use of animal models which portray/simulate all possible comorbidities, but they usually fail to recapitulate exact clinical scenarios.

7. Future Directions and Conclusions

Mucormycosis is a pathogen that the host's immune system suppresses in the microbiota. As it becomes one of the widespread infections among the immunocompromised patients and the therapeutic index is variably decreasing due to multidrug resistance more research is needed to gain a better knowledge of these mechanisms of action and resistance, which could help with the detection of resistant isolates and the development of novel pharmacological targets would aid in the prevention of drug resistance. The continuous upsurge in COVID-19 cases with mucormycosis could favor clinical evaluation strategies for early diagnosis and better evaluate the resistance scenario. It is noteworthy to understand that there is a huge gap in the comprehensive validation of drugs across all the members of Mucorales. Therefore, there is a pressing need to involve promising molecular tools for in-depth evaluation and reducing mortality by combating antifungal resistance. The inclusion of CRISPR-Cas technology will aid in deciphering the antifungal resistance mechanisms and untie the knots due to genetic intractability. Another area for futuristic investigations is to understand the interactions between Mucorales and macrophages. Species-specific differences are observed in the killing of Mucorales spores by macrophages which would add to the resistance landscape. Therefore, disseminating the cellular scenarios facilitating the survival of Mucorales inside the macrophage will expand the knowledge to develop effective treatments against this lethal infection. Overall, appropriate treatment modules at an early stage are essential to decrease the mortality rate in mucormycosis. Additional investigations are essential to delineate the molecular mechanisms of resistance and its role in vivo owing to the inability to correlate in vitro data with clinical outcomes.

Data Availability

The data used in this study were done by the authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Antibiotic resistance's Genotypic and Phenotypic Characteristics and the Frequency of Virulence Factors in *P. aeruginosa* Isolates Isolated from Water Samples in Iran

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Pseudomonas aeruginosa is a pathogenic bacterium that can contaminate water. In this study, 430 water samples were evaluated for *P. aeruginosa*, antibiotic resistance, and the abundance of virulence factors. *P. aeruginosa* was isolated from 28 (6.51%) water samples. Among the types of water, well and spring water showed the highest *P. aeruginosa* with, respectively, 20 (15.6%) and 5 (8.06%) positive samples per type of samples. Drinking water and mineral water showed minor contamination with *P. aeruginosa*. The prevalence of antibiotic resistance against meropenem, imipenem, erythromycin, gentamicin, chloramphenicol, and enrofloxacin was zero. The lowest and highest prevalence of antibiotic resistance was observed in drinking water and well water, respectively. The most abundant genes encoding antibiotic resistance in the *P. aeruginosa* were bla_{TEM}, $bla_{CTX\text{-}M}$, and bla_{SHV}. This study also showed that the most abundant virulence genes in *the Pseudomonas aeruginosa* strain isolated from water were *algD* (15 = 3.49%), *lasB* (11 = 2.56%), *toxA* (10 = 2.32%), and *exoS* (7 = 1.63%). This study suggests that water may be a source of *P. aeruginosa* and contribute to releasing resistance genes through the food chain. Cross-contamination is the water transfer process that can cause contamination with *P. aeruginosa* in water. Therefore, hygienic principles can be effective in reducing water contamination.

1. Introduction

According to international standards, healthy water does not contain any microbial contamination and has some characteristics: no odor, no color, clarity, and impurity. Other specifications must be considered, such as microbial contamination, toxic substances, and the absence of harmful minerals [1].

Water is the natural source of *Pseudomonas aeruginosa* (*P. aeruginosa*) [2]. *P. aeruginosa* is a gram-negative, oxidase-positive, and aerobic bacillus found in soil and water and involved in the breakdown of organic matter. It is the cause of 18-61% of hospital deaths due to nosocomial infections [3]. *Pseudomonas aeruginosa* also contains exotoxin A (*exoA*); alkaline protease (*aprA*); exoenzymes S, U, and T (*exoS*, *exoU*, and *exoT*); elastase; and sialidase. The *exoA* gene and the pathogen *exoS* are secreted by secretory system III [4–6]. Due to the importance of this microorganism in water, the Institute of Standards and Industrial Research of Iran (ISIRI) has determined a test called water quality to identify and count *P. aeruginosa* by membrane filtration method, which includes different types of water, mineral water, and swimming pool water [7].

Some studies have identified the presence of this microorganism as an indicator of surface water contamination, domestic and agricultural effluents, or human feces. According to some researchers, the primary source of *P. aeruginosa* is in the surface waters of domestic wastewater, and it is found in 90% of wastewater samples. Its concentration in surface water receiving wastewater is from 1 to 10,000 cells per 100 ml [8].

However, the intestinal transport rate for *P. aeruginosa* in humans is low, indicating that its presence in water is not necessarily due to sewage contamination. Other sources of this microorganism are agricultural soils, drainage, and municipal wastewater. *P. aeruginosa* is directly related to human activities and is reported in fecal contaminated surface waters [9].

High antibiotic resistance of *P. aeruginosa*, especially beta-lactamases including broad-spectrum cephalosporins, quinolones, chloramphenicol, and tetracycline mainly by several antibiotic resistance genes, including bla_{TEM}, bla_{OXA}, $bla_{CTXM\text{-}1}$, and bla_{VEB}, is coded [10, 11]. The presence of resistance genes is the main reason for antibiotic resistance in *P. aeruginosa* strains isolated from food and clinical samples. Strains isolated from water are less resistant to antibiotics than clinical specimens [12].

In recent years, with the widespread multidrug-resistant (MDR) strains in different countries, identifying resistance-related genes has become particularly important. Therefore, the present study evaluated antibiotic resistance's genotypic and phenotypic characteristics and the frequency of virulence factors in *P. aeruginosa* isolates isolated from water samples in Iran.

2. Materials and Methods

2.1. Sampling. A total of 430 water samples, including 160 drinking water, 80 mineral water, 128 healthy water, and 62 spring water samples, were collected randomly from Isfahan, Ilam, Bushehr, and Mazandaran provinces (Iran) for six months. Water samples were collected to clean glass bottles size of approximately one liter. Samples were placed below 4°C during transportation, and testing was performed immediately after receiving the samples.

2.2. Isolation of P. aeruginosa from Water Samples. To isolate *P. aeruginosa* from samples, we followed ISO-16266-2008 International Organization of Standardization [ISO] (2008) instructions. [13]. Briefly, water sample (250 ml) was filtered through a 0.45-mm membrane (Millipore Co., Billerica, MA, United States) using a portable vacuum pump (Millivac-Mini Vacuum Pump XF54, Millipore, Merck, Germany). The membrane was placed on cetrimide agar (PCA) and incubation at 37°C for 24 hours. The colonies were selected based on color and odor (green-blue pigment with a specific odor). To confirm *P. aeruginosa* species, biochemical tests such as fermentation of lactose, citrate, indole, oxidase, DNase, and hemolysis in blood agar medium were performed. Colonies containing lactose-negative, citrate-positive, indole-negative, oxidase-positive, DNase-negative, and hemolytic bacteria were selected as *Pseudomonas aeruginosa*.

2.3. The Antibiotic Resistance Pattern of Pseudomonas aeruginosa Strains. The antibiotic resistance of *P. aeruginosa* isolated from water samples was tested using the disc diffusion method (Kirby Bauer) according to the Institute of Laboratory and Clinical Standards (CLSI, 2015) instructions. *P. aeruginosa* isolates were concentrated in Müller-Hinton agar, and after incubation at 37° C for 24 hours, bacterial susceptibility or resistance to the antibiotics was determined by the growth inhibition zone. Antibiotics discs used were manufactured by BioRad company, France, and *P. aeruginosa* standard strain (ATCC 10145) was used in this experiment as a positive control.

The antibiotic discs include tetracycline (30 μg/disc), chloramphenicol (30 μg/disc), imipenem (30 μg/disc), sulfamethoxazole (25 μg/disc), gentamicin (10 μg/disc), enrofloxacin (5 mg/disc), cephalothin (30 μg/disc), ciprofloxacin (5 μg/disc), trimethoprim (5 μg/disc), ampicillin (10 units/disc), penicillin (10 μg/disc), and erythromycin (15 μg/disc).

2.4. DNA Extraction and Molecular Detection of Virulence Factors and Antibiotic Resistance Genes. DNA was extracted following the methods of Shahrokhi et al. [14]. The overnight culture of bacteria in brain heart infusion (Merck, Germany) and DNA purification genomic kit (Fermentas Germany) extracted DNA. The DNA samples were then placed at -20° C until polymerase chain reaction (PCR).

PCR technique was used to detect virulence and antibiotic resistance genes. The list of utilized primers and the conditions for the reactions are given in Table 1. Gene amplification process was done in 25 μl of a mixture including 1 unit of Taq DNA Polymerase (Fermentas, Lithuania), 200 μmol dNTP (Fermentas, Lithuania), 2.5 μl of 10× buffer solution (Fermentas, Lithuania), 1 μmol of manganese chloride (Fermentas, Lithuania) 10 picomoles of each primer, 3 μl of template DNA, and 25 μl of sterile distilled water. The thermal program includes the initial denaturation temperature 94°C and 6 minutes, denaturation stage temperature 94° C and 60 seconds, the annealing stage temperature 55° C for 1 minute, the extension stage temperature 72° C for 5.5 minutes, and final extension stage temperature 72° C for 5 minutes.

2.5. PCR Product Electrophoresis. We followed the methods of Shahrokhi et al. [14, 15]. The distilled water was used as a negative control, and *P. aeruginosa* (ATCC 10145) was used as a positive control. PCR products were electrophoresed on 2% agarose gel. The gels were stained by Ethidium Bromide (Fermentase, Germany), and DNA strips were evaluated using UV light.

2.6. Statistical Analysis. The percentage of contamination in different sources and products has been calculated. The differences between groups were analyzed with SPSS statistical software (version 18) using the K2 method. The significant level was determined at $p < 0.05$.

3. Results and Discussion

3.1. Isolation of P. Aeruginosa from Water Samples. In this study, 430 water samples were evaluated for the presence of *P. aeruginosa*. The results of contaminated samples based on culture and molecular method (PCR) are summarized in Table 2. In this study, 28 (6.51%) isolates of *Pseudomonas* by bacteriological and PCR methods from drinking water, mineral water, well water, and spring water specimens were obtained. The highest frequency of the isolates ($n = 20$; 15.6%) was recovered from well water. In addition, all the

Table 1: List of primers used to detect virulence factors in *P. aeruginosa* strains isolated from water [28].

Virulence genes	Sequence (5′–3′)	Size of product (bp)
algD	F: AAGGCGGAAATGCCATCTCC R: AGGGAAGTTCCGGGCGTTTG	275
algU	F: CGCGAACCGCACCATCGCTC R: GCCGCACGTCACGAGC	410
lasB	F: ACACAATACATATCAACTTCGC R: AGTGTGTTTAGAATGGTGATC	284
toxA	F: ATGTGCAGYACCAGTAARGT R: TGGGTRAARTARGTSACCAGA	270
plcH	F: CACACGGAAGGTTAATTCTGA R: CGGTTARACGGCTGAACCTG	608
plcN	F: CGACTTCCATTTCCCGATGC R: GGACTCTGCAACAAATACGC	481
exoS	F: GTGTGCTTTATGCCATGAG R: GGTTTCCTTTTCCAGGTC	444
exoT	F: CATCGTCTACGCCATGAG R: AGCAGCACCTCGGAATAG	1159
exoY	F: GGAATGAACGAAGCGTTCTCCGAC R: TGGCGTCGACGAACACCTCG	1035
exoU	F: CTGCGCGGGTCTATGTGCC R: GATGCTGGACGGGTCGAG	3308
apr	F: GCACGTGGTCATCCTGATGC R: TCCGTAGGCGTCGACGTAC	1017
phzII	F: TCCGTTATCGCAACCAGCCCTACG R: TCGCTGTCGAGCAGGTCGAAC	1036
phzM	F: CGTCGTGTTCAAGCAGATGGTGCTG R: CCGAACCGCTTCACCAGGC	875
phzS	F: CAATCATCTCAGCAGAACCC R: TGTCGTAGAGGATCTCCTG	1752
phzI	F: TATCGACGGTCATCGTCAGGT R: TTGATGCACTCGACCAGCAAG	392
phzH	F: GATTCCATCACAGGCTCG R: CTAGCAATGGCACTAATCG	1752
lasA	F: TGTCCAGCAATTCTCTTGC R: CGTTTTCCACGGTGACC	1075
pvdA	F: GCCAAGGTTTGTTGTCGG R: CGCATTGACGATATGGAAC	1281
pilA	F: ATGGAGAGCGGGATCGACAG R: ATGCGGGTTTCCATCGGCAG	1675
pilB	F: TCGCCATGACCGATACGCTC R: ACAACCTGAGCCAGCCTTCC	408

isolates were confirmed by PCR. The prevalence of *P. aeruginosa* isolates in different water samples is shown in Table 2. The contamination rate of water samples with *P. aeruginosa* was reported 28. Drinking water and mineral water showed the lowest contamination, respectively. Statistically, no significant difference was observed between the samples ($p > 0.05$).

The well water pollution rate was significantly higher than the spring water. Therefore, groundwater is better as raw water than well water. The main reason for this is that healthy water is generally drained from the pipes to the open reservoir and exposed to the air for a long time without effective protective measures [14]. The high rate of *P. aeruginosa* contamination in spring water may be due to irregular production processes during the production process, including inadequate disinfection and formation of *P. aeruginosa* biofilms in the line [15]. Therefore, producers of drinking water should pay attention to controlling and managing the production process to ensure the safety of the water.

Table 2: The prevalence of *P. aeruginosa* in water.

Sample	*N.* samples collected	Bacteriological methods N. samples positive (%)	PCR N. samples positive (%)
Drinking water	160	1 (0.6)	1 (0.6)
Mineral water	80	2 (2.5)	2 (2. 5)
Well water	128	20 (15.6)	20 (15.6)
Spring water	62	5 (8.06)	5 (8.06)
Total	430	28 (6.51)	28 (6.51)

No significant difference was observed between samples ($p > 0.05$).

Szita et al. [2] reported 94 contaminated samples with *P. aeruginosa* out of 160 well water samples [2]. Wei et al. [13] reported 77 cases (24.5%) of *P. aeruginosa* from mineral water and spring water samples collected in China [14]. According to previous research by Wu et al. [16], *P. aeruginosa* contamination in raw water (30.4%) and activated carbon filtered water (38.6%) was significantly higher than in raw water (3.9%) [13]. Stoler et al. [17] reported that 41% of 80 water samples were positive for *P. aeruginosa*. They did not detect any strains of *P. aeruginosa* in the final mineral water [15]. However, the pollution rate of spring water was reported to be 6.9%, which is harmful to consumers' health. Anversa et al. [18] reported 19 cases (7.6%) of *P. aeruginosa* contamination in waters in Brazil [17].

According to the guidelines for the quality of water consumption (98/83/EC), drinking water should not be positive for the prevalence of *P. aeruginosa*, and its prevalence should be systematically controlled in the final water. Water chlorination and activated carbon filter system are the essential treatment processes for drinking water and absorb organic pollutants and microbes for water treatment [16]. Activated carbon filters can be contaminated with microorganisms and the most severe problem of microbial contamination in the whole process of drinking water treatment. Therefore, drinking water manufacturers must regularly clean, disinfect, and replace activated carbon filters [18].

Our results confirmed the contamination of water samples with *P. aeruginosa*. These results are comparable to those of *P. aeruginosa* contamination in other foods. For example, Algammal et al. [19] isolated 31.57% *P. aeruginosa* from 90 fish samples in Egypt [20]. Benie et al. [21] reported the prevalence of *P. aeruginosa* in beef, fresh fish, and smoked fish at 47.8%, 33.1%, and 20%, respectively [22].

Table 3: Antibiotic resistance pattern of *P. aeruginosa* isolates isolated from water.

(a)

Antibiotic	Drinking water	Mineral water	Spring water	Well water
Tetracycline	0	1	1	1
Doxycycline	0	0	0	0
Erythromycin	0	0	0	0
Enrofloxacin	0	0	0	0
Chloramphenicol	0	0	0	0
Cephalothin	0	0	1	1
Imipenem	0	0	0	0
Carbapenem	0	0	0	0
Gentamicin	0	0	0	0
Ciprofloxacin	0	0	1	1
Trimethoprim	0	1	0	0
Sulfamethoxazole	0	0	1	1
Penicillin	0	0	0	0
Ampicillin	0	0	0	0

(b)

Antibiotic	Sensitive	Semisensitive	Resistant	Total
Tetracycline	0	4	3	7
Doxycycline	6	1	0	7
Erythromycin	4	3	0	7
Enrofloxacin	4	3	0	7
Chloramphenicol	7	0	0	7
Cephalothin	4	2	1	7
Imipenem	7	0	0	7
Carbapenem	7	0	0	7
Gentamicin	7	0	0	7
Ciprofloxacin	6	0	1	7
Trimethoprim	6	1	0	7
Sulfamethoxazole	5	0	2	7
Penicillin	4	3	0	7
Ampicillin	5	2	0	7

Benie et al. [23] isolated 153 multidrug-resistant strains of *Pseudomonas aeruginosa* from beef (93), fresh fish (36), and smoked fish (24) [19]. Abd-El-Maogoud et al. [24] isolated *P. aeruginosa* from 65% of frozen mackerel (33%), frozen Sarus (30%), and tilapia samples (23%) [21].

3.2. The Antibiotic Resistance Pattern of Pseudomonas aeruginosa Strains. Table 3 (a, b) summarizes the antibiotic resistance of *P. aeruginosa* strains isolated from water. The results show that the prevalence of antibiotic resistance against meropenem, imipenem, carbapenem, erythromycin, gentamicin, chloramphenicol, and enrofloxacin was reported to be zero. The prevalence of antibiotic resistance against antibiotics used in drinking water was zero. The highest antibiotic resistance was observed in well water.

Table 4: Resistance factors of *Pseudomonas aeruginosa* isolated from water.

Antibiotic resistant gene	Drinking water	Mineral water	Spring water	Well water
bla_{TEM}	0	0	0	5
bla_{SHV}	0	0	0	1
bla_{OXA}	0	0	0	1
$bla_{CTX\text{-}M}$	0	0	0	3
bla_{DHA}	0	0	0	0
bla_{VEB}	0	0	0	0

Table 5: Virulence factors of *Pseudomonas aeruginosa* isolated from water.

Virulence factors	Drinking water	Mineral water	Spring water	Well water
algD	1	1	2	11
algU	0	1	1	1
lasB	0	1	2	8
toxA	1	1	1	7
plcH	0	0	0	0
plcN	0	0	0	0
exoS	0	1	2	4
exoT	1	1	1	2
exoY	0	0	0	2
exoU	0	0	0	2
apr	0	1	1	3
phzII	0	0	0	0
phzM	0	0	1	2
phzS	0	0	0	0
phzI	0	0	0	0
phzH	0	1	1	1
lasA	0	1	1	2
pvdA	0	0	0	0

P. aeruginosa was resistant to tetracycline, cefotaxime, chloramphenicol, imipenem, and penicillin antibiotics. Four semisensitive samples were reported against doxycycline, gentamicin, penicillin, and ampicillin antibiotics.

In Wei et al. (2020) study [13], they determined the prevalence, virulence genes, and antimicrobial resistance of P. aeruginosa in drinking water in China. All P. aeruginosa isolates were sensitive to the 14 antibiotics (ciprofloxacin, levofloxacin, ofloxacin, norfloxacin, gentamicin, tobramycin, amikacin, polymyxin B, imipenem, meropenem, aztreonam, ceftazidime, cefepime, and piperacillin/tazobactam) tested [14]. Silva et al. [25] isolated 30 *P. aeruginosa* from drinking water resistant to one or more antibiotics [23].

Differences in results may be related to different sources of samples. In this study, raw water is mainly supplied from groundwater and less exposed to external factors. These media are rarely contaminated with antibiotics, so antibiotic-resistant *P. aeruginosa* has not been observed.

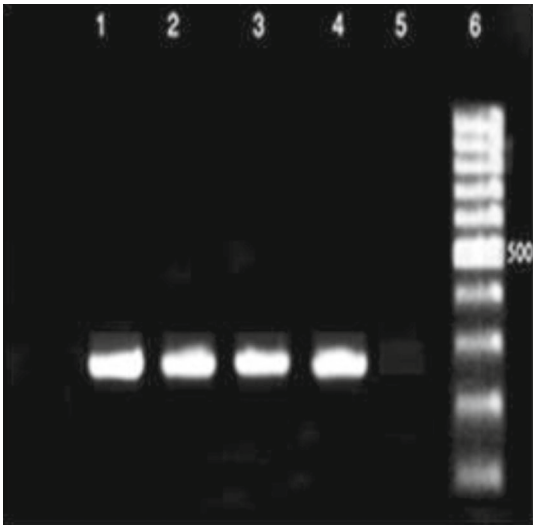

Figure 1: PCR product electrophoresis of 275 bp, *algD* gene (lanes 1–3: positive samples, lane 4: positive control, lane 5: negative control, lane 6: 100 bp gene ruler).

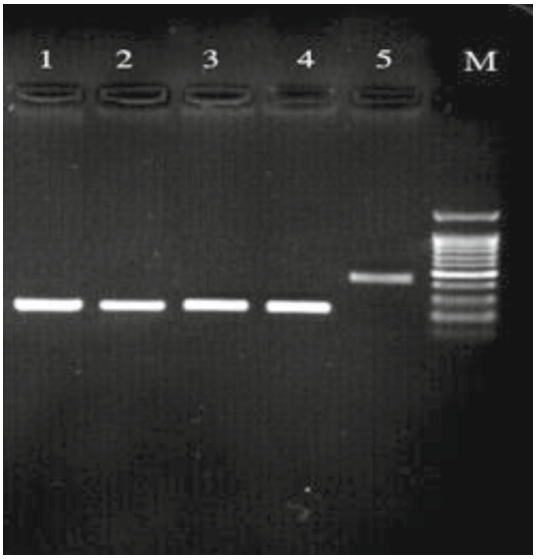

Figure 2: PCR product electrophoresis of 284 bp *lasB* gene and *exoS* 444 bp (lanes 1–3: positive samples 286 bp, 4 positive control, lane 284: *lasB* gene, lane 5: positive sample 444 bp sample *exoS*, lane M: 100 bp gene ruler).

The present study results are comparable to antibiotic resistance *P. aeruginosa* in other foods. For example, *P. aeruginosa* strains isolated in Egypt showed multidrug resistance (MDR) to amoxicillin, cefotaxime, tetracycline, and gentamicin [20]. Carol et al. [26] observed that *P. aeruginosa* had high resistance to amoxicillin, moderate resistance to ampicillin, ceftazidime, nitrofurantoin, and gentamicin sensitivity tobramycin, cefotaxime, methylene, ciprofloxacin, and amikacin [24]. Benie et al. [21] examined the prevalence of *P. aeruginosa* in beef, fresh fish, and smoked fish. They found that *Pseudomonas aeruginosa* strains were mainly resistant to cefepime, imipenem, ceftazidime, ciprofloxacin, and piperacillin [22].

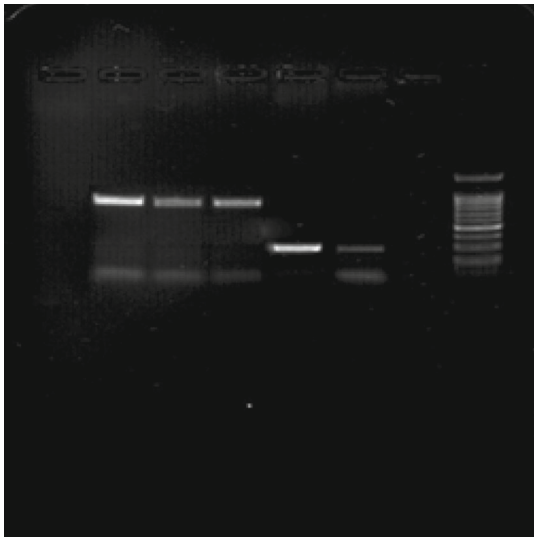

Figure 3: PCR product electrophoresis of 270 bp *toxA* gene and 875 bp *phzM* (from left, lanes 1–3: positive samples of 875 bp *phzM* gene; lanes 4 and 5: positive sample of 270 bp *toxA* gene; lane 6: negative controls; lane 6: 100 bp gene ruler).

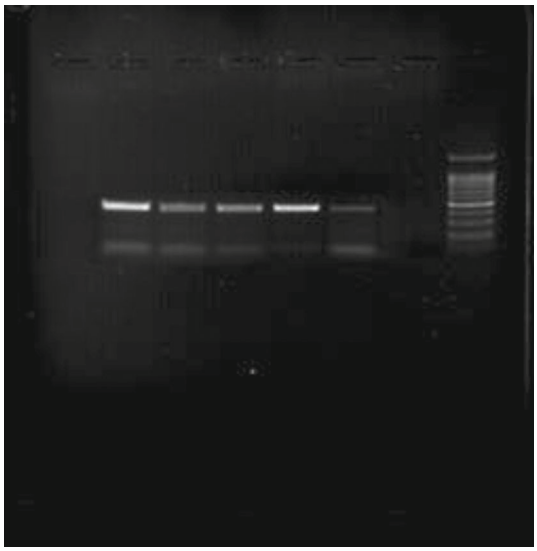

Figure 4: PCR product electrophoresis of 410 bp *algU* gene (from left, lanes 1–4: positive samples, lane 4: positive control, lane 5: negative control, lane 6: 100 bp gene ruler).

3.3. The Virulence and Antibiotic Resistance Genes. This study also showed that the most abundant virulence genes in the *Pseudomonas aeruginosa* strain isolated from water were *algD* (15 = 3.49%), *lasB* (11 = 2.56%), *toxA* (10 = 2.32%), and *exoS* (7 = 1.63%). This study suggests that water may be a source of *P. aeruginosa* and contribute to releasing resistance genes through the food chain. Tables 4 and Table 5 summarize the antibiotic resistance and virulence genes of *P. aeruginosa* isolated from water samples. As can be seen, the most abundant genes encoding antibiotic resistance in *Pseudomonas aeruginosa* strains were bla_{TEM}, $bla_{CTX\text{-}M}$, and bla_{SHV}. The most abundant virulence genes

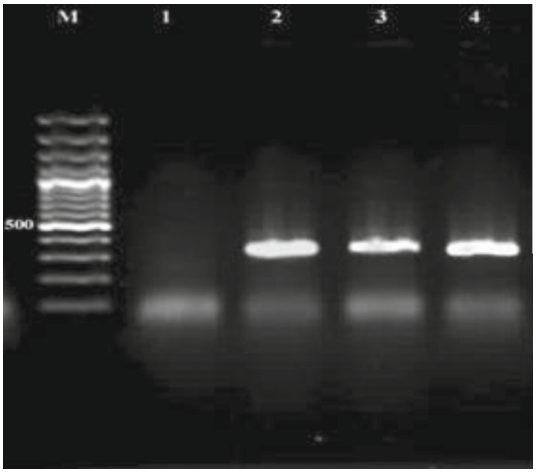

Figure 5: PCR product electrophoresis of 392 bp of *phzI* gene (from left, lane M: 100 bp gene ruler, lane 1: negative control, lanes 2–4: positive samples).

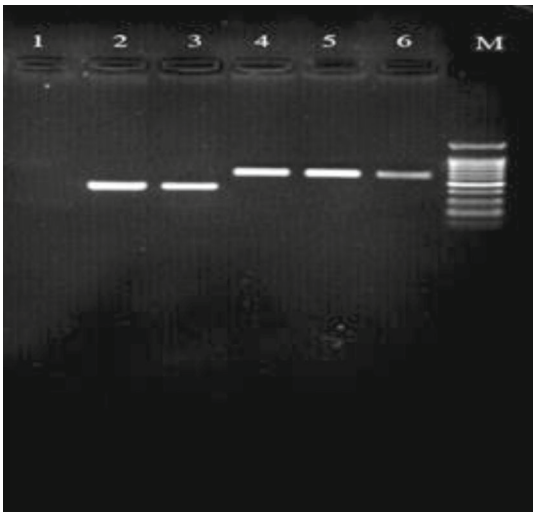

Figure 6: PCR product electrophoresis of 593 bp $bla_{CTX\text{-}M}$ gene and 642 bp bla_{VEB} (from left, lane 1: negative control; lanes 2 and 3: positive samples of 593 bp $bla_{CTX\text{-}M}$ bp; lanes 4 and 5: positive samples, 642 bp sample bla_{VEB} gene; lane 6: positive control; lane M: 100 pb gene ruler).

detected in *P. aeruginosa* were *algD*, *algU*, *lasB*, *toxA*, *exoS*, *exoT*, and *apr*.

Shi et al. [27], using the UP-MPCR method, identified five enterotoxin genes including *ExoU*, *ExoS*, *phzM*, *toxA*, and *lasB* in *P. aeruginosa* isolated from 214 drinking water and environmental isolates [25]. Wei et al. [13] identified the virulence genes *ExoU*, *ExoS*, *phzM*, *toxA*, and *lasB* from mineral water and spring water samples collected in China [14]. Wu et al. [16] identified three virulence genes, *lasB*, *phzM*, *toxA*, *ExoU*, and *ExoS*, widely distributed among *P. aeruginosa* [13]. According to previous research, *ExoU* and *ExoS* viruses have not been detected in identical isolates [6]. *P. aeruginosa* pathogenesis is closely related to the virulence genes *ExoU*, *ExoS*, *phzM*, *toxA*, and *lasB* [26, 27]. The presence of virulence genes in this study is comparable to the results of *P. aeruginosa* contamination in other foods such as fish and beef [19, 20].

3.4. PCR Product Electrophoresis. Figures 1–6 show the PCR product electrophoresis of *P. aeruginosa* isolated from water samples.

4. Conclusion

Pseudomonas aeruginosa is an important pathogen and a significant threat to the microbial safety of drinking water. Isolation of *P. aeruginosa* strains from water is essential because it is a potentially pathogenic bacterium for humans and indicates food quality. This study showed the prevalence of *P. aeruginosa* in well water. Due to the abundance of *P. aeruginosa* in water, especially well water and spring water, special attention should be paid to water treatment methods that will not contribute to the selection of antibiotic-resistant *P. aeruginosa*. In addition, characterization of isolates using molecular techniques can help to develop effective and accurate prevention and control measures against *P. aeruginosa* contamination of the whole drinking water production process.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

Conflicts of Interest

The authors declare no potential conflict of interest.

Authors' Contributions

All authors read and approved the final manuscript.

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Research Article

Proteochemometric Method for pIC50 Prediction of Flaviviridae

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Viruses remain an area of concern despite constant development of antiviral drugs and therapies. One of the contributors is the Flaviviridae family of viruses causing diseases that need attention. Among other anitviral methods, antiviral peptides are being studied as viable candidates. Although antiviral peptides (AVPs) are emerging as potential therapeutics, it is important to assess the efficacy of a given peptide in terms of its bioactivity. Experimental identification of the bioactivity of each potential peptide is an expensive and time consuming task. Computational methods like proteochemometric modeling (PCM) is a promising method for prediction of bioactivity (pIC50) based on peptide and target sequence pair. In this study, we propose a prediction of pIC50 of AVP against the Flaviviridae family that may help make the decision to choose a peptide with desired efficacy. The peptides data was collected from a public database and target sequences were manually curated from literature. Features are calculated using peptide and target sequence PCM descriptors which consist of individual and cross-term features of peptide and respective target. The resultant R^2 and MAPE values are 0.85 and 8.44%, respectively, for prediction of pIC50 value of AVPs.

1. Introduction

Viral diseases have been a cause of multiple epidemic outbreaks in the last few decades. This includes many different viruses like Ebola, Zika, Dengue, SARS, and others. Although belonging to one bigger group of viruses, they differ a lot in their activity, sequence, structure, and function. Viruses are also known to have continuous mutations, which makes it necessary and complex to identify antiviral drug candidates. This leads to the need for continuous drug development. Recently, peptide-based antivirals have gained a lot of importance and have shown promising development [1]. In this work, we study methods to predict the pIC50 value for the Flaviviridae family. Among various types of viruses, publically available data is found majorly for hepatitis C virus (HCV) and dengue virus (DENV). Rajput and Kumar developed an algorithm to identify inhibitory activity of chemicals from ChEMBL and peptides from AVPpred databases against Flaviviruses using QSAR method [2]. Recently, Geoffrey et al. developed machine learning based Auto-QSAR using PubChem data, which generated drug leads for Flaviviruses. For the drug leads and their target proteins in silico modeling was performed [3].

Generally, antiviral peptides are studied based on physicochemical properties, evolutionary properties, and profiles based on only peptide attributes [4]. However, a peptide can have good physicochemical properties that are similar to other bioactive peptides but its efficacy cannot be identified unless its bioactivity is experimentally determined. The inhibition constant (IC) 50 value is commonly used for validating the activity of a peptide. Experimental determination of IC50 is an expensive and tedious task. Taking all the potential peptides for experimental validation might not be feasible. Prediction of IC50 values can reduce the time and effort and help in selecting the most promising peptide for further experimentation. There is very little research done in the area of in silico methods for IC50 value prediction. To understand the IC50 of a peptide, it is necessary to understand the interaction with the target protein. One method for doing so is proteochemometric modelling (PCM).

PCM is a computational method that can predict the bioactivity relations between ligand and targets. It is a

method to incorporate the target interaction into sequence-based analysis. Three types of descriptors are included in PCM-Target descriptor (captures information of target), Ligand descriptor (captures information of ligand), and Cross-term descriptor (captures interaction between the ligand and its target). With the different types of interactions studied, the scope of PCM has expanded to include protein-peptide, protein-DNA, and protein-protein interactions.

1.1. Literature Survey. Recently, Parks et al. using the ChEMBL25 dataset, generated proteochemometric models to predict pIC50 using random forest and feed-forward neural network [5]. The study checked the usability of PCM model to classify binders and nonbinders. For the ChEMBL25 data set, various physicochemical properties like log P, molecular weight, number of specific bonds, and fingerprints were used as descriptors. Yordanov et al. demonstrated the use of PCM for analysing the structure-affinity relationship of antigen peptides binding to HLA-DP proteins [6]. The HLA system plays an important part in the immune system. The HLA proteins bind to a wide range of antigenic peptides, which is essential for the immune recognition of the antigens. The chemical structures of peptides and proteins used were described by three z-scales. Bio-activity modeling of multiple compounds against protein isoforms was done by Rasti et al. using proteochemometrics modeling [7]. They applied PCM to investigate inhibition of Carbon Anhydrase isoforms using a combination of different descriptors (three z-scale, five z-scale and, GRIND). Mutations affect the antimicrobial activity. The PCM model has also been used to identify the mutations. The study by Nabu et al. helped in understanding the impact of physicochemical properties of mutated amino acids on the resistance of penicillin binding proteins [8]. The mutation positions and various chemical descriptors were utilised as protein sequence and ligand descriptors, respectively.

1.2. Approach. In this work, we developed a PCM-based model for the prediction of pIC50 values for peptides against the Flaviviridae family. The peptides and target proteins were cumulatively studied using PCM descriptors which included peptide properties, z-scales for peptides, proteins, and peptide-protein interaction. The complete workflow of the study is shown in Figure 1.

The overall approach of the study includes:

(i) Curation of dataset

(ii) Defining PCM descriptors

(iii) Methodology and training of the machine learning model

(iv) pIC50 prediction algorithm results

2. Materials and Methods

This section elaborates on data, features, and details of the machine learning algorithms.

2.1. Curation of the Dataset. The datasets are made up of publically available antiviral peptide data with their IC50 values and the target proteins collected from the literature. This results in two datasets:

(i) Antiviral peptides with IC50 values (Dataset 1) and

(ii) Antiviral peptides with IC50 values and their target proteins (Dataset 2).

2.1.1. Dataset 1. Antiviral peptide data is taken from the publicly available AVP-IC50 dataset [9]. The dataset consists of AVP sequences, IC50 values in micromolar and their respective viral families. From these, peptides for only the Flaviviridae family are taken, constituting Dataset 1 with total of 130 sequences.

2.1.2. Dataset 2. For Dataset 2, along with the AVP, Flaviviridae target proteins are also taken. The target proteins of the antiviral peptides have been identified in the literature [10–30]. Here, 50 peptide sequences out of 130 have defined targets. These 50 peptide sequences along with their target protein sequences form Dataset 2. Target protein sequences are extracted from Uniprot [31].

2.1.3. pIC50. The IC50 values in the datasets ranged from 0.001 micromolar to 440 micromolar making the distribution very skewed, as shown in Figure 2(a). This would have made it difficult for the model to extract informative patterns to learn from. Thus, the IC50 values were negative log transformed to give the pIC50 value. This is done using the following formula:

$$\mathrm{pIC50} = -\ln\left(\mathrm{IC50} * 10^{-6}\right) \quad (1)$$

The data distribution after converting to pIC50 is shown in Figure 2(b). Hereafter, pIC50 will be used in the rest of the paper.

2.2. Defining PCM Descriptors. PCM modeling works based on descriptors which are mathematical representations of various properties of peptides and their target proteins. Here, we are looking at the following descriptors which are calculated for peptides and their target proteins:

(i) Physicochemical properties of peptides and target protein

(ii) Z-scale for peptides, target protein, and their cross-term

Physicochemical properties. Peptide and target protein properties were calculated from their amino acid sequences using Biopython package [32]

Z-scales. The peptides and target proteins used in this work are represented using five z-scale descriptors (z_1, z_2, z_3, z_4, and z_5) of their amino acid as derived by Sandberg et al. [33]. The z-scale represents their hydrophobicity (z_1), steric bulk properties and polarizability (z_2), polarity (z_3), and electronic effects (z_4 and z_5). These five z-scales are the principal components of 26 computed and measured

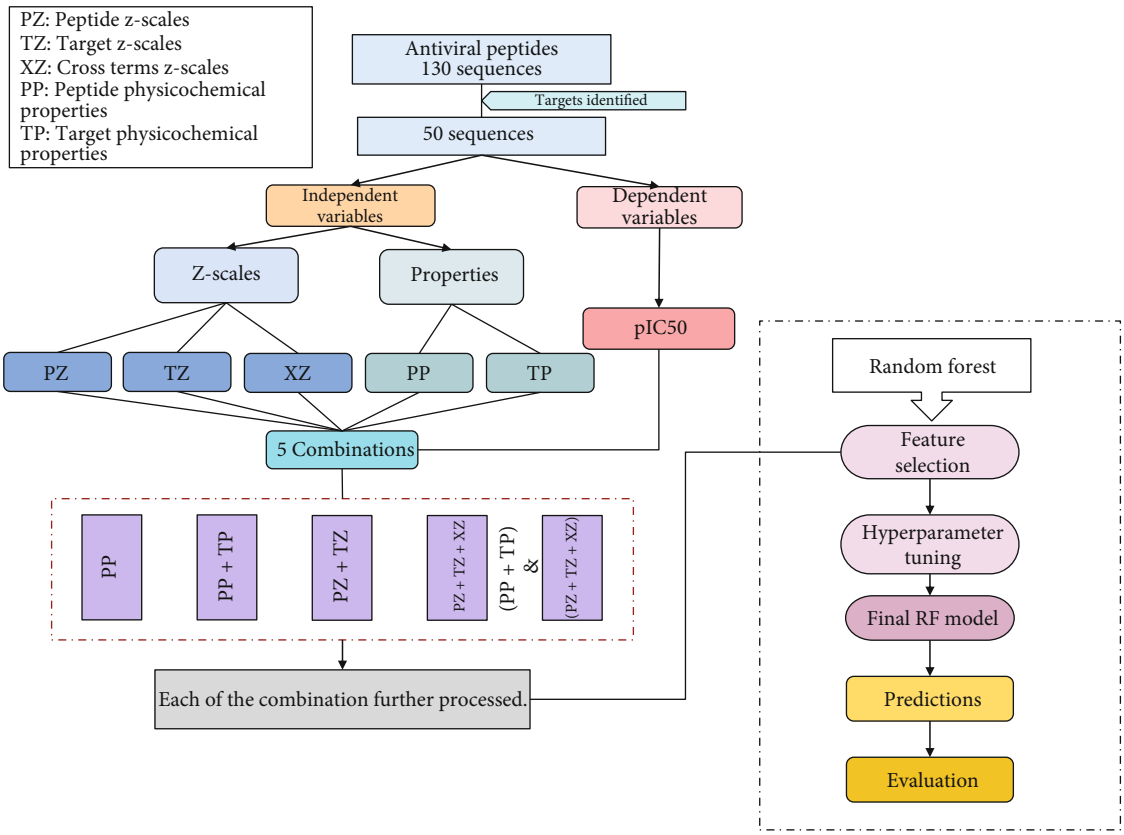
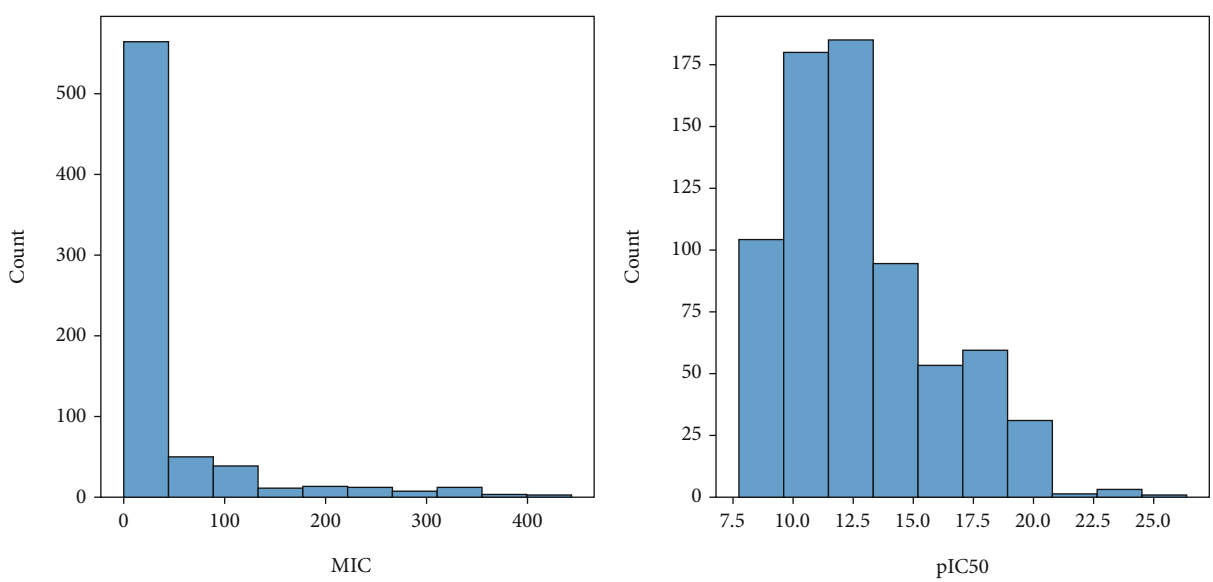


Figure 1: PCM model flowchart. Illustration of the descriptors and multiple combinations of descriptor groups used to predict the pIC50 using Random Forest.



(a) IC50 value distribution

(b) pIC50 value distribution

Figure 2: Distribution of IC50 and pIC50 values.

physicochemical properties of amino acids. To get a z-scale descriptor for a peptide and protein, the average is taken of their amino acid z-scale vectors. The z-scale descriptors for both peptides and proteins are normalized to standard normal. In order to incorporate the information about the interaction between protein and the peptide, cross-term descriptors were also included. This is calculated as flattened out outer product of normalized peptide z-scales

and normalized protein z-scales resulting in 25 (5x5) dimensional vector. As a result, each peptide-protein pair is represented as a 35 dimensional vector (5 peptide z-scale, 5 protein z-scale and 25 cross-term)

Descriptor groups. In order to perform various machine learning experiments, multiple groups of descriptors were created as defined as follows:

(i) Physicochemical properties for peptides (PP)

(ii) Physicochemical properties for target protein (TP)

(iii) Peptide z-scale descriptors (PZ)-5 z-scale descriptors calculated for peptide sequences

(iv) Target protein z-scale descriptors (TZ)-5 z-scale descriptors calculated for target protein sequences

(v) Cross term descriptors (XZ)-Multiplication of peptide and target protein z-scale descriptors generated the cross term descriptors group

2.3. Machine Learning Details. In this section, we discuss the features selection method, machine learning algorithm, and evaluation criteria of the model.

Feature selection. In machine learning, it is important to have useful input features or descriptors. Therefore, in order to remove uninformative predictors, feature selection is carried out. This not only removes noise from the data, but also reduces dimensionality of the data which makes the trained model less complex and more interpretable. The feature selection for this work is carried out in two steps. First, the feature ranking is obtained using Recursive Feature Elimination. This is followed by adding the features iteratively starting from the highest ranking feature and checking for their predictive performance. Further, only those features were added to the final feature set whose addition improved the adjusted-R^2 value. The calculation of adjusted-R^2 based on R^2 value is as follows:

$$\text{adjusted} - R^2 = 1 - \frac{\left(1 - R^2\right)(n-1)}{n-p-1}, \tag{2}$$

where R^2 is sample R-squared value, n is number of examples, and p is number of predictors.

Random Forest. Random Forest is an ensemble learning method for classification and regression. The underlying principle is to construct multiple decision trees and aggregate the output from each decision tree. In case of a regression problem, most common means of aggregating the output is mean of the predictions. In Random Forest, each of the decision tree is trained on a randomly selected subset of features and examples from the training data which causes each tree to learn different patterns from the same training data. This results in a reduced variance, making the model more effective. Random Forest regressor model from scikit-learn library [34] was used

Evaluation criteria. The selection of informative performance metrics is vital in order to measure effectiveness of a prediction model. Therefore, to informatively measure performance of the prediction models, performance measures used in this work is described as follows:

Table 1: Range of values used to tune hyperparameters for Random Forest.

Hyperparameter	Ranges
n_estimators	100, 200, 250, 300, 500, 1000, 1500
min_samples_split	2, 5, 10
min_samples_leaf	1, 2, 4
max_features	auto, sqrt, log2
max_depth	2, 3, 5, 10, 15, 20, None
bootstrap	True, False

(i) Root Mean Squared Error (RMSE):

$$\text{RMSE} = \sqrt{\frac{1}{n}\sum_i (y_i - \hat{y}_i)^2} \tag{3}$$

(ii) Mean Absolute Percentage Error (MAPE):

$$\text{MAPE} = \frac{1}{n}\sum_i \frac{|y_i - \hat{y}_i| * 100}{y_i} \tag{4}$$

(iii) R-squared value (R^2):

$$\begin{aligned} R^2 &= 1 - \frac{SS_{res}}{SS_{tot}}, \\ SS_{res} &= \sum_i (y_i - \hat{y}_i)^2, \\ SS_{res} &= \sum_i (y_i - \bar{y})^2, \end{aligned} \tag{5}$$

where SS_{res} is residual sum of squares, SS_{tot} is total sum of squares

(iv) Pearson's Correlation Coefficient (PCC):

$$PCC = \frac{\sum_i (y_i - \bar{y})\left(\left(\hat{y}_i - \bar{\hat{y}}\right)\right)}{\sqrt{\left(\sum_i (y_i - \bar{y})^2\right)\left(\sum_i \left(\hat{y}_i - \bar{\hat{y}}\right)^2\right)}}, \tag{6}$$

where y is actual pIC50 value, $\hat{y}$ is predicted pIC50 value, $\bar{y}$ is mean of actual pIC50 values, and $\bar{\hat{y}}$ is mean of predicted pIC50 values

2.4. Methodology. This section elaborates on utilizing the curated data, properties, and algorithm for training the

Table 2: Actual and predicted pIC50 values for example sequences.

Sequence	Actual pIC50 value	Predicted pIC50 value
AFLGWIGAIVSTALPQWR	11.289	11.261
ACFPWGNTWCGGK	11.250	11.262
MANAGLQLLGFILAFLGWIGAI	12.429	11.224
RWMVWRHWFHRLRLPYNPGK NKQNQQWP	11.736	11.246
AAQRRGRIGRNPSQVGD	7.934	8.158
RTGRGRRGIYR	10.271	11.294
GELGRLVYLLDGPGYDPIHCSL AYGDASTLVVF	17.678	19.780

Table 3: Results of obtained models.

Descriptor combinations	R^2	MAPE	PCC	MSE
PP	0.48	14.04	0.72	6.88
PP+TP	0.72	11.46	0.84	3.70
PZ+TZ	0.72	11.32	0.85	3.63
PZ+TZ+XZ	0.76	9.48	0.87	3.14
PP+TP and PZ+TZ+XZ	**0.85**	**8.44**	**0.92**	**1.99**

suitable model. As explained in 2.1, properties were calculated for Dataset 1 and Dataset 2. Dataset 1 was studied using physicochemical properties. However, for Dataset 2 all descriptor groups (as explained in Section 2.2) were calculated as they contain both peptide and target protein information. Using only the peptide properties for Dataset 1 to train the model, resulted in lower R^2 value. Considering this, this study further focuses on utilizing Dataset 2.

We see an improvement in the results with the addition of peptide and target property groups in the model. Using multiple sequence descriptors can be helpful for the model to learn. Hence, along with properties, z-scales are used to represent the sequences. The model was trained on peptide (PZ) and target protein z-scales (TZ), which further improved the performance of the model. The PCM approach includes one more cross-term group (XZ). A combination of property groups for peptides (PP), target proteins (TP), and z-scale groups (PZ, TZ, and XZ) performs the best out of all the combinations. The descriptors calculated and the combinations are illustrated in Figure 1. Furthermore, in order to remove the noisy or noninformative features, we performed feature selection as explained in Subsection 2.3. During the feature selection phase, Random Forest with default parameters is used as a base regression model.

The Random Forest model is then tuned on the selected features to achieve the best performance. For this, we use the grid search approach where hyperparameters form the axis of the grid and each point on the grid is a combination of defined values for each hyperparameter. 5 fold cross-validation on Mean Absolute Percentage Error (MAPE) helps to obtain best performing hyperparameter values. The hyperparameters and their values for which the Random Forest model is tuned are mentioned in Table 1. As a last step, the Random Forest training is done on the best preforming feature set and hyperparameters, and its predictive performance is evaluated. Furthermore, to ensure fairness of the results, all performance measures are calculated using Leave-one-out (LOO) cross-validation (CV).

3. Results and Discussion

In this section we look at the results obtained for various combinations of descriptor groups. Results for some of the example sequences are mentioned in Table 2. It can be seen from the results that the predicted pIC50 values are very close to actual pIC50 values. The performance of the model is evaluated using Leave-one-out cross-validation and the results are tabulated in Table 3.

The model is trained using only peptide properties which resulted in the low R-squared value of 0.29 and high mean absolute percentage error of 17.10%. To understand this, we have done further analysis of the dataset. We observed that there are certain peptide sequences in the dataset which are very similar but still have very different pIC50 value and vice versa. Similar is the observation for physico-chemical properties based descriptors. This might make it difficult for the model to learn informative patterns from the peptides alone and needs additional information.

Based on the results in Table 3, the rest of the experimentation are performed using PCM approach. The addition of target protein properties improved the results in R-squared value of 0.30 and MAPE of 18.82. The combination of z-scale for peptide and protein significantly improved the results giving R-squared value of 0.72 and MAPE of 11.32%. The details of peptide and target protein combinations for z-scale and physicochemical properties are detailed in Section 2.2.

In PCM, along with the target and peptide descriptor groups, cross-term z-scales are often considered as they represent the potential of peptide and target protein. The model has been trained using the three descriptor groups (PZ, TZ, and XZ). The addition of peptide protein cross-term descriptor slightly improved the predictive performance of the model further, giving R-squared value of 0.76 and MAPE of 9.48%.

The best results with R-squared value of 0.85 and MAPE of 8.44% are obtained with combinations of z-scale and physicochemical properties.

3.1. Discussion. Despite various scientific discoveries and advancements, viruses continue to be one of the threats to

human health [35]. Antiviral peptides (AVPs) are emerging as one of the interesting alternative therapeutics to viral concerns. Although elusive, antiviral peptides do exhibit certain physicochemical properties which makes them great candidates for antiviral therapeutics [1]. The work done by Surana et al. explains the usage of physicochemical properties [36]. Although physicochemical properties do help in identifying the potential antiviral peptides, IC50 is one of the methods used for further validating the efficacy of candidate peptides [37]. In order to predict the candidate peptides as close to the experimental method as possible, we propose the prediction of pIC50 in our current study.

We initially utilized a combination of peptide properties and IC50 of known peptides to create the algorithm. However, we discovered that there is no direct correlation between the peptides and the IC50 values. The behavior of a peptide depends on its own properties as well as the nature of the target against which the peptide is going to act. This is where PCM methods seem to be the right approach because it does not only take the peptides but also takes the target protein into account.

PCM models give the flexibility to study multiple ligands (peptide in this case) and multiple target setups, which become beneficial in the current study. As for Flaviviridae AVPs, although they are all against flaviviruses, they have different targets that they act upon. As explained earlier, the major features utilized were the physicochemical properties of peptides and target proteins. We observed that adding the physicochemical properties of target proteins improved the pIC50 prediction for the peptides. Along with the basic physicochemical properties, the addition of Z-scores gave a boost to the model performance.

The Z-score includes the hydrophobicity, steric bulk properties, polarity, polarizability, and electronic effects giving additional features to the physicochemical properties. The Z-scores individually cover the features of peptides, target proteins, and cross-descriptors covering the interaction features between the peptide and target proteins. The cross-terms features give an additional edge in terms of covering the ligand-target interactions instead of only features of individual peptides and targets. Multiple combinations of physicochemical properties and Z-score descriptors lead us to the model with the best descriptors giving good predictions. The best descriptors identified were target polarity, cross-term of peptide polarity, and target hydrophobicity along with aromaticity. It can be inferred that aromaticity, along with hydrophobicity and polarity of protein and peptide, best describes the relationship and hence the prediction of IC50.

We further explored the PCM2Vec methodology for IC50 predictions. However, we did not get very good results there, and it needs to be explored further. PCM2Vec can be a promising algorithm which can be explored in future work.

4. Conclusion

In this study we have applied proteochemometric modeling to study the pIC50 values for AVPs against the Flaviviridae family. The activity prediction of probable antiviral peptide brings in additional validation to the efficacy of AVP to choose the peptide for further experiments based on their pIC50 value, and thus reducing the time and cost for the experimental cycle. As most of the peptides or drug candidates are built against specific targets, being able to predict their bioactivity values is one step closer to getting more accurate peptides. This can be further extended to multiple viral families or target-based PCM models. Currently we have considered entire target sequences, however, further study can be done to include binding site residues and their interactions. Taking binding site residues into consideration might further boost the algorithm performance.

Data Availability

The code and data is available at https://github.com/thoughtworks/mic-predictor

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

This paper is available as a preprint on Biorxiv [38].

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Review Article

Multidrug-Resistant Bacteria: Their Mechanism of Action and Prophylaxis

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In the present scenario, resistance to antibiotics is one of the crucial issues related to public health. Earlier, such resistance to antibiotics was limited to nosocomial infections, but it has now become a common phenomenon. Several factors, like extensive development, overexploitation of antibiotics, excessive application of broad-spectrum drugs, and a shortage of target-oriented antimicrobial drugs, could be attributed to this condition. Nowadays, there is a rise in the occurrence of these drug-resistant pathogens due to the availability of a small number of effective antimicrobial agents. It has been estimated that if new novel drugs are not discovered or formulated, there would be no effective antibiotic available to treat these deadly resistant pathogens by 2050. For this reason, we have to look for the formulation of some new novel drugs or other options or substitutes to treat such multidrug-resistant microorganisms (MDR). The current review focuses on the evolution of the most common multidrug-resistant bacteria and discusses how these bacteria escape the effects of targeted antibiotics and become multidrug resistant. In addition, we also discuss some alternative mechanisms to prevent their infection as well.

1. Introduction

In the present situation, an increase in antibiotic-resistant microorganisms has become one of the most vital threats to the healthcare sector. Multidrug-resistant bacteria (MDR) that are deadly pathogenic are rising day by day and pose a very serious threat to human health. Earlier, these types of antibiotic-resistant bacterial strains were rare and limited to only nosocomial-acquired infections, but nowadays, they have become very common. This issue is more prevalent among both Gram-positive and Gram-negative bacterial species, which include *A. baumannii*, *E. coli*, *P. aeruginosa*, and *K. pneumonia* (Gram-negative), along with *S. aureus*, *S. pneumoniae*, *E. faecium*, and *E. faecalis* (Gram-positive). It has been found that this antibiotic resistance occurred among these bacterial species due to the attainment of plasmids through the transfer of resistance genes [1]. To escape from the harmful effects of antibiotics, certain bacterial species develop some special mechanisms like efflux pumps, less permeability of the LPS layer, secretion of degrading enzymes, and alteration of targets [2]. Certain factors that are responsible for increasing this antibiotic resistance may include widespread development, overexploitation of antibiotics, extreme use of broad-spectrum drugs, and scarcity of target-oriented antimicrobial drugs [3].

2. Community, Nosocomial, and Healthcare-Associated Infections

Initially, MDR bacteria were associated with hospital-acquired infections. MDR bacteria have spread and are now the leading cause of community-acquired infections. The spread of multidrug-resistant (MDR) bacteria in society has resulted in an increase in morbidity, mortality, healthcare expenditure, and antibiotic use. According to the IDSA (the Infectious Diseases Society of America), resistance to antimicrobial compounds can be defined as "one of the greatest threats to human health worldwide" [4]. It has

been observed that patients infected with MDR strains of bacteria have more severe consequences than patients infected with other vulnerable organisms [5, 6]. As a result, this increase in the spread of antibacterial resistance leads to posing a serious threat to society as well as to the medicines available and also causes a negative impact on cancer therapy, transplantation, and surgical events [7]. It has been assumed that the occurrence of particular MDR bacterial strains is strongly associated with the application of broad-spectrum antibiotics that consist of empiric along with definitive therapy [8]. Such overexploitation is responsible for the higher incidence of MDR and leads to the development of a vicious cycle.

Infection is an ill-state and could be classified into two types: society-associated and nosocomial. Differentiation among two types of infections is based on whether the beginning of illness was up to the first two days of hospitalization (community-associated) or afterwards (nosocomial type). As per the work initiated by Morin et al. and Friedman et al., community-associated infections can be further classified into healthcare-associated and community-acquired infections [9, 10]. If the patient was in a hospital under critical care for 48 hours or more; within 90 days of the infection; admitted to the hospital or persistent care for an extended period of time; received chemotherapy; intravenous antibiotic treatment; or wound care in the previous month of the existing illness [11], this is considered a healthcare-associated infection. On the other hand, community-acquired infections consist of those patients having a community-onset infection along with those who do not match the above criterion (healthcare-associated infection). Though such criteria are capable of solving and accessing the spread of MDR bacteria in society, they are not enough to explain the complete story. It has been observed that the patients get infected with the same organism with which they were colonized earlier. Henceforth, the moment of colonization is more significant in comparison to the moment of infection diagnosis as far as the origin of MDR bacteria is concerned.

3. Transitioning from a Hospital-Acquired Pathogen to a Society-Associated Pathogen

It has been noticed in various studies that the frequent use of antibiotics always becomes a threat to mankind. The increase in antibacterial resistance patterns can vary according to the threat associated with the particular antibiotic class. Here, an attempt has been made to explain the link between the application of a particular antibacterial agent and the origin of resistance to a specific antibiotic. The application of tigecycline to individuals suffering from *Klebsiella pneumonia* containing carbapenem-resistant infection was observed to develop tigecycline resistance in the same bacterial strain, which was a very crucial example [12]. From this instance, it has become evident that we have to manage and control this antibacterial resistance in all aspects, i.e., at the level of nosocomial infections, societal level infections, and nonmedical antibiotics. Moreover, healthcare devices pose a supplementary risk factor for multidrug-resistant bacteria. Certain medical devices, like endotracheal tubes, urinary catheters, vascular lines, and feeding tubes, are also recognized as high-risk factors [13]. In addition to it, other risk factors responsible for the illness or establishment of multidrug-resistant bacteria may include immunocompromised conditions like transplantation of any organ or hematopoietic stem cell along with other factors like kidney failure [14]. Among the microorganisms, such resistance genes are found to be beneficial for the organism and have implausibly spread in society, except when the compensatory genetic material is collected or through the application of antibiotics, the expression of the resistance gene gets completely induced. One good example of such an event is erythromycin resistance methylase (erm) genes that act as inducible genes and are present in *S. aureus* and *Mycobacterim*. Such genes are expressed only when the bacteria are treated with the particular antibiotic that results in the development of antibiotic resistance among the bacteria.

Among the bacterial community, the development of biofilm is a unique characteristic of their life cycle. The formation of biofilm is a necessary constituent of periodontal illness and epigastric illness with *H. pylori* [15]. On the community level, the MDR become more numerous and have the capability to develop biofilm when foreign material is absent. Moreover, it has been noticed that community-associated MDR has the capability to infect healthy individuals if the immunocompromised individuals are not available. Immunity genes of the host develop specific polymorphisms that facilitate colonization of selected bacteria. In the case of the *Staphylococcus aureus* in nasal carriage, human genes have even been classified as the dominant decision [16].

3.1. MRSA. The most common example of MDR bacteria is MRSA (methicillin-resistant *S. aureus*), i.e., transmitted efficiently from strict hospital-acquired infection to community-associated spread. However, the epidemiology of such CA-MRSA (community-associated methicillin-resistant *S. aureus*) has already been extensively evaluated [17, 18]. Numerous nonlactam antibiotics were found to be effective against such CA-MRSA strains. In the USA, a novel strain of CA-MRSA was discovered, i.e., known as the CA-MRSA-USA300 strain, that successfully substituted the previous USA400 CA-MRSA strain in the early 2000s [17]. It has been observed that this USA300 strain is distinguished by the existence of staphylococcal cassette chromosome mec (SCCmec) type IV along with the genes responsible for the secretion of Panton-Valentine leucocidin (PVL) toxins [19]. *P. aeruginosa* is well-known for causing nosocomial infections with symptoms such as pneumonia and bloodstream illness. Favorable environments for *P. aeruginosa* are moist places and can be found mostly in washing sinks, aerators, equipment like respiratory gear, and unhygienic solutions in the hospital environment [20]. Moreover, fewer patients have experienced the chronic biofilm-associated pseudomonal establishment along with cystic fibrosis (CF) [21]. Among such patients, repetitive applications of antibiotics lead to the origin of MDR bacterial strains. Community-associated infections with MDR strains of *P. aeruginosa* are found to be uncommon [22, 23]. In a

group of 60 patients suffering from community-acquired bloodstream illness due to *P. aeruginosa*, 100% of isolates are meropenem susceptible, and 95% are susceptible to ceftazidime, tazobactam, and piperacillin [24].

3.2. Vancomycin-Resistant Enterococci (VRE). One more antibiotic-resistant bacterial strain was developed in the late 1980s, i.e., VRE (vancomycin-resistant *Enterococci*), which is responsible for the major source of hospital-acquired infections in 1990. It has also become evident in the European study that such vancomycin-resistant *Enterococci* were isolated in the fecal material of healthy individuals [25]. Due to this variation between Europe and the USA, avoparcin is widely used. Avoparcin is a glycopeptide antibiotic, i.e., used to enhance the growth of animals as food additives. It has become evident that avoparcin was not permitted for use in the USA or Canada, but it was widely used in Europe until 1997 [26]. When the use of avoparcin was strictly prohibited in animal husbandry, the prevalence of vancomycin-resistant *Enterococci* was decreased in both animal samples and human volunteer samples [25, 26]. These examples show that there is a strong link between the use of antibiotics in food production and the rate of antimicrobial resistance in people.

Afterwards, such VRE were collected from the wastewater from a semi-closed agri-food system [27]. However, VRE was found to be an unusual infectious agent among community-associated infections. Among the 289 individuals suffering from community-associated VRE, 85% of individuals undergo hospitalization, while 71% of individuals undergo antimicrobial contact within 3 months, respectively [28]. Moreover, conventional risk factors associated with antimicrobial exposure can be attained through serious infection, indwelling medical equipment, cancerous growth, and a weakened immune system [28, 29]. The increased prevalence of vancomycin-resistant *Enterococci* (VRE) in society is caused by either a constant influx of VRE into the food chain via shared community gut microbiome or increased antibiotic pressure. In terms of inducible resistance, the fitness costs of VRE for *Enterococci* were found to be almost nothing [30, 31].

It has been noticed that the majority of community-associated infections like UTI and bacteremia are mainly caused by members of the *Enterobacteriaceae*. Among the widespread ESBL groups (extended-spectrum beta-lactamases), 91% belong to the CTX-M group, while the rest are SHV (8%) and CMY-2 (1%). Among them, the majority of isolates (54%) belonged to the ST131 clonal group [32]. It has been observed that the community-onset infection by ESBL-producing *E. coli* is the most recurrent type, occurring mostly in Asia, the Middle East, South America, and a few regions of Europe. This infection, however, is less common in New Zealand, Northern Europe, and Australia. In such less frequently occurring areas, there is a particular risk factor associated with community-onset ESBL-producing *E. coli* infections. In a finding conducted in Australia and New Zealand, it was observed that individuals born in the Indian subcontinent or on a tour of India, China, Africa, or the Middle East were at high risk for community-onset third-generation cephalosporin-resistant *E. coli* infections [33].

Among Asian continents, an important issue is related to the dissemination of infection with *K. pneumoniae* strains. Such "hypermucoviscous" varieties have the tendency to develop community-onset pyogenic liver abscess along with metastatic illness and meningitis [34]. Such bacterial strains are mostly susceptible to a variety of antibiotics; community-onset ESBL-secreting strains are becoming more common [35]. Carbapenem is now the preferred drug recommended by physicians against the fatal infection of ESBL. Henceforth, the application of empiric carbapenem has improved remarkably to treat the widespread infection of such organisms within society.

3.3. Carbapenem-Resistant Acinetobacter baumannii (CRAB). A. baumannii is a Gram-negative bacillus that causes infections in hospitals and intensive care units [36]. Moreover, in Asia and Australia, the community associated with *A. baumannii* illness has been well-recognized [37]. In the majority of cases, such infections are coupled with pharyngeal carriage along with addiction to alcohol and smoking [37]. These infections are extremely lethal, accounting for approximately 56% of people suffering from bacteremia and/or pneumonia [37]. Certain natural resources like soil, fruits, and vegetables, along with animal and human skin and throat tissue, may serve as community reservoirs for *A. baumannii.* In addition to this, *A. baumannii* has also been isolated from human lice [38].

However, resistance to carbapenem may take place due to the accumulation of carbapenemases like IMP, associated oxacillinases (OXA), and carbapenemases [39]. The data of a study in Australia clearly reflects that out of 36 individuals suffering from community-onset bacteremic *A. pneumonia*, all were found vulnerable to carbapenems [40]. In another study in China, more troublesome results were obtained when out of 32 individuals suffering from community-acquired pneumonia caused by *A. baumannii*, and 3 and 6 isolates were nonsusceptible to meropenem and imipenem, respectively [41].

It has been noticed that *A. baumannii* having community-associated carbapenem-resistant infections is unusual and causes infection in natural habitats. It has become evident that carbapenem-resistant *Enterobacteriaceae* pose a serious threat to human health. From now on, immediate and timely action is required [1, 42]. In addition to it, carbapenem-resistant *E. coli* (CREC), i.e., an integral component of the CRE subset, was found to have a major epidemic in the USA [42, 43]. This CREC is not widespread in Asia, and such strains have been isolated from drinking water supplies in India and food-producing cattle in China [44].

But still, in such a populated country, people become afraid of the threat of CRE colonization [45, 46]. Moreover, much research data is available on carbapenemase-producing *E. coli* ST131 [47]. In India, research was conducted to compare the clinical isolates of ST131 with non-ST131. The findings of this study clearly showed that approximately 20% of clinical isolates tested positive for

metallo-lactamases such as blaNDM-1, which was distributed evenly between ST131 and non-ST131 *E. coli* [48]. Moreover, it has been noticed that the distribution pattern of ESBL is found to be 10 years ahead of the carbapenemases. Henceforth, it is evident that community-associated carbapenem-resistant ST131 *E. coli* will pose a hazard in the near future.

4. Antibiotic Resistance Mechanisms in Gram-Positive Bacteria

The major threat in the present scenario is an increase in MDR bacteria and the unavailability of novel antibiotics to kill them. Nowadays, the research is mainly focused on searching for novel methods to treat such MDR. In this direction, the major step has been laid down by the WHO by releasing a document containing the names of all deadly MDR which are resistant to all the available methods. Moreover, the WHO appeals to all nations to develop new novel drugs and other treatment methods to handle MDR successfully. In this document, certain Gram-positive bacteria like methicillin-resistant *Staphylococcus aureus*, drug-resistant *Streptococcus pneumonia*, vancomycin-resistant *Enterococcus faecium*, and many more are listed that pose a serious threat to societal health [1]. It has been noticed that such resistance develops in bacteria due to genetic mutations and/or acquired genomes [49] (Table 1).

It has been observed that a rise in the number of MDR is resulting in the nonavailability of any alternate to treat such strains, which henceforth is responsible for the high incidence of morbidity and mortality [50]. As discussed earlier, the WHO has drafted a list of such MDR and classifies them on the basis of severity of infection as medium, high, and critical antibiotic-resistant bacteria [51]. As per the WHO, there is an immediate requirement for some novel treatments against such MDR. Among such deadly pathogens, β-lactamase-resistant *Streptococcus pneumonia*, methicillin-resistant *Staphylococcus aureus* (MRSA), and vancomycin-resistant *Enterococcus faecium* (VRE) are major Gram-positive bacteria associated with multidrug resistance and posing a serious threat to society [1, 52]. There is some difference in the cell wall composition of Gram-positive and Gram-negative bacteria, as Gram-positive bacteria possess a thick layer of peptidoglycan over the cytoplasmic membrane that provides protection against adverse environmental conditions, and Gram-negative bacteria lack the LPS layer [53]. It is well-known that Gram-positive bacteria have mainly two basic morphologic forms, like cocci (e.g., *Staphylococcus*) and bacilli (e.g., *Bacillus*) [54]. Moreover, Gram-positive bacteria have teichoic acids, which are long anionic polymers, membrane proteins that make possible the incoming and outgoing of various molecules and capsular polysaccharides that are covalently linked to peptidoglycan [55]. There are two main mechanisms through which Gram-positive bacteria develop resistance to antibiotics. These are as follows:

(i) Secretion of β-lactamases, i.e., responsible for the enzymatic breakdown of antibiotics

(ii) By reducing the affinity and susceptibility of their target site, e.g., PBP (penicillin-binding protein), through either attainment of exogenous DNA or through mutation among native PBP genes [56, 57]

Different antibiotics have diverse mechanisms of action. A few such examples are given below.

(i) Penicillin-binding proteins were targeted by β-lactams which bring about the end step of cell wall synthesis that ultimately cause cell death. β-lactamases are responsible for the deactivation of the antibiotic and hence develop resistance. The major source for the development of penicillin resistance takes place due to horizontal distribution of penicillinase plasmids through bacteriophages or through horizontal gene transfer which engage the genes for penicillin-binding proteins. In addition to this, methicillin resistance can develop from extra penicillin-binding proteins like PBP2/2a, i.e., obtained from foreign DNA elements [56–58]

(i) Vancomycin and teicoplanin are glycopeptides. These antibiotics also inhibit the end step of cell wall synthesis. Attainment of the van gene cluster (VanA, VanB, VanC, VanD, VanE, and VanG) is mainly responsible for the development of resistance that ultimately causes less binding affinity to glycoproteins [57–59]

(ii) Other antibiotics like quinolones act on the DNA gyrase and topoisomerase IV enzymes and inhibit their function. This results in bacterial death. Resistance to quinolones develops due to the mutations occurring in the subunits of these two enzymes (grIA/grIB and gyrA/gyrB) [60, 61]

(iii) Moreover, aminoglycosides have affinity for 30S ribosomal subunits that prevent the translocation process and hence produce nonfunctional proteins that upset the membrane structure and enhance aminoglycoside penetration. Resistance develops through the attainment of certain modifying enzymes like phophotransferases, nucleotidyltransferases, acetyltransferases, or through mutation and efflux mechanisms [62]

(iv) On the other hand, macrolides have affinity for the 50S ribosome and prevent protein synthesis. Resistance developed as a result of various mechanisms such as mutations in the 23S rRNA and protein L4, methylation of the 23S rRNA, and efflux systems Mef (A) and Msr (A) [57, 63]

(v) Oxazolidinones, e.g., linezolid, have affinity for the 50S ribosome subunit and prevent protein synthesis. Resistance was achieved through mutations in 23S rRNA and G2576T in DNA [56, 64]

4.1. Methicillin-Resistant Staphylococcus aureus. *Staphylococcus aureus* is Gram-positive cocci that belong to the

Table 1: Mechanism of antibiotic resistance.

S. No.	Kind of antibiotic resistance	Mechanism involved in resistance
1	*Restricting entry of antibiotics*	Antibiotics spread out in the cell through the occurrence of mutations in the gene which specifically encodes the outer membrane porin protein, and this results in the change in OMPK36 variant porin which shows less permeability for the antibiotics in *Klebsiella pneumonia* [159]. Because of the downregulation of the main porin protein or refilling the cell membrane with some another selected protein channel, the permeability of membrane for antibiotics in some bacteria such as *E. coli* and *Acinetobacter* is decreased [160].
2	*Accession of various efflux pump genes related to chromosomal and plasmid*	Through strong efflux pumping, the numbers of antimicrobials are launched out of the cell. Their overexpression allows resistance to formerly effective antibiotics example—MDR efflux pump in *Pseudomonas aeruginosa* and *E. coli* [161].
3	*Moderation and defense of antibiotic target*	By changing the arrangement of the targets, the binding affinity of antibiotics can be reduced. *Klebsiella pneumonia* and *Staphylococcus aureus* are found to be resistant to linezolid, and this is achieved by the mutation in allele which encodes the 23 s rRNA ribosomal subunit [162]. Development of resistance to the certain drugs such as macrolids, lincosamines, and streptograminscan be attained by doing methylation of their binding site and the 16 s rRNA by the action of enzyme called erythromycin ribosomal methylase and family. Resistance to the several other group of drugs such as penicillin, pleuromutilins, lincosamides, and oxazolidons can be achieved with the help of enzyme chloramphenicol florfenicol resistance methyltransferase through the incorporation of CH_3 to A2503 in the 23 s rRNA [163]. Resistance to methicillin in *S. aureus* is because of the genetic discovery of chromosomal mec A which records a single binding protein for extra penicillin, PBP2a, with a less affinity for all β-lactam [164].
4	*Antibiotic opposition via hydrolytic enzymes*	The resistance is achieved by chromosomal detection, and plasmid-mediated encoding genetic enzyme degrades with antibiotics, for example, β-lactamase which includes penicillinase only degrading penicillin, cephalosporinases deactivate cephalosporins and aminopenicillins, and expanded beta-lactamases, which play an important role in digesting all β-lactam, but carbapenemase and carbapenem disabled the whole β-lactamase [165].
5	*Moderation of antibiotic resistance*	Detection of gene enzymes is deactivated by antibiotics with the addition of an active functional group. For instance, resistance to aminoglycolides in *Campylobacter coli* (*C. coli*) that is microaerophilic Gram-negative bacteria is caused by nucleotidylation, acetylation, and phosphorylation of its -OH and CO-NH groups by acetyl transferases, phosphotransferases, and nucleotidyltransferases [166].

Staphylococcaceae family. It is one of the major human pathogens responsible for fetal illness and increased mortality rates. It causes several deadly diseases, including infective endocarditis, skin, respiratory tract, and soft tissue infections, and infections of pleuro-pulmonary-related devices [65, 66]. It also has a high level of resistance to several antibiotics. Penicillin-resistant *S. aureus* secretes a plasmid-mediated penicillinase, which breaks the β-lactam ring of penicillin, which is required for its antimicrobial activity [67]. Celbenin, now known as methicillin, is a derivative of penicillin and was introduced to neutralize the bacterial resistance mechanism. Resistance develops as a result of the production of extra penicillin-binding proteins like PBP2a, which reduces the affinity for penicillin and β-lactam antibiotics [65, 68, 69].

4.2. Vancomycin Intermediate and Resistant Staphylococcus aureus. MRSA is a deadly pathogenic MDR, and it has been noticed that VISA and VRSA have originated from MRSA itself. Due to variation among the resistance mechanisms, VRSA does not develop into VISA. Vancomycin kills the bacterial cell by breaking the PPCB (pentapeptide cross bridge) bond between two NAM units (D-Ala-D-Ala residues) [70, 71]. It is well-known that the cell walls of Gram-positive bacteria are very thick. Henceforth, these D-Ala-D-Ala residues serve as decoy targets in the thickened cell wall and block vancomycin on the outer surface of the cell wall, causing misreading in identifying its true targets. Moreover, VRSA attained this genetic resistance from vancomycin-resistant *Enterococci* (VRE). Until now, six VRSA strains have been isolated and characterized in

the USA, and each one has attained the vanA genes, i.e., the genes responsible for providing a high degree of resistance to teicoplanin, glycopeptides, and vancomycin. It has been noticed that VanA genes are responsible for the manufacturing of modified peptidoglycan precursors having a terminal D-Ala-D-Lac, where vancomycin reflects much less affinity in comparison to the terminal wild-type D-Ala-D-Ala [69] (Figure 1).

4.3. Ampicillin/Penicillin and Cephalosporin Resistance Enterococcus faecium: faecium. E. faecium is Gram-positive cocci from the *Enterococaceae* family. *E. faecium* develops resistance against β-lactams, e.g. penicillin, as it is linked with the pbp5 chromosomal gene, i.e., responsible for the secretion of a low binding affinity class B PBP for ampicillin/penicillin and the cephalosporins. In addition, mutations in penicillin-binding proteins and hypersecretion of β-lactamase enzymes lead to the development of resistance to β-lactam antibiotics. Furthermore, other mechanisms are found to be associated with the resistance to cephalosporins as they undergo similar types of response regulator, CroR, serine/threonine kinase designated IreK, and phosphatase IreP [72, 73].

4.4. E. faecium (Vancomycin-Resistant). E. faecium is a Gram-positive bacterium that attained special genes that are present in the plasmid (extra chromosomal DNA), which were classified into 6 of the 19 families and transposons like Tn1547 that provide resistance to vancomycin [74]. Vancomycin, like penicillin, binds to the D-alanyl-D-alanine (D-Ala-D-Ala) terminus and inhibits cell wall synthesis. It has been observed that gene clusters responsible for vancomycin resistance like van A, B, D, and M carry out the substitution of D-Ala-D-Ala by D-alanyl-D-lactate termini which, i.e., are accountable for the low binding affinity of vancomycin. Among them, the Van A gene cluster was discovered to be the most effective and to be present on a transposon, i.e., linked to Tn1546 [75].

4.4.1. Other Mechanisms of Resistance. As already discussed, fecal resistance to vancomycin is widespread. Moreover, this bacterium also has resistance to aminoglycosides such as gentamicin, tobramycin, and kanamycin because it poses aminoglycoside-modifying enzymes (AMEs) containing aminoglycoside nucleotidyltransferases (ANTs), aminoglycoside acetyltransferases (AACs), and aminoglycoside phosphotransferases (APHs). In addition to that, attainment of genes responsible for encoding ANT(3")-Ia or ANT(6)-Ia enzymes along with mutation in S12 ribosomal protein develops a high degree of resistance against streptomycin. Besides it, *E. faecium* also reflects strong resistance to fluoroquinolones through making point mutations in gyrA and parC genes, i.e., those responsible for the encoding subunits A of and topoisomerase IV or DNA gyrase or NorA-like efflux pump. Additionally, *E. faecium* also reflects resistance to quinupristin–dalfopristin (a streptogramin drug) that inhibits bacterial protein synthesis through binding to 23-S rRNA of the 50S ribosomal subunit and stops its function [76].

4.5. Streptococcus pneumoniae (Penicillin-Nonsusceptible). Among Gram-positive bacteria, the most common pathogenic bacteria is *S. pneumonia*, which adheres to the upper respiratory tract and is capable of causing infections such as pneumonia, meningitis, bronchitis, and sinusitis. It reflects penicillin resistance by causing changes in one or more of the six penicillin-binding proteins found in its cell membrane. This occurs because of chromosomal mutations or may be attributed to the usual transformation process in which the DNA is taken up from other bacteria and is inserted inside the *Pneumococci* (host) DNA. This *Pneumococci* is capable of causing infection in older people. Children and daycare workers are more prone to this resistant *Pneumococci* infection [77, 78]. Moreover, *Pneumococci* not only undergo resistance to penicillin but also to erythromycin and trimethoprim-sulfamethoxazole (TMP-SMX). Furthermore, the erm(B) gene, which is responsible for methylase secretion, is responsible for resistance to macrolide-lincosamide-streptogramin B. Similarly, the mef(A) gene also undergoes similar resistance through an antibiotic efflux pump. Some other mechanisms for resistance have also been noticed, which include mutations in ribosomal proteins L4 and L22 leading to the dysfunction of ribosomal RNA (23S rRNA). Antibiotic resistance was also discovered against fluoroquinolones, tetracyclines, and chloramphenicol [79, 80].

4.6. Other Resistant Gram-Positive Bacteria and their Resistant Mechanisms. The tendency to develop biofilms and the presence of exopolysaccharide matrix are a few mechanisms that develop resistance in *Staphylococcus epidermidis* through decreasing penetration and diffusion of antibiotics. *S. epidermidis* is primarily responsible for hospital-acquired infections due to the transport of resistant mecA genes that encode PBP2a. Moreover, they also show resistance to vancomycin and quinolones [81]. The major cause of uncomplicated UTIs (urinary tract infections) is caused by *Staphylococcus saprophyticus.* This bacterium undergoes resistance against several antibiotics like ciprofloxacin, ampicillin, cephalexin, and ceftriaxone [82].

The most common commensal bacterium, *Streptococcus viridians*, which lives in the human upper respiratory tract, develops resistance to antibiotics such as penicillin and other β-lactam drugs by modifying the penicillin-binding protein. In a few studies, it has been mentioned that *S. viridans* act as reservoirs for resistance genes like mef (E) and mel genes that show resistance to macrolide-lincosamide-streptogramin B (MLS (B)) antibiotics [83, 84]. Another bacterial pathogen that is established in the upper respiratory tract and skin is *Streptococcus pyogenes* that shows resistance against streptogramins, macrolides, and lincosamides. Moreover, it also reflects resistance to aminoglycosides, fluoroquinolones, and tetracyclines [85]. It has been noticed that most neonatal infections in humans are caused by *Streptococcus agalactiae* or group B *Streptococcus* (GBS), i.e., Gram-positive cocci. During delivery, this pathogen might be transformed from mother to baby. Resistance to erythromycin and other macrolides occurs in *S. agalactiae* via changes in ribosomal function, mediated by erm genes, or via efflux pumps encoded by mefA genes. Apart from this,

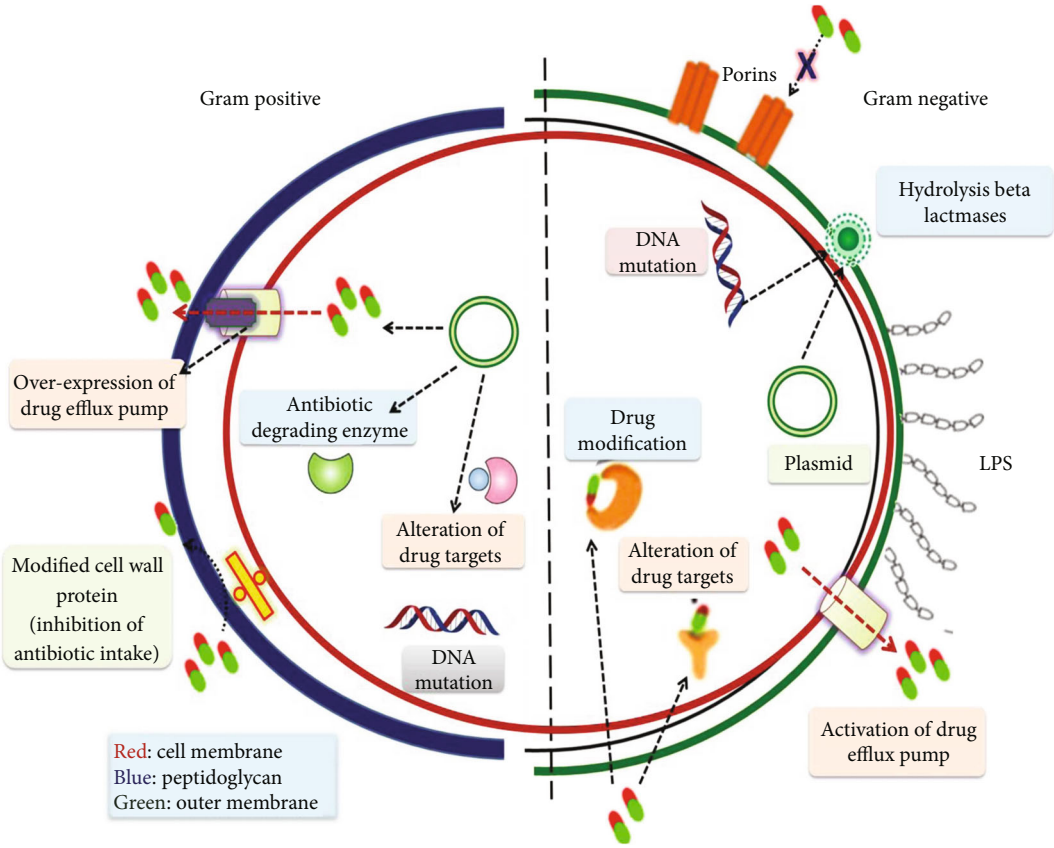

Figure 1: Resistance mechanisms found in Gram-positive and Gram-negative bacteria.

linB genes are responsible for inhibiting the ribosomal translocation that develops clindamycin resistance in GBS [86].

5. Gram-Negative Bacterial Resistance to Antibacterial Agents and Methods to Overcome It

Natural products have been used to cure many ailments since ancient times. For example, the cinchona tree contains quinine, which is used to treat malaria. Since Fleming discovered penicillin in 1929, a plethora of antibacterial medicines have been developed, all of which have had a significant influence on human health and death rates across the world [87]. Antibiotics have been overused and misused, resulting in the emergence of new resistant forms. People in impoverished nations can readily obtain antibiotics without a prescription, so public awareness of the issue is critical [88]. Bacteria can evade antibiotics by a variety of processes, including neutralizing antibiotics, pumping them outside the cell, or altering their outer structure, resulting in drug inhibition. All of these strategies lead to the evolution of antibiotic resistance in bacteria. There are four types of antibiotic resistance mechanisms in bacteria: intrinsic resistance, where bacteria modify their structure and components, and acquired resistance, when bacteria acquire new resistant genes or DNA from other bacteria. Additionally, certain genetic alterations in the gene can result in protein modifications, resulting in new components and receptors for antibiotics to identify, and lastly, DNA is transmitted across bacteria by combination, transduction, or transformation [53].

We employ the complex formed by the addition of crystal violet-iodine followed by safranin as a counter stain to discriminate between Gram-positive and Gram-negative bacteria, as developed by Christian Gram. Gram-positive bacteria keep their complex stain and look purple, but Gram-negative bacteria lose their complex stain and appear pink owing to counter stain. The change in cell wall composition between two species of bacteria is the cause of this discrepancy [87, 88]. Three layers make up the envelope carried by Gram-negative bacteria. The outer membrane, which serves as a protective barrier, has a unique characteristic that distinguishes Gram-positive bacteria from Gram-negative bacteria. Lipo-polysaccharides are bound in the inner leaflets of the outer membrane, whereas phospholipids are bound in the outer leaflets. Furthermore, the outer membrane contains proteins such as porins and others that enable diverse molecules to flow through it (Figure 1). The bacterial cell wall, i.e., composed of peptidoglycan, is made up of repeating units of NAM and NAG, which help in regulating the cell shape [53]. The second layer, discovered in the subsequent layer, the inner membrane, is composed of phospholipids that serve various roles, including structure,

biosynthesis, and transport. It also serves as a location for DNA anchoring and aids in the separation of sister chromosomes [89].

Antibiotic resistance has been linked to the outer membrane of bacteria, which includes β-lactase, quinilons, colistins, and other antibiotics. Antibiotics must be able to pass through the cell wall to reach the target, much as hydrophilic medications can pass via porins and hydrophobic drugs may pass through diffusion. Any change or mutation in the outer membrane can lead to the development of resistance. Gram-negative bacteria become more antibiotic resistant than Gram-positive bacteria because Gram-positive bacteria lack this layer [90, 91]. Gram-negative bacteria are frequently observed causing illness in humans, particularly in immunocompromised people. Due to resistance, Gram-negative bacteria produce the most difficult nosocomial infections [92]. Antibiotic inactivating enzymes are the main cause of antibiotic resistance in Gram-negative bacteria, which can be acquired by plasmids, aminoglycoside-modifying enzymes, or other mobile genetic elements carrying resistance genes or as a result of increasing intrinsic resistance due to mutations in chromosomal genes. A nonenzymatic pathway for fluoroquinolone resistance among *Enterobacteriaceae* was also observed, such as a plasmid-borne quinolone resistance gene [93].

5.1. Cephalosporin of the Third Generation for Enterobacteriaceae. The synthesis of beta-lactamses by resistant *Enterobacteriaceae* causes resistance to third-generation cephalosporins. ESBLs, for example, can hydrolyze broad-spectrum cephalosporin monobactams and penicillins. Resistance to early generation cephalosporins, ampicillin, and amoxicillin is caused by class A beta-lactamase such as SHV-1, TEM-1, and TEM-2. Resistance to third-generation cephalosporins develops when mutations in genes encoding TEM-2, TEM-1, or SHV-1 produce novel beta-lactams capable of hydrolyzing them. Carbapenem-resistant bacteria CRE is an *Enterobacteriaceae* isolate that is resistant to all carbapenem antimicrobials, including ertapenem, imipenem, and meropenem. Due to the synthesis of AmpC beta-lactamases and the loss of outer membrane protein, the initial isolates developed resistance. Carbapenem-producing CRE (CP-CRE), whose genes are found on mobile genetic elements, and noncarbapenem-producing CRE (non-CP-CRE) are the two forms of CRE that are generally recognized [12]. The following are the five main carbapenemases:

(i) OXA-48, a carbapenemase-like class D OXA

(ii) Pneumocystis pneumonia

(iii) New Delhi metallo-lactamases, class B (NDM)

(iv) Metallo-lactamases encoded by Verona integrin (VIM)

(v) On imipenem, IMP is active. *Morganella morganii*, *Proteus* spp., and *Providencia* spp. are among the *Enterobacteriaceae species* with inherent imipenem resistance [94]

5.2. Acinetobacter baumannii. A. baumannii is a Gram-negative bacterium that is aerobic in nature and is one of the most dangerous species among *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Enterobacter species* (*ESKAPE*) declared by the WHO to be capable of neutralizing antibiotic effects [95]. *A. baumannii* has also been linked to nosocomial infections all over the world.

Mechanisms via which it can acquire antibiotic resistance quickly:

(i) The inactivation of beta-lactams by beta-lactamases is a common MDR mechanism. *A. baumannii* has demonstrated that all four β-lactam classes, A, B, C, and D, can integrate foreign DNA into their genomes and can quickly recognize a large number of β-lactams. Some of these enzymes are narrow-spectrum beta-lactamases, whereas others are involved in ESBL hydrolysis, which can reduce carbapenem sensitivity

(ii) Multidrug efflux pumps, which are resistant to a variety of antibiotics, including imipenem, are another source of resistance in *A. baumannii*. The four recognized types of efflux pumps are multidrug and toxic compound extrusion (MATE), resistance nodulation division (RND) superfamily, major facilitator superfamily (MFS), and small multidrug resistance (SMR) family transporters [95]

(iii) The three types of enzymes present, acetyltransferases, adenyltransferases, and phosphotransferases, are important in mediating *A. baumannii's* resistance to aminoglycosides. These enzymes change the chemical structure of aminoglycosides. Coding genes can be transferred by transposons, integrons, and plasmids

(iv) Changes in the envelope's permeability have an impact. Porins, which are proteins, are found in the outer membrane and form channels that allow molecules to pass through. This is a crucial part of the resistance process. Some porins, such as Caro and Omp22-33, have been linked to carbapenem resistance in *A. baumannii*

5.3. Pseudomonas aeruginosa. Pseudomonas aeruginosa is one of the most dangerous pathogens in the ESKAPE group. It is a Gram-negative bacterium that is present in the typical flora of the intestine. In critically unwell patients, it is also responsible for ICU-acquired infections. Innate resistance mechanisms, such as overexpression of the efflux pump and decreased permeability of the outer membrane, as well as acquired resistance mechanisms, such as the acquisition of resistance genes and mutations in genes that encode for proteins called porins and other proteins, can make this bacterium difficult to treat. Antibiotics like penicillin, carbapenem, and cephalosporin disrupt the bacterial peptidoglycan cell wall production. Ceftazidime and cefepime, which belong to the third and fourth generations of cephalosporins,

are two of the most effective β-lactams for treating *Pseudomonas aeruginosa* [96].

β-lactamases break the amide link in the beta-lactam ring, rendering medicines ineffective. A, B, C, and D are the four primary classes of β-lactamases discovered in *Pseudomonas aeruginosa*. The enzymatic activity of serine residues suppresses β-lactams in classes A, C, and D, whereas zinc cation is required for class B's effect. *Pseudomonas aeruginosa* can also gain resistance by gene mutation, which can result in AmpC β-lactamase overexpression. Amp G encodes for a transmembrane protein that works as an A permease for 1,6-anhydromurapeptides that induce ampC, while amp D eventually encodes for a cytosolic N-acetyl-anhydromuramyl-L-alanine amidase that acts as an abridge for Amp C production.

Pseudomonas resistance to aminoglycosides is caused by transferable aminoglycoside-modifying enzymes. These enzymes are divided into three groups: aminoglycoside acetyltransferases (AAC), aminoglycoside phosphoryl transferases (APHs), and aminoglycoside adenylyl transferases (AADs). They are able to inactivate aminoglycosides by joining a phosphate, adenyl, or acetyl radical to the present antibiotic molecule, reducing their ability to bind with their target in the bacterial cell. Besides beta-lactams, colistine has been reported to be more effective against MDR *Pseudomonas* when combined with an anti-*Pseudomonas* medication such as ceftazidime, ciprofloxacin, or imipenem. Fosfomycin therapy in combination with aminoglycosides, cephalosporins, and penicillins has also been shown to be effective in the treatment of MDR *Pseudomonas aeruginosa* [96].

5.4. Helicobacter pylori-Clavithromycin-Resistant. *Helicobacter pylorus* is a Gram-negative bacterium that has been identified as one of the most significant pathogens in humans, causing gastritis, peptic ulcers, and stomach cancer. It has been stated that the efficacy of *Helicobacter pylori* is increased as a result of the rapid development of resistance to antibiotic therapy, and therefore, the treatment efficiency is reduced [97, 98]. Overexpression of efflux and the translation initiation factor IF-2 with ribosomal protein L22 have also been linked to the development of resistance.

5.5. Fluoroquinolone Resistance in Salmonella spp. Bacteria that are Gram-negative Salmonellae are split into two types: typhoidal *Salmonella* and nontyphoidal *Salmonella*, both of which are pathogenic to humans. MDR in *Salmonella* has been documented against ampicillin, chloramphenicol, and sulfamethoxazole, prompting the use of FQ-ciprofloxacin and ceftriaxone, the third generation of cephalosporins, which has resulted in the fast development of resistance to these medications. This has been cited as one of the main reasons for the World Health Organization's designation of FQ-resistant *Salmonella* as an important pathogen for research and development of novel antibiotics in 2017. Mutations in the quinolone resistance determining areas of the chromosomal gyr and par genes have been shown to cause quinolone resistance. As a result of this, quinolone has poor binding affinity for topoisomerase enzymes. Another method worth mentioning is plasmid-mediated quinolone resistance (PMQR), where physical protection is supplied by genes like Qnr, the Aac-60-lbcr gene reduces FQ action, and quinolone efflux pumps are encoded by oqxAB and qepA [99].

6. Alternative Techniques for Controlling MDR Infections

6.1. Use of Nanotechnology to Create anti-MDR-Resistant Nanobacterial Agents. According to one study, nanoparticles can be used to replace antibiotics and certain disinfectants. Liposome nanoparticles made of both inorganic and organic materials, such as silver, zinc, gold, and copper, can be used as antibiotics and disinfectants. The majority of examples of nanoparticles being used as antibiotics include preventing catheter-associated urinary tract infection (CAUTI) and biofilm development. Nanoparticles have also been used in antibacterial wound dressings and coatings [100–106]. Antimicrobial resistance to a therapeutic treatment in bacterial pathogens is not difficult to develop, as resistance can be generated even through basic genetic changes and modifications [107]. The antibiotic combinatorial method is thus one of the most promising strategies for limiting antimicrobial resistance [108].

Nanodelivery systems increase the vulnerability of MDR bacterial strains to specific antibiotics by protecting them from bacterial hydrolytic enzymes and suppressing resistance mechanisms such as changes in outer membrane porin proteins that result in antibiotic impermeability [109]. Individual nanoantibiotic complexes have different working procedures and configurations than individual antibiotics. When nanoparticles are conjugated to therapeutic drugs like antibiotics, a synergistic antibacterial effect may be obtained; they can also create nanocarrier antimicrobial complexes with bactericide properties [110]. Antimicrobial agents that are unstable, such as bacteriophages, phytochemicals, peptides, and antibiotics, can be delivered by utilizing both organic and inorganic materials, such as chitosan and gold nanocarrier systems and drug nanovectors [111]. In an experiment [112], pomegranate ring extract (PGRE) has also been shown to inhibit the release of bacterial flagellins.

The restoration of the chemicals inside the nanohybrid has been documented in both experiments. The polyelectrolyte (PAH) surface-modified gold nanoparticles and silver nanoparticles had a symbiotic repressive effect of about 100% on Bacillus Calmette-Guerin and *E. coli* [113], while the silver nanoparticles conjugated with curcumin had a combined antimicrobial effect on *Pseudomonas aeruginosa* and *E. coli* [114]. In rat models, monotype chemoattractant protein-1 (MCP-1) nanocoated on orthopedic implants with interleukin-12 p70 (IL-12p70) and multilayer polypeptide nanoscale coatings with IL-12 [115] powerfully arouse the body's innate defense against open fracture [116]. In another investigation, a ceragenin-coated iron oxide magnetic nanoparticles (MNP CSA-13) hybrid was created. Despite its significant antibacterial action, ceragenin (CSA-13), a synthetic peptide, has been restricted in usage because of its nonselective toxicity.

At extremely high concentrations of around 100 micrograms/ml, the MNP-CSA-13 nanocomposite produced by conjugating iron oxide magnetic nanoparticles (MNP) with CSA-13 apparently targets primarily *P. aeruginosa* biofilms and free live cells. The absence of erythrocyte hemolysis indicates that conjugating synthetic peptides with nanocarriers reduces host cytotoxicity [117]. Glutaraldehyde [118] was used to establish a relationship between MNPs and CSA-13. The functional amino terminal silica on MNPs' surface was created by reacting glutaraldehyde with 3-aminopropyl trimethoxy silane (APTMS). CSA-13 binds to the MNP surface's terminal aldehyde groups, which react with CSA-13's main amine groups. MNP-CSA-13 may become dissociated at low pH due to inflammation and infection. Due to the hydrolysis of the imine link at low pH, the MNP-CSA-13 nanocomplex dissociates, and the CSA-13 antimicrobial peptide is liberated. As a result, the MNP-CSA-13 nanocomplex offers a promising nanodrug vector for pH-dependent standard antimicrobial administration to kill bacteria at infection sites where the pH is less than six [119].

6.1.1. Nanoparticles' Efficacy against Bacterial Biofilms and Spores. Because bacterial biofilms enable the conjugation of plasmids containing antibiotic genes and the biofilm matrix protects bacterial cells in lower films from antibiotics, assertions that bacterial biofilms are extremely tolerant and resistant to antibiotics [120] have been made. Organic and inorganic nano-ordered surfaces and coatings are now the most popular alternatives for preventing biofilm growth on medical devices [121]. The experiments of [122, 123] show that zeolitic imidazolate framework (ZIF), nanodragger arrays, and nanostructured polyurethane can prevent the formation of *Staphylococcus epidermis*, *S. aureus*, *E. coli*, and *P. mirabilis* biofilms by allowing bacterial cells to adhere to the topography of the nanolayered surface.

6.2. Essential Oils and Mono-/Bi-/Tri-Metallic Nanocomposites as Alternate Sources of Antibacterial Agents in the Fight against Multidrug-Resistant Pathogenic Microorganisms. Plants' active phytochemicals, bioactive substances, secondary metabolites, and essential oils are thought to be important in combating antibiotic resistance in pathogenic bacteria. Some alternative antimicrobial medicines can help to slow the spread and development of resistance to certain diseases. Essential oils are natural substances made up of volatile secondary metabolites extracted from various parts of plants, including flowers, seeds, buds, twigs, leaves, barks, herbs, roots, and fruits [124, 125]. Some of the most common chemical constituents of essential oils are flavonols, flavonoids, phenols, terpenoids, polyphenols, tannins, quinones, flavons, coumarins, alkaloids, lectins, and polypeptides, which have potential biological activities such as antioxidants, insecticidal, antiseptic, antiallergic, anti-inflammatory, antiviral, or antimicrobial properties. Many plants' essential oils are utilized in aromatherapy, food flavoring and additives, cosmetics, polymers and resins, and perfumes [126]. Many essential oil components have antibacterial characteristics, with terpenes including carvacrol, geraniol, menthol, and thymol having the strongest antimicrobial qualities.

Essential oils have a wide spectrum of inhibitory actions against many bacterial pathogens [127], since they may readily enter the lipid component of the bacterial cell membrane and break the cell wall structure [128]. The loss of integrity and cellular content caused by the combination of essential oils and lipids leads to cell death [129]. Few essential oil components, such as terpene-4-ol terpenol isomers, inhibit cellular respiration and render the cell membrane ineffective as a permeable barrier [130, 131]. The amount of bioactive components found in various essential oils, for example, has a significant and unique function; essential oils isolated from cinnamon and black pepper, for example, damage cell membranes and inhibit *E. coli* and *S. aureus* metabolic activity [132, 133]. Another example is *Dipterocarpus gracious* essential oil, which inhibits the development of *P. mirabilis* and *B. cereus* by infecting their cytoplasm membrane. Essential oils acquired from *Lawsonia inermis*, *Zanthophylum alatum*, *Ammodaucus leucotrichus*, *Marrubium globosum*, *Citrus sinensis*, and *Zanthophylum alatum* have shown significant antioxidant activity along with *Menta spicata* L. and *Eremanthus erythropappus* M [134–139].

6.2.1. Metallic Nanoparticles with Antimicrobial Properties. Aluminum oxide, iron oxide, titanium dioxide, copper oxide, zinc oxide, nickel oxide, zirconium dioxide, and chromium oxide nanoparticles have all been shown to have antibacterial properties. Metal and metal oxide base nanoparticles bind to the cell membrane, releasing metallic ions into the bacterial cell wall's proteins and enzymes. In addition, nanoparticles can harm the bacterial cell wall in a variety of ways, including electrostatic attraction, Van der Waals forces, and hydrophobic interactions [140–142].

6.2.2. Essential Oil Nanoencapsulation Efficiency. Capturing essential oils in innovative nano-based delivery systems such as nanoemulsions, microemulsions, solid lipid nanoparticles, and liposomes are examples of how natural bioactive substances may be encapsulated to boost antibacterial activity. As nanoemulsions, lime essential oils encapsulated with chitosan showed enhanced antibacterial activity against *S. aureus*, *L. monocytogenes*, *Shigilla dysenterias*, and *E. coli* [143, 144]. *Aspergillus parasiticus* and *Schinus Moller* use chitosan [145, 146], lipid phase, and orecirol as solid lipid nanoparticles against *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus ochraceus*, *Alternaria solani*, *Rhizopus stolonifer*, and *Rhizoctonia solani* [147]. Furthermore, chitosan with cardamom essential oil as nanoencapsulation against *S. aureus* and *E. coli* [148], chitosan with cardamom essential oil as nanocomposites against *S. aureus* and *E. coli* [149], and *Siparuna guianensis* with chitosan as nanoencapsulation against *Aedes aegypti* as nanoencapsulation [150].

6.3. Nonribosomal Antibacterial Peptides (NRAPs) That Target Multidrug-Resistant Bacteria. NRAPs are a subclass of nonribosomal peptides that are produced by gigantic nonribosomal peptide synthetases (NRPSs) [151]. The NRPSs are made up of several modular sections, each of which is in charge of fusing amino acids into peptide-like products

[152, 153]. The variable biosynthetic pathway of NRAPs results in the formation of molecules with structural diversity.

The rapid advancement of DNA sequencing technology has aided genomic data availability. Bioinformatics tools like NRPS Predictor 2, 122, Minowal 123, and PRISM 121 [154] may be used to identify and study possible BGCs from stored genomic sequences. These are frequently used, publicly accessible algorithms for predicting genetically encoded NRPs, such as PRISM, which predicted a cyclic telomycin derived from *Streptomyces canus* by mining biosynthetic scaffolds and characterized it with a novel antibacterial action by targeting cardiolipin [155]. Humimycin was also created via solid phase peptide synthesis, which is based on the investigation of the human microbiome using bioinformatics techniques and has a novel antibacterial action that targets lipid II lipase in MRSA and other bacteria [156].

The NRAP of baecaucein without lipid modification has demonstrated specific antibacterial action against MRSA in both *in vivo* and *in vitro* settings. Baecaucein-1 has all L-type amino acids, and the cationic guanodino group plays a major role in its action under physiological conditions, suggesting that the design of a linear peptide can be utilized as a basis for next-generation antibiotics [157]. The shortest known natural tripeptide with antibacterial action has been discovered [158], and the NRAP makes them more accessible.

Ethical Approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Conflicts of Interest

Authors have no conflict of interest to declare.

Authors' Contributions

(1) Dr. Alok Bharadwaj has contributed to the conceptualization, writing original draft, review, and editing. (2) Ms. Amisha Rastogi has contributed to the data collection, analysis, and interpretation of results. (3) Ms. Swadha Pandey has contributed to the data collection, analysis, and interpretation of results. (4) Dr. Saurabh Gupta has contributed to the conceptualization, writing original draft, review, and editing. (5) Dr. Jagdip Singh Sohal∗ has contributed to the critical revision of the article and editing.

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Research Article

Phytochemical Analysis and Antioxidant, Antibacterial, and Antifungal Effects of Essential Oil of Black Caraway (*Nigella sativa* L.) Seeds against Drug-Resistant Clinically Pathogenic Microorganisms

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Nigella sativa (NS) is a plant that has long been utilized in traditional medicine as a treatment for certain diseases. The aim of this work was to valorize the essential oil (EO) of this species by phytochemical analysis and antimicrobial and antioxidant evaluation. EO was extracted by hydrodistillation from the seeds of *Nigella sativa* (EO-NS). Phytochemical content of EO-NS was evaluated by use of gas chromatography coupled to mass spectrometry (GC-MS/MS). Antioxidant ability was *in vitro* determined by use of three assays: 2.2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing power (FRAP), and total antioxidant capacity (TAC) relative to two synthetic antioxidants: BHT and quercetin. Antimicrobial effect was evaluated against four clinically important bacterial strains (*Staphylococcus aureus*, ATCC 6633; *Escherichia coli*, K12; *Bacillus subtilis*, DSM 6333; and *Proteus mirabilis*, ATCC 29906) and against four fungal strains (*Candida albicans*, ATCC 10231; *Aspergillus niger*, MTCC 282; *Aspergillus flavus*, MTCC 9606; and *Fusarium oxysporum*, MTCC 9913). Fifteen constituents that accounted for the majority of the mass of the EO-NS were identified and quantified by use of GC-MSMS. The main component was *O*-cymene (37.82%), followed by carvacrol (17.68%), α-pinene (10.09%), trans-sabinene hydrate (9.90%), and 4-terpineol (7.15%). EO-NS exhibited significant antioxidant activity with IC_{50}, EC_{50}, and total antioxidant capacity (TAC) of 0.017 ± 0.0002, 0.1196 ± 0.012, and 114.059 ± 0.97 mg EAA/g, respectively. Additionally, EO-NS exhibited promising antibacterial activity on all strains under investigation, especially on *E. coli* K12 resulting in inhibition diameter of 38.67 ± 0.58 mm and a minimum inhibitory concentration

(MIC) of $1.34 \pm 0.00\,\mu$g/mL. Also, EO-NS had significant antifungal efficacy, with a percentage of inhibition of $67.45 \pm 2.31\%$ and MIC of $2.69 \pm 0.00\,\mu$g/mL against *F. oxysporum*, MTCC 9913 and with a diameter of inhibition 42 ± 0.00 mm and MIC of $0.67 \pm 0.00\,\mu$g/mL against *C. albicans*. To minimize development of antibiotic-resistant bacteria, EO-NS can be utilized as a natural, alternative to synthetic antibiotics and antioxidants to treat free radicals implicated in microbial infection-related inflammatory reactions.

1. Introduction

Excessive generation of free radicals damages biological components directly by oxidation of DNA, proteins, lipids, and carbohydrates, as well as causing secondary damage, due to cytotoxic and mutagenic effects of metabolites released [1]. Due to the diversity and severity of medical problems caused by oxidative stress [2], and the fact that use of synthetic antioxidants is no longer recommended because of their carcinogenic potential [3], in order to minimize oxidative stress and its associated pathologies, new antioxidants have been sought [4]. In particular, natural products of plants are regarded as having potential as antioxidant compounds for protection of cells against damage caused by free radicals [5, 6].

Antimicrobial resistance (AMR) is a major and ongoing global challenge and a threat to public health. It is estimated that by 2050, AMR will be responsible for ten million deaths with a total cost of 100 trillion dollars [7]. Faced with this problem, alternative therapeutic solutions, based on natural resources, particularly medicinal plants, have been the subject of extensive research to develop new antibiotics or new therapeutic modalities and to seek alternatives to currently used antibiotics and develop alternative molecules effective against infectious diseases [8–10]. Aromatic and medicinal plants are an important source of bioactive compounds, such as essential oils (EOs) that could be applied as therapies for infectious diseases [11–14]. EOs have been shown to be valuable as a nontraditional sources of natural, bioactive antioxidants and antimicrobials to combat antibiotic-resistant bacteria and harmful reactive oxygen species (ROS) and are involved in inflammatory immune responses associated with infection. EO derived from seeds of black caraway *Nigella sativa*, a flowering plant in the family Ranunculaceae (EO-NS), was recently studied for biological activities [15, 16]. *N. sativa* is also often referred to as black cumin, nigella, or kalonji. The aim of the current study was to examine the chemical composition of EO-NS as well as their antioxidant and antimicrobial potential against antibiotic-resistant pathogenic and phytopathogenic microorganisms.

2. Materials and Methods

2.1. Plant Material. Seeds of the black caraway (*N. sativa*) were collected in the Souk El Arbaa area of Morocco (34°39′57 ″N 5°58′54 ″W).

2.2. Extraction and Identification of Constituents of EO-NS. The EO of the crushed seeds was extracted through hydrodistillation for a period of 4 h at 100°C by use of Clevenger apparatus (Haborne, 1984) [17]. The chemical profiling of EO-NS was conducted by GC coupled to spectrometer. Varian capillary was employed (Model: TR5- CPSIL-5CB) with length, diameter, and film thickness of 50 m, 0.32 mm, and 1.25 μm, respectively. Temperature programming of the column was in the range of 45-290°C increasing with a steady rate of 4°C/min. While the injector had a fixed temperature of 280°C, temperature of the detector (MS-PolarisQ) was 200°C. The flow rate of helium (carrier gas) was set to 1 mL/min. The injection volume for EO was 1 μL, after having been diluted in organic solvent (hexane) according to the technique of splitless injection. In electronic ionization mode, the ionization energy was 70 eV. The ion source and interface temperatures were 200°C and 350°C, respectively. The range employed for scanning mass was 30-650 m/z. Identification of EO-NS phytoconstituents was conducted by the comparison of their Kovats index values, which were calculated compared to the retention times of a group of linear alkanes (C4-C29), with the values of those standard references collected by Adams library and NIST-MS V2.0 search.

2.3. In Vitro Antioxidant Activity of EO-NS

2.3.1. DPPH Assay. Antioxidant effects of EO-NS were evaluated by use of previously published methods [18]. Briefly, 800 μL of DPPH (0.2 mM, in methanol) was added to 200 μL of various serial dilutions of EO-NS ranging from 0 (in the control) to 1 mg/mL. The obtained mixture was then kept in the dark for 30 min at room temperature (RT). Absorbances were measured at 517 nm against a control consisting of 800 μL of DPPH and methanol solution. Positive controls of quercetin or BHT and blank control were prepared under the same conditions. Antioxidant activity was expressed as percent of inhibition (PI) of the absorbance at 517 nm.

$$\mathrm{PI}(\%) = \left(1 - \frac{\text{Sample absorbance}}{\text{Control absorbance}}\right) * 100. \quad (1)$$

The IC_{50} is the concentration of either EO-NS or ascorbic acid, necessary to reduce free radicals in the reaction medium by 50%. The abscissa represents the concentration values of the tested compound and the ordinate represents the percentage inhibition, with IC_{50} values obtained by linear regression and interpolation (PI%).

2.3.2. Total Antioxidant Capacity (TAC). The total antioxidant capacity (TAC) of EO-NS was measured by use of the phosphomolybdenum method. Briefly, 100 μL of various concentrations of EO-NS was added to 1000 μL of H_2SO_4, Na_2PO_4, and ammonium molybdate reagent mixture such that their concentration was in the range of 0.6 M, 28 mM, and 4 mM, respectively. The tubes were placed at a

Table 1: Phytochemical constituents of essential oil extracted from seeds of black caraway *Nigella sativa* (EO-NS).

P	R.T	Name	C.C	RI		Area (%)
				Cal	*Lit*	
1	7.68	α-Thujene	MO	902	930	10.09
2	7.90	α-Pinene	MO	948	939	2.57
3	9.03	α-Phellandrene	MO	994	1002	0.97
4	9.19	β-Pinene	MO	972	979	2.33
5	10.35	α-Terpinene	MO	998	1017	0.95
6	10.59	O-cymene	MO	1042	1026	46.36
7	10.72	Cis-chrysanthenyl acetate	O	1256	1265	2.56
8	11.60	Limonene	MO	998	1029	0.90
9	12.74	Cis-sabinene hydrate	O	1040	1070	0.72
10	12.81	Linalool	MO	1082	1096	0.54
11	13.43	Trans-sabinene hydrate	O	1070	1098	8.71
12	15.26	Terpinen-4-ol	MO	1148	1177	5.98
13	18.56	Carvacrol	MO	1274	1299	14.82
14	21.66	Longifolene	ST	1398	1390	1.95
15	38.23	Widdrol	ST	1604	1599	0.55
Chemical classes (C.C)						
Monoterpene (MO)						85.51
Sesquiterpene (ST)						2.50
Others (O)						11.99
Total						100

P: peak; R.T: retention time; C.C: chemical classes; RI: retention index; Cal: calculate; Lit: literature; O: others; MO: monoterpene; ST: sesquiterpene.

temperature of about 95°C for 90 minutes. After cooling, the absorbance was read at 695 nm. The control consisted of 100 μL of methanol mixed with 1000 μL of reagent mixture [19]. Samples and controls are incubated under identical conditions. The results obtained are represented as mg of ascorbic acid equivalents per gram (mg EAA/g).

2.3.3. Reduced Ferric Assay (FRAP). The ferric reduction process relies on antioxidants to reduce ferric iron to iron salt, which results in formation of a blue solution. Briefly, 200 μL of different concentrations of EO-NS and 500 μL of 0.2 M phosphate buffer (pH 6.6) were added to glass tubes, followed by 500 μL of 1% potassium hexacyanoferrate ($K_3Fe(CN)_6$) in distilled water. The mixture was heated to 50°C for 20 minutes in a water bath. A 500 μL volume of trichloroacetic acid (10%) was pipetted, and the solution was subjected to centrifugation at 3000 rpm for 10 min. A 500 μL aliquot of the supernatant was transferred to another tube to which 500 μL of double-distilled water (ddH_2O) and 100 μL of 1% $FeCl_3$, freshly prepared, in ddH_2O were added. A blank without an EO sample was also prepared similarly by replacing the EO-NS with methanol. The absorbance was read at 594 nm with reference to the blank, replacing the EO-NS with methanol, which allows calibrating the apparatus (UV-VIS spectrophotometer). Solution of standard antioxidants, either BHT or quercetin, whose absorbances were read in a similar fashion as with the samples, served as positive controls [20].

2.4. In Vitro Antimicrobial Activity of EO-NS

2.4.1. Microbial Strains. The antimicrobial capacity of EO-NS was assessed against four clinically important fungal strains (*Candida albicans*, ATCC 10231; *Aspergillus niger*, MTCC 282; *Aspergillus flavus*, MTCC 9606; and *Fusarium oxysporum*, MTCC 9913) and four bacterial strain (*Staphylococcus aureus*, ATCC 6633; *Escherichia coli*, K12; *Bacillus subtilis*, DSM 6333; and *Proteus mirabilis*, ATCC 29906), which were obtained by Sidi Mohammed Ben Abdellah University (Fez, Morocco).

2.4.2. Method for Assessing Antimicrobial Activity. Antimicrobial activity of EO-NS was assessed by use of the disc diffusion method [21]. Briefly, Petri dishes containing Mueller-Hinton and malt extract were inoculated with the four bacterial strains and *C. albicans*, respectively, using the double-layer method. From fresh cultures grown in Mueller-Hinton and malt extract media, serial dilutions were established in sterilized saline solution (NaCl, 0.9%) until obtaining turbidity of 0.5 McFarland (10^6 to10^8 CFU/mL). Then, 100 μL was added to tubes containing 5 mL of soft agar (0.5% agar), and the inoculated tubes were plated into Petri dishes containing Mueller-Hinton and malt extract media. Whatman paper discs No. 4, with a diameter of 6 mm, were impregnated with 20 μL of EO-NS. For the fungal strains *A. niger*, *A. flavus*, and *F. oxysporum*, the antifungal potency was determined by use of the direct confrontation assay in the malt extract medium between EO-NS and

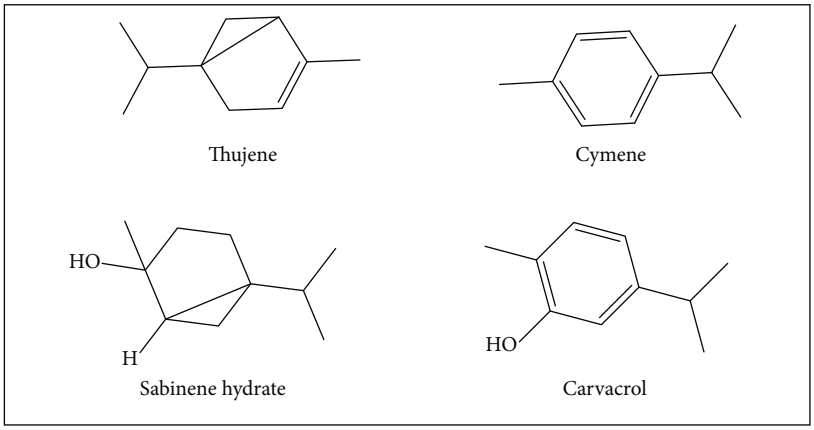


Figure 1: Structures of four of the major phytoconstituents of essential oil extracted from seeds of black caraway *Nigella sativa* (EO-NS).

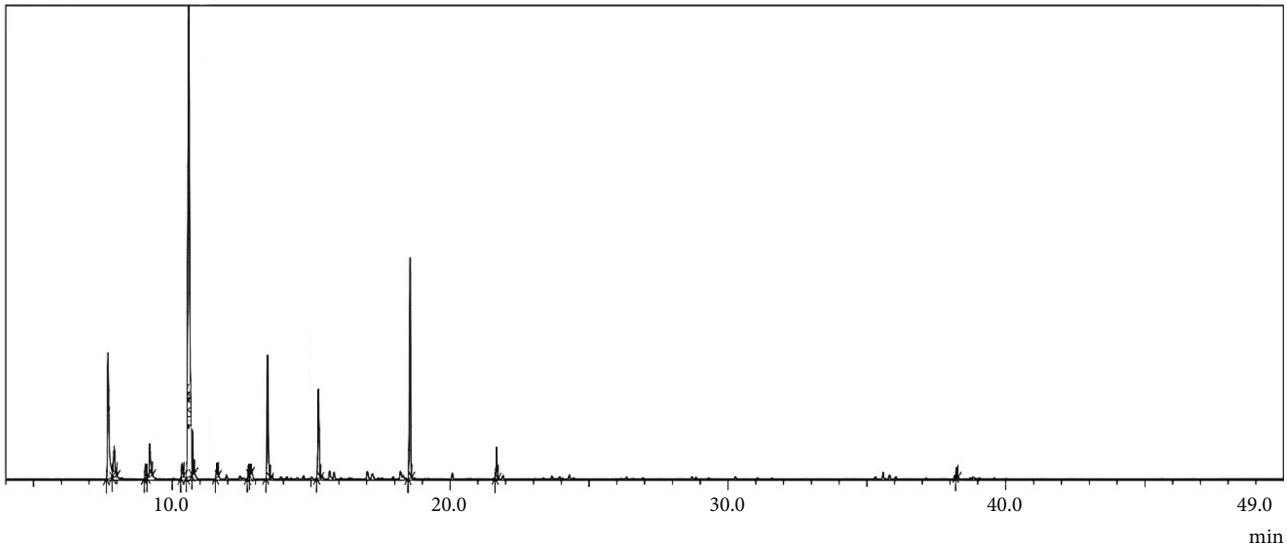


Figure 2: GC-MS/MS chromatogram of essential oil extracted from seeds of black caraway *Nigella sativa* (EO-NS).

the fungal strains tested. Briefly, Whatman paper discs No. 4 with a diameter of 6 mm were soaked with 20 μL of EO-NS, and an agar plate of the fungal strain was positioned 1 cm from the disc containing EO-NS. To assess the efficacy of EO-NS negative controls and positive controls containing conventional antimicrobial drugs, streptomycin and oxacillin for bacterial strains and fluconazole for fungal strains were performed in the same way as the tests. Petri dishes, which had been inoculated with the strain, were placed in an incubator at 30°C or 37°C for fungi or bacteria and *C. albicans*, respectively. Diameters and percentages of inhibition were measured after 24 h, bacteria; 48 h, *C. albicans*; and 7 days, *A. niger*, *A. flavus*, and *F. oxysporum* [22, 23].

2.4.3. Minimum Inhibitory Concentration. Minimum inhibitory concentration (MIC) of EO-NS against each of the four strains of bacteria and fungi was determined by use of previously described methods for microdilution [23]. Briefly, sterile 96-well microplates were premarked, under aseptic conditions; then, 100 μL of EO-NS prepared in DMSO (10%, *v*/*v*) was added to the first row of the plate. The following volumes were subsequently pipetted into all remaining wells, 50 μL sterile Mueller-Hinton and 50 μL sterile malt extract for bacterial and fungal strains, respectively. Multichannel pipette was utilized to make serial dilutions. Finally, 30 μL of bacterial or fungal suspensions of each strain was pipetted into each well. Following a 24 h of incubation for bacteria, 48 h for *C. albicans*, and 7 days for *A. niger*, *A. flavus*, and *F. oxysporum* at 37°C and 30°C, respectively [21–23], the MIC end point was assessed by close observation of the growth inside the wells or *via* colorimetric determination (0.2% TTC, *w*/*v*) [23].

2.5. Statistical Analysis. Results were represented as means of triplicates ± standard deviation (SD). GraphPad Prism (version.8.0.1) was utilized to perform statistical analyses by use of the Shapiro-Wilk tests to verify the normality of the variables as well as Levene's test to assess the homogeneity of variances. Statistical differences between the means were calculated by analysis of variance (One way-ANOVA)

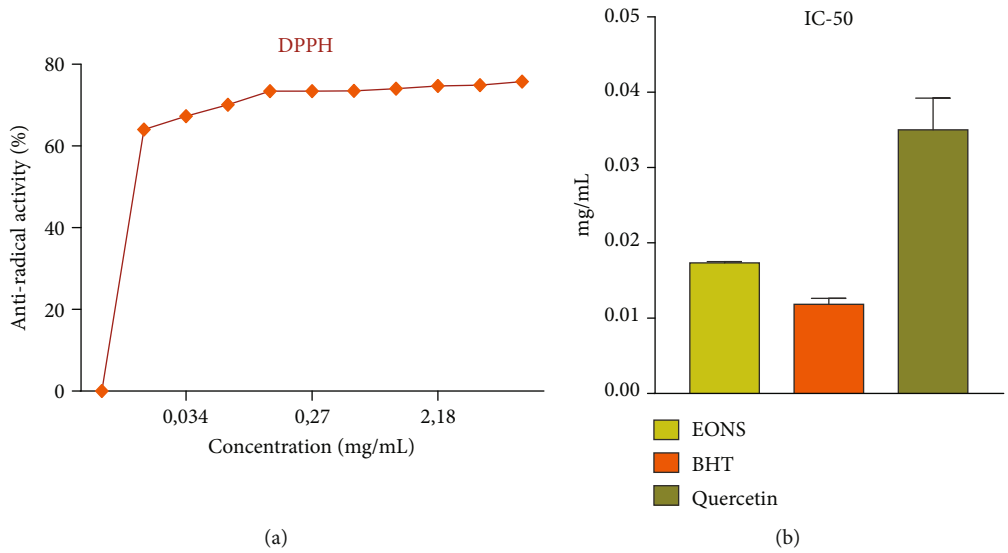


Figure 3: Antiradical activity of essential oil derived from seeds of *Nigella sativa* (EO-NS), by DPPH test (a), and IC_{50} values of antiradical activity of EO-NS and BHT and quercetin (b).

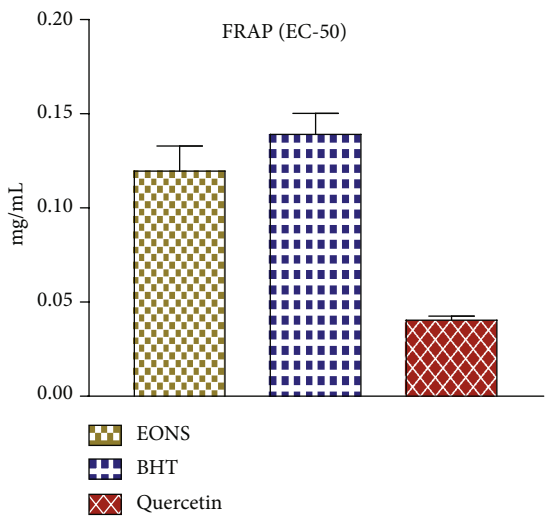


Figure 4: EC_{50} of ferric-reducing antioxidant power (FRAP) values of essential oil derived from seeds of black caraway *Nigella sativa* (EO-NS) and controls (BHT or quercetin).

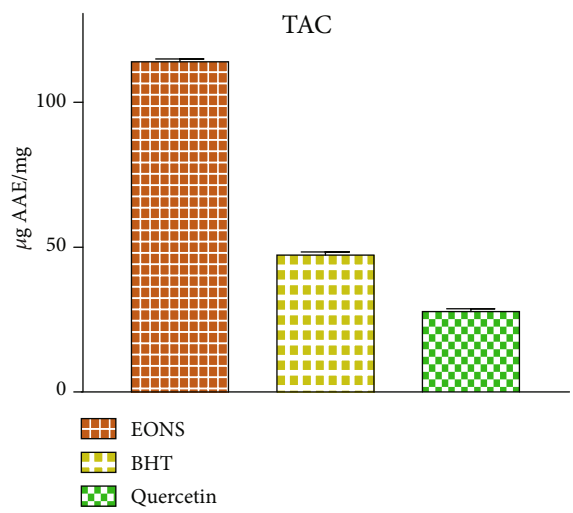


Figure 5: Total antioxidant capacity (TAC) of essential oils extracted from seeds of black caraway *Nigella sativa* (EO-NS) and controls (BHT or quercetin).

and Tukey's test for multiple comparison. Significance of differences was considered at a probability cut-off level of $p \leq 0.05$.

3. Results and Discussion

3.1. Extraction of EO-NS. The yield of EO achieved by the hydrodistillation, expressed on mass of seed, was about $0.8 \pm 0.02\%$, with characteristic transparent yellow color with aromatic odor. This yield was similar to that of $0.832 \pm 0.025\%$ found previously for EO-NS from Beni Mellal, Morocco [24]. Several studies have the EO-NS content of *N. sativa* seeds [25] and revealed that EO-NS extracted by hydrodistillation was about 0.08%, while EO-NS extracted by microwave distillation was approximately 0.11%. Yields of EO-NS of seeds of *N. sativa* from five different countries, namely, Saudi Arabia, Syria, Morocco, India, and France, were achieved by hydrodistillation ranging from 0.047% to 1.7% [26, 27]. Variability among yields of EO-NS can be ascribed for slight differences in extraction procedures as well as other factors, such as geographic origin, ecological factors, agronomic practices, and storage conditions [27–29].

3.2. GC-MS/MS Studies. Based on GC-MS/MS analysis of EO-NS extracted from seeds of Moroccan origin, the following compounds and respective proportions of the total mass of EO-NS were determined. Among the 15 compounds

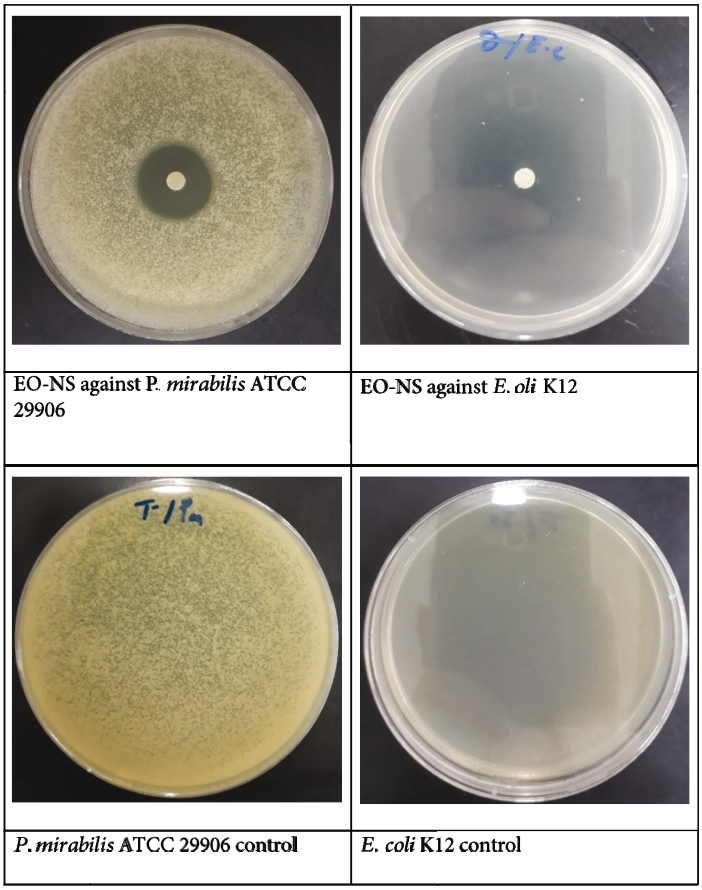


Figure 6: *In vitro* antibacterial activity of essential oil extracted from seeds of black caraway *Nigella sativa* (EO-NS).

identified, 10 were basic monoterpenoids, which accounted for 85.51% of total masses of constituents. These are mainly terpenoid hydrocarbons, including α-pinene, β-pinene, sabinene, γ-terpinene, α-terpinene, and *O*-cymene. Terpenoid alcohols represented 6.52% of total constituents EO-NS, with terpinene-4-ol and linalool comprising 5.98% and 0.54%, respectively. Terpenoid phenols, including carvacrol comprised 14.82%. Other components of EO-NS were chrysanthenyl acetate, trans and cis-sabinene hydrate, two sesquiterpenoids, which represented 2.50% of total mass of EO-NS, including longifolene and widdrol that accounted for 0.55% and 1.95%, respectively. Monoterpenes were dominant, with *O*-cymene being the major component in EO-NS of Moroccan origin. The chemical components of EO-NS and structures of main constituents of EO-NS are presented (Table 1 and Figure 1) and GC-MS/MS chromatograms (Figure 2).

3.3. Antioxidant Activity of EO-NS

3.3.1. DPPH Assay. EO-NS exhibited significant antioxidant activity against the DPPH free radical, which was used to evaluate its antiradical efficacy (Figures 3 and 4), exhibiting an IC_{50} value of 0.017 ± 0.001 mg/mL, compared to BHT or quercetin which exhibited IC_{50} values of 0.0118 ± 0.007 and 0.035 ± 0.004 mg/mL, respectively. In comparison, the IC_{50} values of the EO-NS studied showed a greater

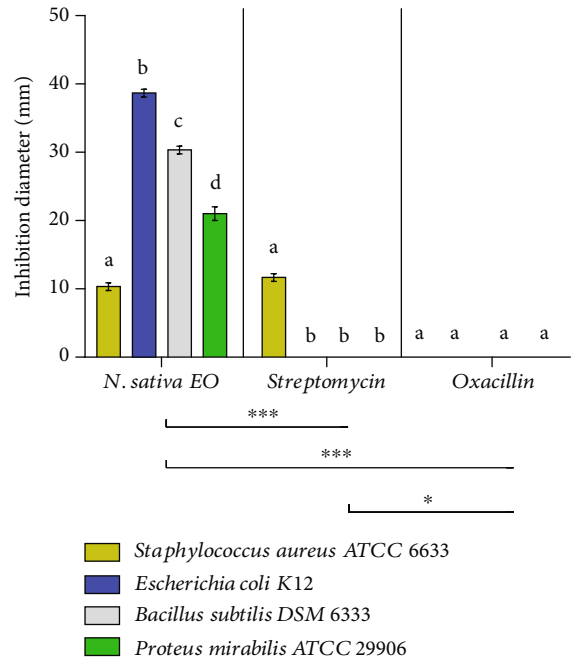


Figure 7: Antibacterial potency of essential oil extracted from seeds of black caraway *Nigella sativa* (EO-NS). Means (± SD, $n = 3$) with the same letter denote no evident significant differences based on Tukey's multiple range tests $p \leq 0.05$.

Table 2: Minimum inhibition concentration (MIC) of essential oil extracted from seeds of black caraway *Nigella sativa* (EO-NS).

	Staphylococcus aureus ATCC 6633	*Escherichia coli* K12	*Bacillus subtilis* DSM 6333	*Proteus mirabilis* ATCC 29906
EO-NS (μg/mL)	2.69 ± 0.00^a	1.34 ± 0.00^b	1.34 ± 0.00^b	2.69 ± 0.00^a
Streptomycin (μg/mL)	1.56 ± 0.00	Rs	Rs	Rs

Means (± SD, $n = 3$) labeled with different letters in same row are considered significantly different according to one-way ANOVA and Tukey's test; $p \leq 0.05$).

Table 3: Antifungal activity and the MIC of essential oils extracted from seeds of black caraway *Nigella sativa* (EO-NS).

	Candida albicans ATCC 10231	*Aspergillus niger MTCC 282*	*Aspergillus flavus MTCC 9606*	*Fusarium oxysporum MTCC 9913*
EO-NS				
Antifungal activity	42 ± 0.00 mm^a	$0.0 \pm 0.0\%^b$	$0.0 \pm 0.0\%^b$	$67.45 \pm 2.315\%^c$
CMI (μg/mL)	0.67 ± 0.00^a	—	—	2.69 ± 0.00^b
Fluconazole				
Antifungal activity	0.0 ± 0.0 mm^a	$8.20 \pm 2.02\%^b$	$0.0 \pm 0.0\%^a$	$30.77 \pm 0.58\%^c$
CMI (μg/mL)	—	7.125^a	—	3.125^b

Means (± SD, $n = 3$) labelled by different letters within the same row are considered significantly different (one-way ANOVA; Tukey's test, $p \leq 0.05$).

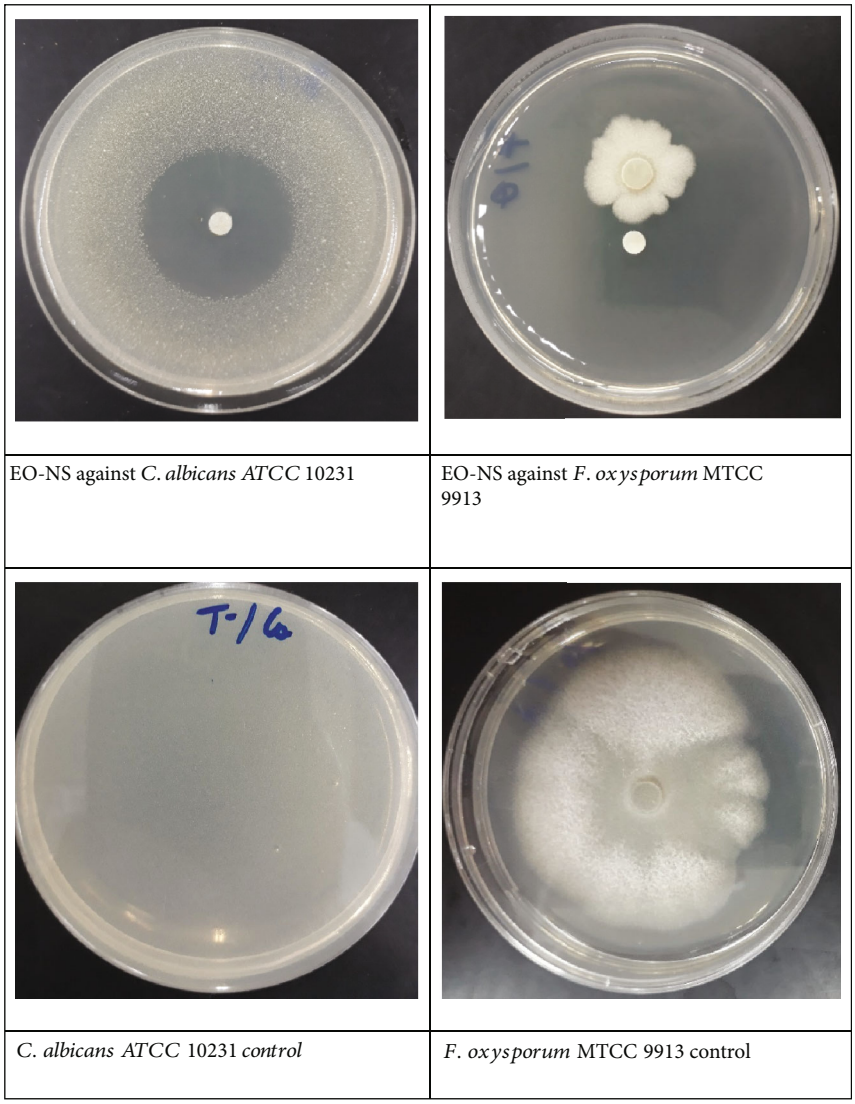
antioxidant capacity than [30, 31] exhibiting IC_{50} values of 36.90 μg/mL and 19 ± 0.7 μg/mL, respectively.

Various components in EOs, including terpenes, sesquiterpenes, and phenolic compounds, which function in diverse modes, can be linked to their antioxidant properties. The principal phytoconstituents EO-NS were *O*-cymene, carvacrol, 4-terpineol, and longifolene, which were responsible for antioxidant properties of EO-NS [15, 32, 33].

3.3.2. FRAP Assay. Results of the FRAP assay revealed that EO-NS exhibited significant dose-dependent, reducing activity exhibiting an EC_{50} of 0.119 ± 0.013 mg/mL (Figure 4). This potency was comparable with that of BHT with EC_{50} of 0.139 ± 0.0110 mg/mL, but less than that of quercetin with an EC_{50} of 0.040 ± 0.002 mg/mL. Reducing power of EO-NS was probably due to the presence of chemically bioactive compounds. Indeed, results of previous studies on reducing power of EO-NS revealed that EO-NS possess considerable reducing power [30, 34]. Specifically, the reducing power of EO-NA can be attributed to phenolic compounds especially carvacrol, which have a hydroxyl-OH group that can donate a hydrogen atom [35, 36]. Other chemicals that operate synergistically with EOs, such as alcohols (linalool), ethers, and hydrocarbons (α-terpinene and γ-terpinene), can contribute to their antioxidant potency. [37–39].

3.3.3. Total Antioxidant Capacity (TAC). Based on results of the TAC assay, EO-NS exhibited antioxidant activity equivalent to 114.059 ± 0.972 μg EAA/mg EO-NS (Figure 5). This result can be attributed to the presence of active antioxidant substances [40]. The antioxidant activity is probably attributed to the monoterpene compounds in essential oils [41]. Generally, EOs with greater terpene content possess powerful antioxidant potential [42]. Indeed, EO-NS seem to be effective antioxidant [31]. However, minor compounds are more likely than the major compounds to provide a pivotal role in the observed antioxidant potency [43]. It is well documented that synergies between various chemicals must be taken into consideration when predicting their biological activities [44, 45].

3.4. Antibacterial Activity of EO-NS. The *N. sativa* EO-NS exhibited measurable antibacterial efficacy against all bacterial strains tested (Figures 6 and 7) (Table 2) and showed promising antibacterial activity compared to two commercially available antibiotics (streptomycin and oxacillin), especially against *E. coli* K12 with a diameter of inhibition of 38.67 ± 0.58 mm and a MIC of 1.34 ± 0.00 μg/mL. Differences in inhibition diameters obtained could be due to differences in chemical compositions of the EO, and the antibacterial activity could be due mainly to the majority compound (*O-cymene*) or to a combination of less predominant compounds found in EO-NS. Results of several studies have indicated that EOs from *N. sativa* and their single compounds are effective against infections caused by bateria. Results of the current study reported here are in agreement with those of Harzallah et al. [46], which demonstrated that EO-NS from *N. sativa* collected in Tunisia and its bioactive compound, thymoquinone, had significant antibacterial activity. Other studies have found that the seed extract derived from *N. sativa* has strong antibacterial potency against *B. subtilis*, IMG 22 with an inhibition diameter of 27 mm, against *E. coli* DM with an inhibition diameter of 19.3 mm [47]. Another study found that *N. sativa* extract has antibacterial activity against *S. aureus* (ATCC 103207) with an inhibition diameter of 19 mm and against *B. subtilis* (ATCC 27853) with an inhibition diameter of 26 mm and against *E. coli* (ATCC 12079), with an inhibition diameter of 21 mm [48]. The results presented here demonstrate greater than that or the previous studies [49], which found that ethyl alcohol extract of *N. sativa* exhibited antibacterial



EO-NS against *C. albicans ATCC* 10231

EO-NS against *F. oxysporum* MTCC 9913

C. albicans ATCC 10231 *control*

F. oxysporum MTCC 9913 control

Figure 8: *In vitro* antifungal activity of essential oil extracted from seeds of black caraway *Nigella sativa* (EO-NS).

potency against *B. subtilis* with an inhibition diameter of 7 mm. The results of the study presented here are also more potent than that reported previously [32], which indicated that the antibacterial potency of EO-NS was greater on *S. aureus* (MTCC 9542) with an inhibition diameter of 16 mm than *Vibrio harveyi* (MTCC 7771) with an inhibition diameter of 5 mm for the 10 mg/mL concentration. EO-NS exhibits potent antibacterial activities against antibiotic-resistant bacterial strains (gram-negative and gram-positive), thereby advocating the utility of the bioactive molecules contained in EO-NS as an alternative to commercially available antibiotics to combat bacterial resistance.

The antibacterial potency of EO-NS on the bacterial strains that we have highlighted in this study is supported by literature data showing the action of EOs rich in carvacrol, whose antimicrobial efficacy is explained by the actual position of the hydroxyl group on the phenolic structure of these molecules [50–52] and which modify permeability and cause leaking of intracellular components through the specific binding to the amine and hydroxylamine groups of bacterial membrane-bound proteins [53]. Because of their cheap cost, biocompatibility, antibacterial and resistance reversal potential, lack or low toxicity to eukaryotic cells, and decreased toxicity to eukaryotic cells and the environment, these volatile compounds are termed green antimicrobials [54]; as a result, they are regarded as an efficient approach for addressing AMR in underdeveloped nations as well as in bacterial strains, including ESKAPEE members [54].

3.4.1. Antifungal Activity of EO-NS. Evaluation of the *in vitro* antifungal activity of EO-NS against *A. niger*, *A. flavus*, *F. oxysporum*, and *C. albicans* by the disc diffusion method has revealed promising antifungal activity with an inhibition percentage of 67.45 ± 2.315% and MIC of 2.69 ± 0.00 μg/mL against *F. oxysporum*, MTCC 9913 and with an inhibition diameter of 42 ± 0.00 mm and MIC of 0.67 ± 0.00 μg/mL against *C. albicans*, compared to the control and the antibiotic fluconazole (Table 3 and Figure 8). Furthermore, EO-NS exhibits fungicidal efficacy against both *F. oxysporum*

and *C. albicans*. However, EO-NS did not exhibit significant antifungal potency against *A. niger*, MTCC 282 or *A. flavus*, MTCC 9606. The current results indicated that EO-NS has an inhibitory effect against pathogenic and phytopathogenic fungi, which might be attributed for its chemical composition, especially the presence of *O-cymene*, *carvacrol, α-thujene*, and *trans-sabinene hydrate*, all of which exhibit significant antifungal efficacies [55, 56]. The significant antifungal activity against *C. albicans* might be due to the presence of *O-cymene*, a conclusion that is in agreement with results of previous studies [57, 58], which reported that *O-cymene* component of EO-NS presents antifungal potency on *C. albicans* and other pathogenic fungal strains. The results reported here are different from those of Khosravi et al. [59], which showed that EO-NS exhibits antifungal activity against *A. flavus*. For cytotoxicity of EO-NS, the study of Mahmoud et al. [60] demonstrates that EO-NS did not show significant cytotoxicity on macrophages, and results of the current study indicate promising antifungal activity of EO-NS without causing cytotoxicity, which suggests its usage as a good alternative to substitute commercially available antifungals to combat fungal resistances.

4. Conclusion

The results of the present study show that EO-NS was effective as antioxidants, antibacterial, and antifungal that could be used as an alternative to currently available synthetic molecules. Notably, GC/MS-MS analysis of EO-NS revealed the richness of these oils in potentially bioactive compounds with a dominance of O-cymene and carvacrol. The antioxidant activity of EO-NS was confirmed by three tests (DPPH, FRAP, and TAC) even at low concentrations. EO-NS acts as a promising antibacterial agent on almost all the studied strains, with a more pronouncing effect on *E. coli (K12)*. Antifungal activity indicated that EO-NS had broad spectrum of action against almost all the studied strains. In further studies, the focus will be on testing purified compounds of EO-NS along with investigating their mode of action. Prior to any prospective application of EO-NS as a natural agent to control microorganisms, we expect to assess the possible adverse consequences on nontarget creatures, as well as clinical trials on both humans and nonhuman primates.

Data Availability

Data used to support the findings are included within the article.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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Research Article

Inhibition of *Staphylococcus aureus* Efflux Pump by O-Eugenol and Its Toxicity in *Drosophila melanogaster* Animal Model

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Background. Efflux pumps are transmembrane proteins that expel drugs out of a bacterial cell contributing to microorganism drug resistance. Several studies addressing the use of natural products with medicinal properties have intensified given the above. Thus, the aim of the present study was to investigate the antibacterial activity and the O-eugenol potential in *Staphylococcus aureus* resistance reversal by efflux pump inhibition, as well as to evaluate its toxicity in the *Drosophila melanogaster* arthropod model. The broth microdilution method was used to determine the minimum inhibitory concentration (MIC) and the O-eugenol efflux pump inhibition. For the *D. melanogaster* toxicity assays, mortality and locomotor system damage were performed using the fumigation method. *Results*. O-eugenol presented a MIC of 1024 μg/mL against *S. aureus*. The association of this compound with the antibiotic tetracycline demonstrated a synergistic effect ($p < 0.0001$), this also being observed when the antibiotic was associated with ethidium bromide ($p < 0.0001$); thus, these results may be attributable to an efflux pump inhibition. The *D. melanogaster* mortality and geotaxis assays revealed the compound is toxic, with an EC_{50} of 18 μg/mL within 48 hours of exposure. *Conclusions*. While we can conclude that the tested product has an efflux pump inhibitory effect, further studies are needed to elucidate its mechanisms of action, in addition to assays using other strains to verify whether the substance has the same inhibitory effect.

1. Introduction

Efflux pumps are one of the resistance mechanisms used by pathogenic microorganisms, this being characterized by actively expelling drugs from the bacterial cell, thus collaborating with the appearance of multidrug-resistant (MDR) phenotypes in strains with clinical interest [1, 2], where the genes that code for efflux pumps can be located on the chromosomes or plasmids of these microorganisms [3].

Staphylococcus aureus is among these infectious pathogens, being easily contracted by humans due to its ample capacity to synthesize extracellular toxins, as well as for presenting known virulence factors such as staphylococcal enterotoxins [4]. Moreover, *S. aureus* is able to acquire

resistance to a variety of antimicrobial agents [5]. The IS-58 strain, which carries the TetK efflux pump, is among the strains of clinical interest [6]. This pump has the efflux protein that confers resistance to tetracyclines and is part of the major facilitator superfamily (MFS) family that uses energy from a proton gradient to extrude the antibiotic [7].

Therefore, the search for new natural bacterial resistance modifiers has been intensified, aiming at the reintroduction of ineffective therapeutic antibiotics in clinical practice [8]. Thus, some phytochemicals can act as adjuvants, inhibiting target-modifying and drug-degrading enzymes, or as inhibitors of efflux pumps [9]. Phenolic compounds stand out among these phytochemicals given their several bioactivities, such as antioxidant, anti-inflammatory, antiallergic, antithrombotic, antimicrobial, and antineoplastic activity [10, 11]. O-eugenol (2-allyl-6-methoxyphenol) is defined as a phenolic derivative and has a hydroxyl group moved to the carbon that is situated between the methoxy and allyl groups [12, 13].

While many of these phenolic compounds can present antimicrobial activities, these can have a high toxicity and be harmful to eukaryotic cells [14]. *Drosophila melanogaster*, an organism that has a low maintenance cost in the laboratory and a short reproductive cycle, in addition to being highly sensitive to the presence of toxic substances at minimal concentrations, is one of the models used to assess the toxicity of these compounds [15, 16].

With this in mind, the objective of the present study was to investigate the antibacterial activity of the isolated O-eugenol compound and its potential for reversing *Staphylococcus aureus* resistance by efflux pump inhibition, as well as to evaluate its toxicity in the *D. melanogaster* arthropod model.

2. Materials and Methods

2.1. Culture Media and Microbial Strains. The IS-58 *Staphylococcus aureus* strain, endowed with the PT181 plasmid carrying the TetK, tetracycline efflux protein, gene was used. The strain was provided by Prof. S. Gibbons (University of London). The bacteria were kept in blood agar base supplements with the antibiotic tetracycline to maintain the plasmid (Laboratórios Difco Ltda., Brazil) and then transferred and kept in glycerol -80°C. Heart Infusion Agar (HIA, Difco laboratorises Ltda.), prepared according to the manufacturer, and 10% Brain Heart Infusion (BHI Acumedia Manufacturers Inc.) were used as the culture media in the assays.

2.2. Substances. Tetracycline was the antibiotic used, this being specific to the strain carrying the TetK pump. The antibiotic and O-eugenol were diluted in dimethyl sulfoxide (DMSO), then in sterile water. Chlorpromazine and ethidium bromide (EtBr) were dissolved in distilled sterile water; carbonyl cyanide m-chlorophenylhydrazone (CCCP) was dissolved in methanol/water (1 : 1, *v*/*v*). All substances were diluted to a concentration of 1024 μg/mL, stored at 20°C, and protected from light. All substances were purchased from Sigma-Aldrich Brazil, except chlorpromazine, which was purchased from a commercial pharmacy.

2.3. Determination of the Minimum Inhibitory Concentration (MIC). MIC is defined as the lowest concentration that inhibits the visible growth of a microorganism [17]. The MIC was determined for O-eugenol and tetracycline using the broth microdilution method [18]. The stock strains were sprayed in HIA medium and incubated at 37°C for a period of 24 hours. The inoculants were prepared in test tubes containing 3 mL of sterile saline solution, these being compared to the 0.5 McFarland scale which corresponds to 10^6 CFU (colony forming units). Then, Eppendorfs® containing 1.440 μL of the BHI liquid culture medium and 160 μL of the bacterial inoculum were prepared, forming a final volume of 1.6 mL. After mixing 1,140 μL of BHI plus 160 μL of the inoculum (~10^5 CFU/mL), the inoculum was diluted 10 times. Subsequently, microdilution plates were filled, with rows 7 and 8 being growth controls and rows 9 and 10 sterility controls. Microdilution was performed with O-eugenol (100 μL) in the first 6 rows, where concentrations ranged from 512 μg/mL to 4 μg/mL. The plates were incubated in a bacteriological incubator at 37°C for 24 hours. The assay was finished by adding 20 μL of resazurin [19], a redox dye to evaluate the presence of cell metabolism, with the color change of the medium from blue to red being an indicative of the bacterial growth [20].

2.4. Antibiotic Modulatory Effect and Efflux Pump Inhibition Evaluation by an Ethidium Bromide (EtBr) Modulatory Effect. For this, Eppendorfs® were filled with 160 μL of the inoculum, O-eugenol at a subinhibitory concentration (MIC/8), and completed with BHI until reaching a volume of 1.6 mL. A modulation control was prepared with 160 μL of the inoculum and 1.440 μL of BHI without the O-eugenol, and 100 μL of the antibiotic was sequentially diluted. Microdilution plates were then filled, where rows G and H were reserved for bacterial growth controls. Sterility controls were performed on separate plates. Subsequently, a microdilution was performed with the antibiotic (100 μL) to assess the modulatory effect of the antibiotic, with concentrations ranging between 0.25 and 512 μg/mL.

The efflux pump inhibition assays were performed by evaluating the decrease in the MIC of ethidium bromide, since the efflux pumps are the only mechanism responsible for the extrusion of EtBr. Microdilution was performed with 100 μL of EtBr for the inhibitory evaluation of the efflux pump. A modulation control was prepared with 160 μL of the inoculum and 1.440 μL of BHI without the O-eugenol, and 100 μL of the EtBr was sequentially diluted. Concentrations ranged from 512 μg/mL to 0.25 μg/mL [21]. After 24 h, readings were performed by adding 20 μL of resazurin [19].

2.5. Drosophila melanogaster Stock. D. melanogaster (Harwich strain) was obtained from the National Species Stock Center, Bowling Green, OH. The flies were cultivated in 340 mL glass bottles grown with the medium containing: 83% corn mass, 4% sugar, 4% lyophilized milk, 4% soy bran, 4% wheat bran or oats, and 1% salt. 1 g of Nipagin (Methylparaben) was added when cooking the mixture. Following a cooling period in the growth flasks, 1 mL of a solution containing *Saccharomyces cerevisiae* was added to the flask.

The flies were grown in photoperiod BOD greenhouses at a temperature of 25°C ± 1°C and 60% relative humidity.

2.6. Mortality Assays. Drosophila melanogaster is widely used to assay in vivo toxicity, because through its sensitivity to harmful substances in minimal concentrations, it is an important model to assess the toxic activity of these substances [22]. The fumigation bioassay methodology was used to evaluate the O-eugenol toxicity, where adult flies (males and females aged approximately 3 to 5 days) were placed in 130 mL flasks in multiples of 20, previously prepared with 1 mL of a sucrose solution in distilled water, at a concentration of 20%, allowing the flies to feed *ad libitum*. This solution was soaked in a paper and placed on the bottom of the glass, while the glass cover had a filter paper. The control was prepared with 20 μL of acetone. The compound O-eugenol was diluted in acetone according to its molecular weight obtaining a stock solution of 213.6 μg/mL. After that, volumes of 20, 10, and 5 μL of this stock solution were withdrawn, resulting in final concentrations of 33, 16, and 8 μg/mL air using 130 mL air bottles, respectively. All bioassays were conducted in a BOD-type greenhouse with a 12-hour light and dark cycle, with the temperature controlled at 25°C ± 1°C and 60% relative humidity. The tests were performed in triplicates, and mortality rate readings were made at 3, 6, 12, 24, 36, and 48 hours [15].

2.7. Negative Geotaxis Assay. Damage to the locomotor system was determined by a negative geotaxis test, which consists of counting the number of flies that rise above 3 cm in the glass column of the experiment itself in a 5-second time interval, this being repeated 2 times at 1-minute intervals [23]. This test was performed every 3, 6, 12, 24, 36, and 48 hours. The results were presented as the mean time (s) ± SE obtained from two independent experiments.

2.8. Statistical Analysis. Statistical analysis for the microbiological tests was performed using a two-way ANOVA followed by Bonferroni's *post hoc* test, using the GraphPad Prism 7.0 software. For the toxicity data analysis, a two-way ANOVA followed by Tukey's multiple comparisons test was performed. No statistical differences were observed with the same concentration as a function of time.

3. Results

3.1. Efflux Pump Inhibition by Antibiotic and Ethidium Bromide MIC Reductions. O-eugenol demonstrated a MIC of 1024 μg/mL against the IS-58 *S. aureus* strain. The association between O-eugenol and the antibiotic tetracycline revealed a reduction in the antibiotic's MIC, indicating a potentiation of antibiotic activity, as observed in Figure 1. When the antibiotic was tested in association with standard inhibitors at subinhibitory concentrations, the MIC values for chlorpromazine did not differ from the antibiotic control, whereas the MIC for CCCP presented a marked synergism. CCCP and chlorpromazine are standard efflux pump inhibitors, but have no direct effect on the efflux pump. They act inhibiting by modifying the transmembrane electrochemical potential of the bacteria [24].

Table 1 represents the association values of the specific antibiotic tetracycline and EtBr with standard inhibitors (CCCP and chlorpromazine). In terms of efflux pump inhibitory assays based on the reduction of the ethidium bromide MIC, its association with O-eugenol (MIC/8) presented a decrease in the MIC of EtBr from 32 to 16 μg/mL, as seen in Table 1, this being characterized as a synergistic action. Figure 2 shows that similar results were observed for the standard inhibitors.

3.2. Drosophila melanogaster Toxicity. O-eugenol obtained an EC_{50} of 18 μg/mL within 48 hours of exposure. The results obtained with the *Drosophila melanogaster* toxicity test showed that O-eugenol presented moderate toxicity at the 33 μg/mL concentration after 3 hours of exposure, with the mortality rate increasing following the hours of exposure (Figure 3). Significant mortality was observed after 36 hours of exposure to the compound for the 16 μg/mL concentration (Figure 3).

In the negative geotaxis assays where possible damage to the locomotor apparatus is verified, a significant locomotor deficit was observed in the flies ($p < 0.0001$) at the 33 μg/mL air concentration at a 3-hour exposure period when compared to the control (Figure 4). This effect was intensified over the following hours of exposure, and a marked damage to the locomotor apparatus was observed at the 24-hour reading, as the alive flies showed difficulty in locomotion (Figure 4).

4. Discussion

The MIC is defined as the lowest concentration capable of completely inhibiting microbial growth [25] and thus is considered clinically irrelevant when it is insufficient to inhibit bacterial growth. There are no studies evaluating the antimicrobial activity of O-eugenol on bacterial strains in the literature. Furthermore, in the present study, the antibacterial activity of O-eugenol on the IS-58 strain of *S. aureus* was not verified. The phenolic compounds caffeic acid and gallic acid demonstrated a MIC of 1024 μg/mL for the IS-58 *S. aureus* strain [26].

However, other phenolic compounds have already shown antibacterial activity, as the results observed in experiments with quercetin, which showed antimicrobial activity on *S. aureus*, obtaining a MIC value of 6.25 μg/mL [27]. A MIC value considered to be relevant was also found in assays with eugenol against the *S. aureus* ATCC 25923 strain, with this MIC value being 256 μg/mL [28].

In addition to the antimicrobial action of isolated phenolic compounds, the action of plant extracts with high total phenolic and flavonoid contents has also been reported in the literature, for example, the *Corymbia ficifolia* (Eucalyptus) extract with the following compounds, gentisic acid, chlorogenic acids, p-coumaric, hyperoside, isoquercitrin, rutin, and quercitrin, presented an antibacterial activity against the *S. aureus* strain with a MIC value of 20 μg/mL [29].

Although O-eugenol did not show direct antibacterial activity against the IS-58 strain of *S. aureus*, synergism was observed when associated with the antibiotic tetracycline, as shown in Figure 1. Assays using quercetin and its morin

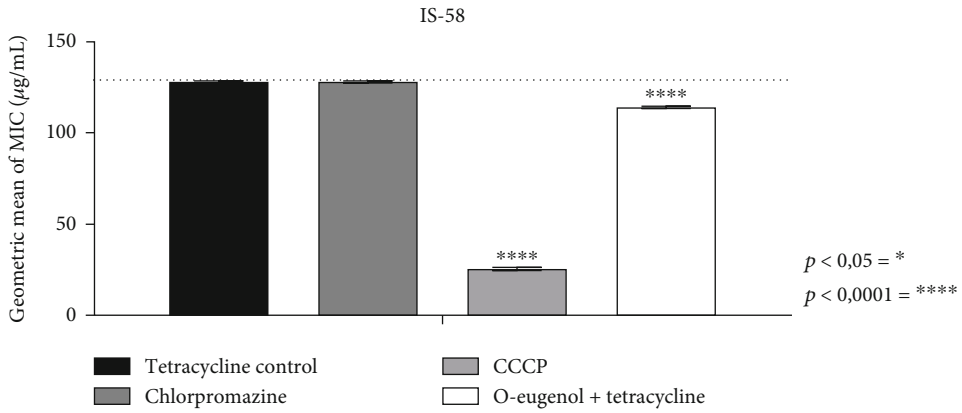

Figure 1: MIC of tetracycline in isolation and in combination with standard inhibitors and O-eugenol. CCCP: carbonyl cyanide m-chlorophenylhydrazone. * represents statistical significance compared to the control.

Table 1: Minimum inhibitory concentrations of the associations between O-eugenol and standard inhibitors and ethidium bromide against the IS-58 *S. aureus* strain.

Substance	Control	Chlorpromazine	CCCP	O-eugenol
Tetracycline	128.0000	128.0000	25.39842	114.035
EtBr	32.0000	2.00000	16.0000	16.0000

EtBr: ethidium bromide; standard inhibitors: (CCCP) carbonyl cyanide m-chlorophenylhydrazone and chlorpromazine.

isomer showed potential to reduce 3 to 16 times the MIC of tetracycline on methicillin-resistant *S. aureus* strains (MSSA-MSRA) [30].

Efflux pumps are associated with pathogenic resistance phenotypes, thus representing an important threat to the effective treatment of diseases triggered by Gram-negative and Gram-positive bacteria [31]. In the IS-58 strain of *S. aureus*, the TetK efflux pump is responsible for the mechanism of bacterial resistance to tetracycline, which acts by extruding the antibiotic out of the bacterial cell [32]. By this fact, it is extremely important to identify and produce efflux pump inhibitors (EPIs) from natural sources, such as plants which have secondary bioactive metabolites [33]. These EPIs can act by triggering an energy depletion process, by preventing binding to ATP or altering the proton gradient [34].

Although our results show that there was no direct antibacterial activity on the IS-58 strain of *S. aureus*, synergism was observed when O-eugenol was associated with the antibiotic and EtBr, decreasing their MICs, indicating that the compound acts on the resistance mechanism characterized as active efflux, which is promoted by efflux pumps that actively expel EtBr to the outside of the cell, decreasing its toxicity on bacterial cells [35].

The use of ethidium bromide as a substrate for efflux pumps is well described in the literature, and as shown in Figure 2, O-eugenol presented a behavior similar to that of standard inhibitors, pointing to a similar pump inhibitory mechanism against the analyzed strain. The TetK efflux pump present in the IS-58 strain of *S. aureus* is responsible for therapeutic resistance to antibiotics of the tetracycline class. To combat bacterial resistance, a wide range of studies has been conducted to verify whether natural products act as adjuvants to antibiotics. In this perspective, products obtained from plant sources, rich in phytochemicals such as phenolic compounds, flavonoids, tannins, and phenolic acids, can act synergistically when associated with antibiotics against *S. aureus* strains that carry efflux pumps. It is important to note that there are no studies reporting the inhibitory effects of O-eugenol on the IS-58 (TetK) pump in *S. aureus*, with this study being the first to report this activity. However, there are other studies in the literature analyzing other phenolic compounds as possible efflux pump inhibitors, for example, assays performed with caffeic acid and gallic acid. Caffeic acid when combined with ethidium bromide reduced the MIC against strains carrying the TetK, MrsA, and NorA pumps; however, it only inhibited the action of the *S. aureus* MrsA and NorA efflux pumps. On the other hand, gallic acid reduced the MIC of ethidium bromide against *S. aureus* strains that had the TetK, MrsA, and NorA pumps; however, bacterial resistance reversal by efflux pump inhibition was only observed in the strain with the NorA pump [26].

Another study evaluates the antibacterial activity of natural products on *S. aureus* strains carrying the TetK efflux protein. Recent research has evaluated the potential of two natural compounds, α-bisabolol and β-cyclodextrin, and it was observed that these compounds when associated with the antibiotic tetracycline showed synergistic action, in other words, reduced the MIC of tetracycline [36].

Another study in the literature demonstrates the antibacterial effect of menadione (vitamin K) on this same strain (IS-58) of *S. aureus* with an MIC value of 64 μg/mL. And when menadione was associated with ethidium bromide, there was a reduction in the MIC of BrEt, indicating inhibition of the efflux pump mechanism [34]. The essential oil from *Chenopodium ambrosioides* L. leaves also reduced the MIC of ethidium bromide, demonstrating inhibition on the TetK efflux pump [37].

Experiments using tannic acid showed its potential to decrease the MIC of antibiotics and ethidium bromide against *S. aureus* strains carrying MrsA and TetK efflux pumps, indicating this substance can inhibit the resistance mechanisms to

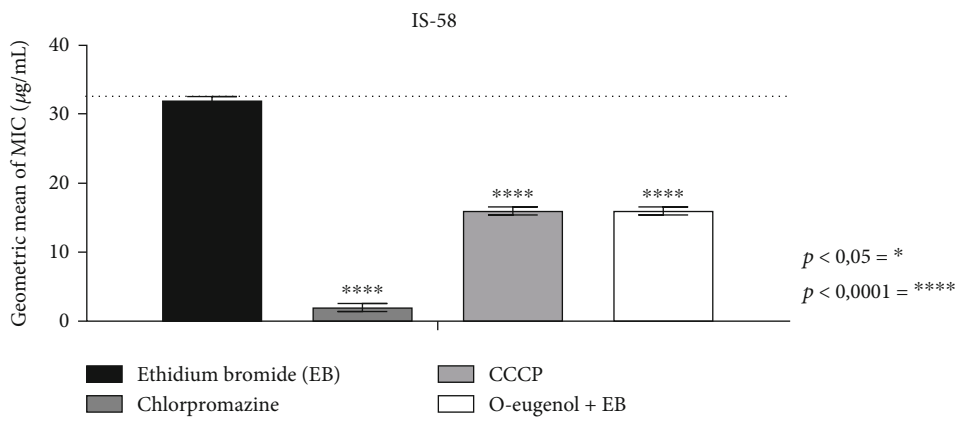

Figure 2: MIC of ethidium bromide in isolation and in association with standard inhibitors and O-eugenol. CCCP: carbonyl cyanide m-chlorophenylhydrazone; * represents statistical significance compared to the control.

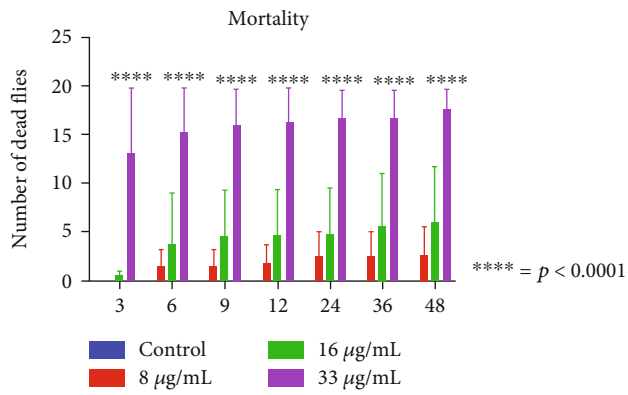

Figure 3: *Drosophila melanogaster* mortality assays with the O-eugenol compound. $^{*}p < 0.05$ compared to the control.

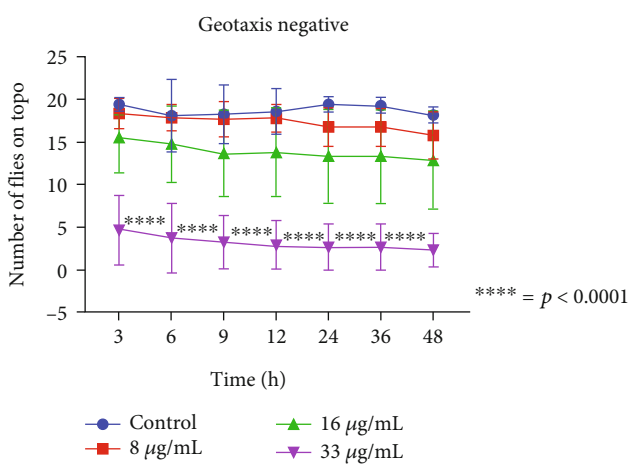

Figure 4: Negative geotaxis assays with the *Drosophila melanogaster* model.

these antibiotics [31]. The antimicrobial effects of phenolic acids are attributable to their chemical structure, especially to the length of the saturated chain, and position and number of substitutions in the benzene ring of the nucleus [38].

An analysis of twelve flavonoids showed that four of these (naringenin, phloretin, diosmetin, and myricitrin) decreased the MIC of the antibiotic norfloxacin from 128 μg/mL to 32 μg/mL, this equating to a fourfold reduction, while hesperetin resulted in a reduction from 128 μg/mL to 8 μg/mL, a sixteenfold decrease, against *S. aureus* SA-1199B. For association assays with ethidium bromide, naringenin stood out by decreasing the MIC of EtBr from 32 μg/mL to 8 μg/mL, a fourfold reduction [39].

The results obtained in this study suggest that O-eugenol may present antibacterial effect modifier activity; in other words, decreasing the antibiotic concentration required to suppress the growth of bacterial pathogens such as *S. aureus*, when used as adjuvants in antibiotic therapy against these pathogens. Recent reports have evidenced that the association of some terpenes with conventional antibiotics resulted in increased antibiotic activity, reversing the antibiotic resistance observed experimentally [40].

The bioactivities of a plant or an isolated compound are considered excellent when toxicity or adverse events considered lethal in experimental models are not observed. For this reason, toxicity evaluation studies are of paramount importance [14]. Invertebrate models, such as *D. melanogaster*, have been widely used in studies to assess the toxicity and genotoxicity of natural products since this model has many signaling pathways similar to those of humans, in addition to having drug target homology with vertebrate models, such as rodents and other small mammals [41]. There are no reports of studies in the literature evaluating the toxicity of the O-eugenol compound on the *D. melanogaster* arthropod model, this study being the first to report this effect.

D. melanogaster is characterized as an alternative eukaryotic model that has been widely used to verify the toxicity of substances due to aspects that favor its use, namely, its high sensitivity to low concentrations of substances, easy maintenance in the laboratory, short reproductive cycle, and high number of offspring [42]. In the literature, there are several studies that associate the antibacterial activity and toxicity of substances using *D. melanogaster* as a result of the factors mentioned above.

The fruit fly has been used to analyze the toxicity of several compounds, including eugenol and isoeugenol, which showed a high toxicity against *D. melanogaster* obtaining LC_{50}s of 0.03 and 0.05 μL/L, respectively [43]. Studies have shown that the *Eugenia uniflora* leaf (Pitanga) essential oil has toxicity against *D. melanogaster* at concentrations of 3 μg/mL, 15 μg/mL, and 30 μg/mL, with mortality rates of 51, 79, and 78%, respectively [44].

Tests investigating the toxicity of the *Psidium guajava* (Goiaba) essential oil on *D. melanogaster* using the fumigation method found a significant increase in mortality, with this effect being associated with the time and concentration that the organism was exposed to, where the 23.5 and 30 μg/mL concentrations presented the highest toxicity [45].

Toxicity assays evaluated through mortality and negative geotaxis are used as toxicity indicators of natural or synthetic chemicals, because they indicate through the mortality rate and damage to the locomotor apparatus physiological changes in the test organism, considering that the behavior is integrated to the subcellular and cellular processes of these organisms [46]. In addition, some studies in the literature perform biochemical assays to demonstrate physiological changes triggered by the substances in this alternative model, for example, we can cite oxidative stress as the main condition that is associated with the toxic profile of a substance, promoting an imbalance in the oxidant and antioxidant system of these organisms [47]. However, in this study, biochemical assays were not performed due to limitations in the technical facilities of the laboratory.

5. Conclusion

The present study demonstrated the reversal of resistance by efflux pump inhibition in *Staphylococcus aureus* carrying the TetK pump, which confers resistance to tetracyclines, by O-eugenol by ethidium bromide MIC reduction. Mortality and geotaxis assays with *Drosophila melanogaster* revealed the compound has moderate toxicity. This study is the first to analyze the antimicrobial activity of O-eugenol, as well as its toxicity. Investigating the mechanisms of action of natural compounds that present antibacterial effects by evaluating the gene expression profile is a fundamental requirement for drug discovery and development; however, due to technical limitations, these assays were not conducted in this study. Thus, future research should be encouraged to correlate gene expression in treated bacterial strains and possible molecular targets of the tested compounds.

Data Availability

The data will be available after request to the corresponding authors.

Disclosure

All authors declared the agreement with this submission and publication.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Authors' Contributions

Nair Silva Macêdo is responsible for investigation, writing—original draft, and writing—revision and editing; Zildene de Sousa Silveira for investigation and writing—revision and editing; Paula Patrícia Marques Cordeiro for writing—revision and editing; José Pinto Siqueira Júnior for donation of bacterial strains; Lucindo José Quintans Júnior for review of writing content; Henrique Douglas Melo Coutinho for resources and funding acquisition; Bonglee Kim for project management; Abolghasem Siyadatpanah for resources; Francisco Assis Bezerra da Cunha for resources, project management, funding acquisition, and supervision; and Márcia Vanusa da Silva for supervision.

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Retraction

Retracted: Neuroglobin Is Involved in the Hypoxic Stress Response in the Brain

BioMed Research International

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This article has been retracted by Hindawi following an investigation undertaken by the publisher [1]. This investigation has uncovered evidence of one or more of the following indicators of systematic manipulation of the publication process:

(1) Discrepancies in scope

(2) Discrepancies in the description of the research reported

(3) Discrepancies between the availability of data and the research described

(4) Inappropriate citations

(5) Incoherent, meaningless and/or irrelevant content included in the article

(6) Manipulated or compromised peer review

The presence of these indicators undermines our confidence in the integrity of the article's content and we cannot, therefore, vouch for its reliability. Please note that this notice is intended solely to alert readers that the content of this article is unreliable. We have not investigated whether authors were aware of or involved in the systematic manipulation of the publication process.

Wiley and Hindawi regrets that the usual quality checks did not identify these issues before publication and have since put additional measures in place to safeguard research integrity.

We wish to credit our own Research Integrity and Research Publishing teams and anonymous and named external researchers and research integrity experts for contributing to this investigation.

The corresponding author, as the representative of all authors, has been given the opportunity to register their agreement or disagreement to this retraction. We have kept a record of any response received.

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[1] L. Shang, D. Mao, Z. Li, X. Gao, and J. Deng, "Neuroglobin Is Involved in the Hypoxic Stress Response in the Brain," *BioMed Research International*, vol. 2022, Article ID 8263373, 11 pages, 2022.

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Research Article

Neuroglobin Is Involved in the Hypoxic Stress Response in the Brain

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Neuroglobin is an oxygen-binding heme protein expressed predominantly in the brain. Despite many years of research, the exact distribution and expression of neuroglobin in the neocortical development and under mild hypoxia stress still remain unclear. Therefore, we aim to explore the expression of neuroglobin during neocortex expansion and under mild hypoxia stress *in vivo*. We used Kunming mice to examine the expression of Ngb protein during neocortex expansion. In addition, we analyzed the density of Ngb-positive neural stem cells using the Image-Pro PLUS (v.6) computer software program (Media Cybernetics, Inc.). Our data indicated that the density of the neuroglobin-positive neurons in mice cerebral cortex displayed a downward trend after birth compared with high expression of neuroglobin in a prenatal period. Similarly, we identified that neurons were capable of ascending neuroglobin levels in response to mild hypoxic stress compared with the no intervention group. These findings suggest that neuroglobin behaves as a compensatory protein regulating oxygen provision in the process of neocortical development or under physiological hypoxia, further contributing to the discovery of novel therapeutic methods for neurological disorders, which is clinically important.

1. Introduction

The globins are widely expressed in many organisms where they display a variety of functions. Hemoglobin (Hb), which consists of two α and two β subunits, is a better known globin presenting not only in the brain but also in different tissues and organs. The expression of Hb α- and β-chains is fundamental for Hb to function, mainly supporting the transport of oxygen and carbon dioxide in the blood [1]. Meanwhile, altered Hb levels have been detected the neurodegenerative diseases in post-mortem brains, which suggests that Hb functions are not exclusively restricted to the blood but may play multiple roles in health and diseases [2]. Lately, neuroglobin (Ngb), the third member of the globin family, was identified in a wide range of vertebrates. As a crucial molecule in hypoxia-induced signaling, the prospective neuroprotective properties of Ngb have aroused the concern of scholars [3]. Nevertheless, the evidence of Ngb expression in the development of cerebral cortex still remains unclear.

Ngb, an oxygen-binding heme protein, is involved in transporting oxygen and expressed predominantly in the cerebral cortex, hippocampus, thalamus, hypothalamus, and cerebellum of the brain [4]. Previous studies have demonstrated that Ngb, as an endogenous neuroprotective factor, could be triggered by hypoxic stress, contributing to regulating the death or survival of neural cells [5, 6]. Sun et al. [6] found that overexpression of Ngb in vivo could help the brain resist neuronal injury from experimentally induced stroke [7]. Soon after, Ngb has been reported to be a neuroprotective protein that is involved in age-related neurodegenerative disorders such as Alzheimer's disease [8]. In line with these ideas, evidence indicated that Ngb also has neuroprotective effects on a traumatic brain injury mouse model [9]. As we know, the expansion of mammalian neocortex is a hallmark of human evolution [10]. To be more specific,

the development of the mammalian neocortex involves the increased proliferation of a limited number of neural stem cells (NSCs), and the radial migration of NSCs further contributes to the production of cortical neurons [11–14]. Perturbation of any step during the process will give rise to organizational anomalies, leading to severe brain damage [15]. Although accumulated evidence indicated that Ngb acts as an oxygen-dependent neuroprotectant expressed in the brain and exerted antiapoptotic effects, the exact expression sites of Ngb during brain development are still a matter of debate [3, 6, 16].

Some *in vitro* studies have proved that upregulation of Ngb levels in response to hypoxia in various neuronal cell lines indicates the involvement of Ngb in neuronal response to low oxygen [6, 17–19]. Meanwhile, *in vivo* investigations have demonstrated that neuronal survival after hypoxia or ischemia decreased by inhibition of Ngb, but enhanced by Ngb overexpression [6, 20, 21]. In addition, Ngb has been considered as a nitrite reductase, further preventing mitochondrial respiration in hypoxia [22]. Fago et al. [23] and Raychaudhuri et al. [24] also pointed out that Ngb may interact with cytochrome c, thus inhibiting the intrinsic apoptotic pathway. However, although these studies have suggested a relationship between Ngb and cerebrovascular diseases, many of which mainly focused on the expression of Ngb in the brain of adult individuals, the specific distribution and function of Ngb in the development of cerebral cortex under mild hypoxia stress remain unclear.

To gain further insights into the biological functions of Ngb in the development of cerebral cortex, in the current study, Kunming mice were used for examining the expression of Ngb protein during neocortex expansion and under mild hypoxia stress, helping to better understand the role of Ngb in the development of central nervous system. Further, these results may have significant implications in the physiology and pathology of the brain and may contribute to the discovery of novel therapeutic methods for neurological disorders, which is clinically important.

2. Materials and Methods

2.1. Animals and Study Design. Kunming mice were obtained from Laboratory animal center of Henan Province, China, and had free access to food and water. All animals were maintained according to the guidelines approved by Animal Care and Use Committee of Henan University. The animals were housed in climate-controlled quarters with a 12-hour light/dark cycle. Embryonic or postnatal offspring were produced from timed pregnancies. E referred to embryonic day, and E0 meant the day of vaginal plug in mated females. Postnatal day was represented by P, and P0 was defined as the first 24 hours after birth. Mice were grouped according to the following ages: E16, E18, P1, P3, P7, P14, P30, P90, P180, and P360. From E16 to P360, a total of 126 Kunming mice were used in this study. Each group contained at least ten mice.

Mild hypoxia in was imitated with burden swimming exercise. Briefly, Kunming mice were randomly assigned to the control group and experiment group at age P120. Control mice were fed conventionally without any interventions. Experimental mice were subjected to adaptable swimming exercises for two days in advance, and nonswimming mice were excluded. Then, burden swimming exercise was conducted with mice carrying a load of 5% of their body weight. Weight-loaded swimming training was executed in a cylinder (100 × 60 cm) with a smooth inner wall, which was filled with water (the water depth according to the size of the mice, so that it cannot touch the bottom of the container, and water temperature maintained at 26 ± 2°C). Weight-loaded swimming exercise was done in a quiet environment for up to one hour, and mice were sacrificed under sodium pentobarbital anesthesia immediately.

To get the samples of embryonic mice and at specific stages, pregnant dams were anesthetized and fetuses at E16 and E18 were harvested by cesarean section. The brains were carefully separated and fixed with 4% w/v paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C. In addition, from P1 to P360, postnatal mice (without mild hypoxia stress treatment) were anesthetized and perfused transcardially with 4% paraformaldehyde, and brains were fixed with the same fixative. Similarly, the brain samples from the mild hypoxia stress group (both control and weight-loaded swimming exercise group) were isolated and were fixed in the fixative for 24 hours at 4°C and processed for immunofluorescence.

2.2. Immunocytochemistry. One of the most distinct characteristics in the development of the cerebral cortex is the structure of lamination [25]. Typically, the neocortex has six layers, known as layers I to VI in an inside-out manner. Layer I consists of axons and dendritic tufts, and newborn neurons migrate through the IZ and ultimately give rise to laminae II to VI [26]. Coronal sections of mice brains were deparaffinized and were rinsed in 0.01 M phosphate buffer and preincubated in 5% normal goat serum for 30 minutes. The slices were incubated with primary antibodies at 4°C overnight. Antibodies to detect Neuroglobin (sc-22001) and Nestin (SC-33677) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Sox2 (AB97959) was purchased from Abcam (Cambridge, UK). Moreover, anti-NeuN (MAB377B) was obtained from Merck Millipore (Massachusetts, USA). Then, the sections were incubated with secondary antibodies for 3 hours at room temperature after multiple washes in 0.01 M phosphate buffer. Alexa Fluro 568 donkey anti-goat IgG (A11055), Alexa Fluro 488 donkey anti-rabbit IgG (A10042), and Alexa Fluro 488 donkey anti-mouse (A21202) were obtained from Invitrogen (Carlsbad, USA). Then, cover slips were mounted under 65% glycerol with 1 : 60,000 4′,6-diamidino-2-phenylindole (DAPI) for counterstaining. Slices were photographed with an epifluorescence microscope (BX61, Olympus, Tokyo, Japan) under rhodamine, fluorescein isothiocyanate, or ultraviolet filter sets. High-quality sections were imaged using a laser confocal microscope (FV1000, Olympus, Tokyo, Japan), using separate scans at 568 nm (red) and 488 nm (green).

2.3. Statistical Analysis. The density of Ngb positive neural stem cells was analyzed using the Image-Pro PLUS (v.6) computer software program (Media Cybernetics, Inc.). All quantitative data are expressed as the mean values ± SD of at least

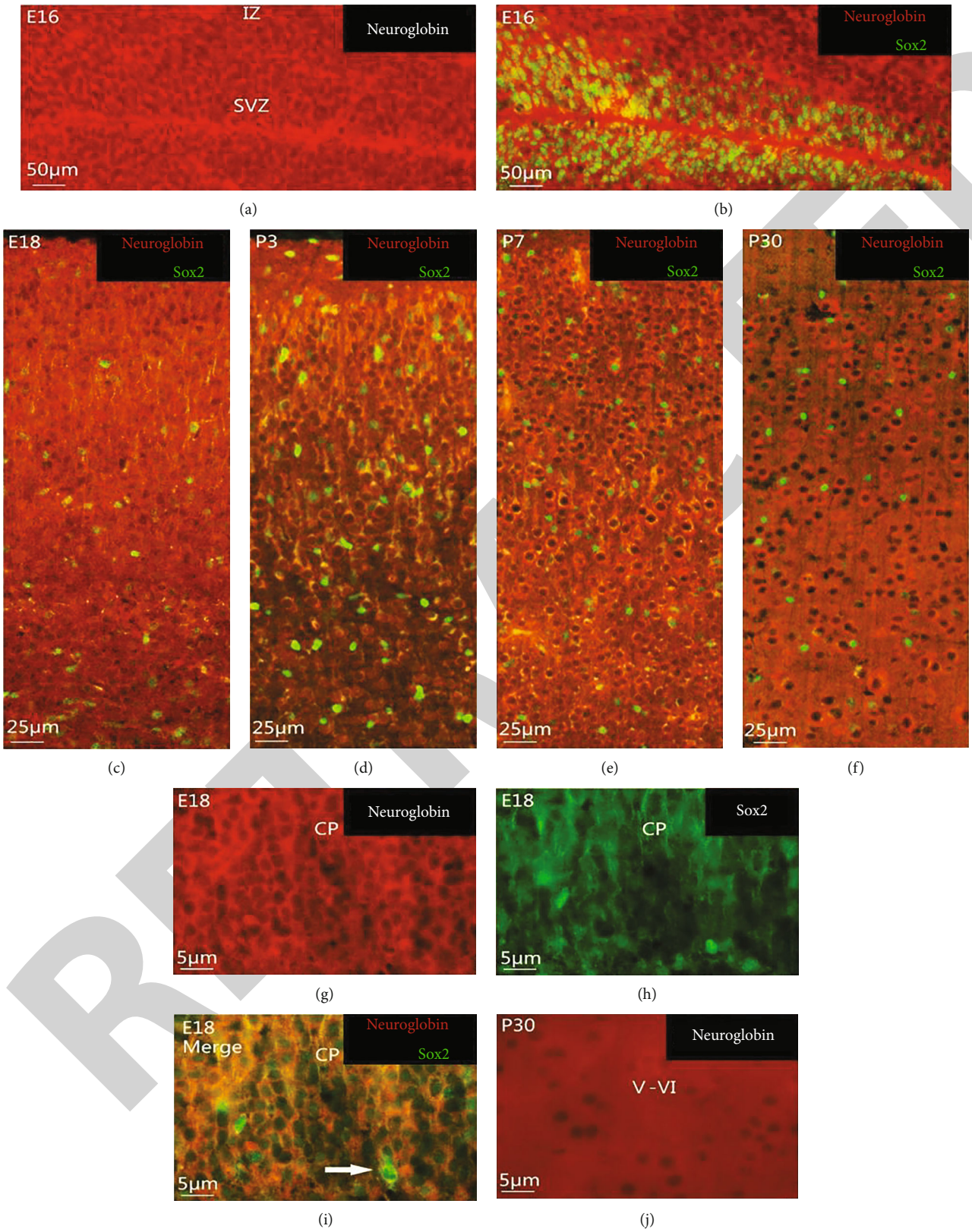
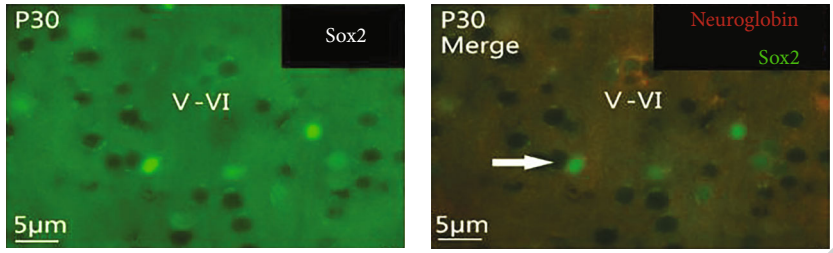


Figure 1: Continued.



(k) (l)

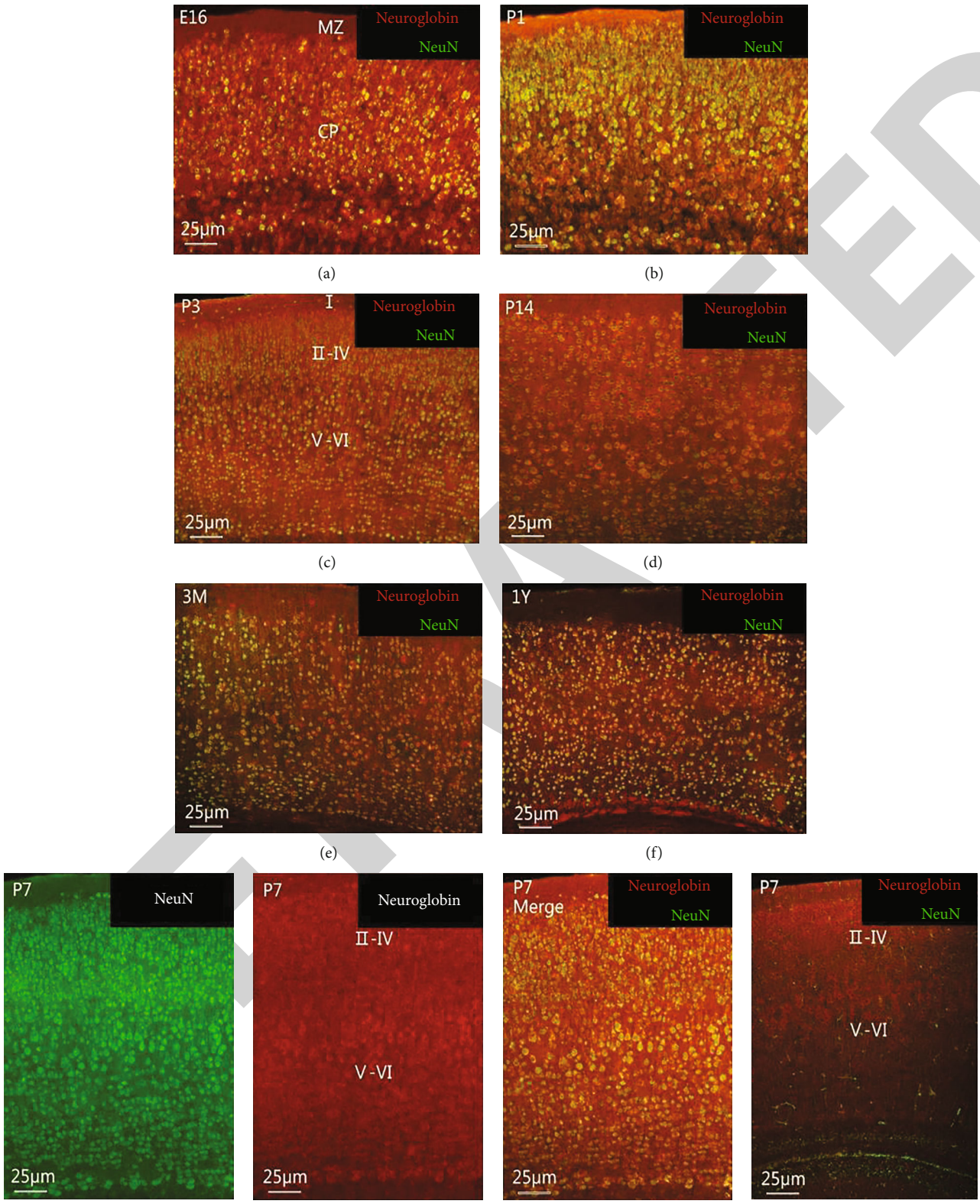
Figure 1: Expression of neuroglobin protein in cortical neural stem cells during neocortical development. (a) Ngb-positive cells (red) were prominently distributed in the cytoplasm of IZ and VZ at E16. (b) Costaining of Ngb (red) with Sox2 (green) demonstrating Ngb colocalized with Sox2 in the cytoplasm of SVZ at E16, supported a potential role of Ngb in NSC migration, proliferation, and neurogenesis. (c) At E18, NSCs were mainly identified in IZ and CP, and Ngb was expressed in almost all NSCs. (d) NSCs increased at P3, and almost NSCs expressed Ngb. (e) At P7, the NSCs continued to migrate upwards widely distributed in the neocortex and nearly half of the NSCs displayed lower or undetectable level of Ngb protein. (f) the NSCs showed little or no Ngb immunostaining at P30. (g–i) Ngb and Sox2 double labeling showed Ngb-positive neural stem cells in CP at E18. (j–l) Ngb-immunoreactive cells were barely observed in the NSCs at P30 in the cortical V-VI layers. Scale bar = 50 μm in (a, b); scale bar = 25 μm in (c–f); scale bar = 5 μm in (g–l).

three independent experiments. The density of Ngb-positive cells were graphed using GraphPad Prism 6.0 (GraphPad Software, USA), and significant differences were determined by one-way ANOVA or fitting curve. The regression equation was calculated with the following formula: $Y = -123.1X3 + 2206.4X2 - 12629X + 24933$ ($R^2 = 0.9945$) in the neocortex II-IV layers, and the formula $Y = 19.921X3 - 285.43X2 + 617.06X + 4518.6$ ($R^2 = 0.8291$) was used in the neocortex V-VI layers. A probability value of $p < 0.05$ was used as the criterion for statistical significance.

3. Results

3.1. Expression of Neuroglobin Protein in Cortical Neural Stem Cells during Neocortex Expansion. To better understand the distribution and development regulation of Ngb in mouse neocortex expansion, we evaluated Ngb expression in cortical neural stem cells. Ngb immunolabeling was detectable in the cytoplasm of intermediate zone (IZ), subventricular zone (SVZ), and ventricular zone (VZ) at E16. Both SVZ and VZ had relatively high levels of neuroglobin-positive cells, while the IZ had lower levels (Figure 1(a)). Meanwhile, we found that Ngb colocalized with Sox2, the marker of neural stem cells, in the cytoplasm of SVZ at E16, supported a potential role of Ngb in NSC migration, proliferation, and neurogenesis (Figure 1(b)). At E18, NSCs could be identified in the cytoplasm of IZ and cortical plate (CP) of the mouse brain, and Ngb expression was readily demonstrable in almost all NSCs (Figures 1(c) and 1(g)–1(i)). Gradually, neural stem cells in the neocortex increased with the birth of the mice, and most of the NSCs expressed Ngb at P3 (Figure 1(d)). At P7, the neural stem cells continued to migrate upwards and were widely distributed in the neocortex. Interestingly, nearly half of the neural stem cells in the neocortex weakly expressed or not expressed Ngb protein (Figure 1(e)). Notably, Ngb-immunoreactive cells were barely observed in the NSCs at P30 (Figures 1(f) and 1(j)–1(l)). In brief, there was a strong correlation between Ngb immunoreactivity and NSCs. Ngb immunoreactivity was detectable in the cytoplasm of NSCs in the SVZ of the brain neocortex at E16, and Ngb levels were the highest in the NSCs at E18. Then, the number of Ngb-positive neural stem cells dropped gradually in the process of upmigration of the neocortex from the ependymal layer. Eventually, the NSCs showed little or no Ngb immunostaining at P30.

3.2. Expression of Neuroglobin Protein in Neurons during the Development of Cerebral Cortical. In the present study, Ngb labeling combined with NeuN was applied to observe the changes of Ngb-positive neurons during cerebral cortical development. At E16, the majority of Ngb-positive neurons were presented in the cortex plate and subcortical plate and were less expressed in the shallow cortical, IZ, and other parts of the cerebral cortex (Figure 2(a)). At P1, Ngb-immunoreactive neurons were detectable in all cortical layers, especially highly expressed in laminae V and VI (Figure 2(b)). Apparently, Ngb-positive cells increased in layers II to VI consistent with the migration of the neurons at P3, and it was clear that the Ngb protein was generally expressed in the cytoplasm of the neurons under the oil microscope (Figures 2(c) and 2(k)–2(m)). Because the stratification of the neocortex had basically formed at P7, the boundaries among each stratum were easy to be identified. Specifically, small tight-knit neuronal soma was seen in laminae II to IV, whereas larger neurons were stained in layers V and VI (Figure 2(g)–2(i)). In the meantime, strong Ngb-immunoreactive neurons were detected close to the molecular layer in layer II-IV, while neurons near the deep cortical layer showed moderate Ngb labelling (Figures 2(g)–2(i)). Moreover, in laminar V to VI, Ngb-positive neurons are strongly expressed near the shallow cortical and weakly expressed in the VZ (Figures 2(g)–2(i)). At P14, the laminated structure of the neocortex had fully formed, and Ngb-positive neurons showed cytoplasmic staining in all layers of the neocortex (Figure 2(d)). The lamination of the neocortex has entered its mature stage at P30, and the layers were easy to be distinguished between II-IV and V-VI according to the different sizes of the aligned Ngb-positive neurons.



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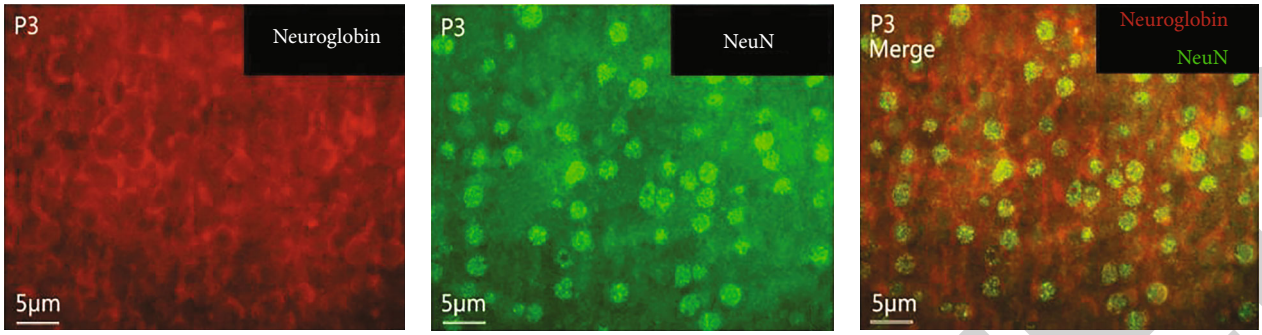


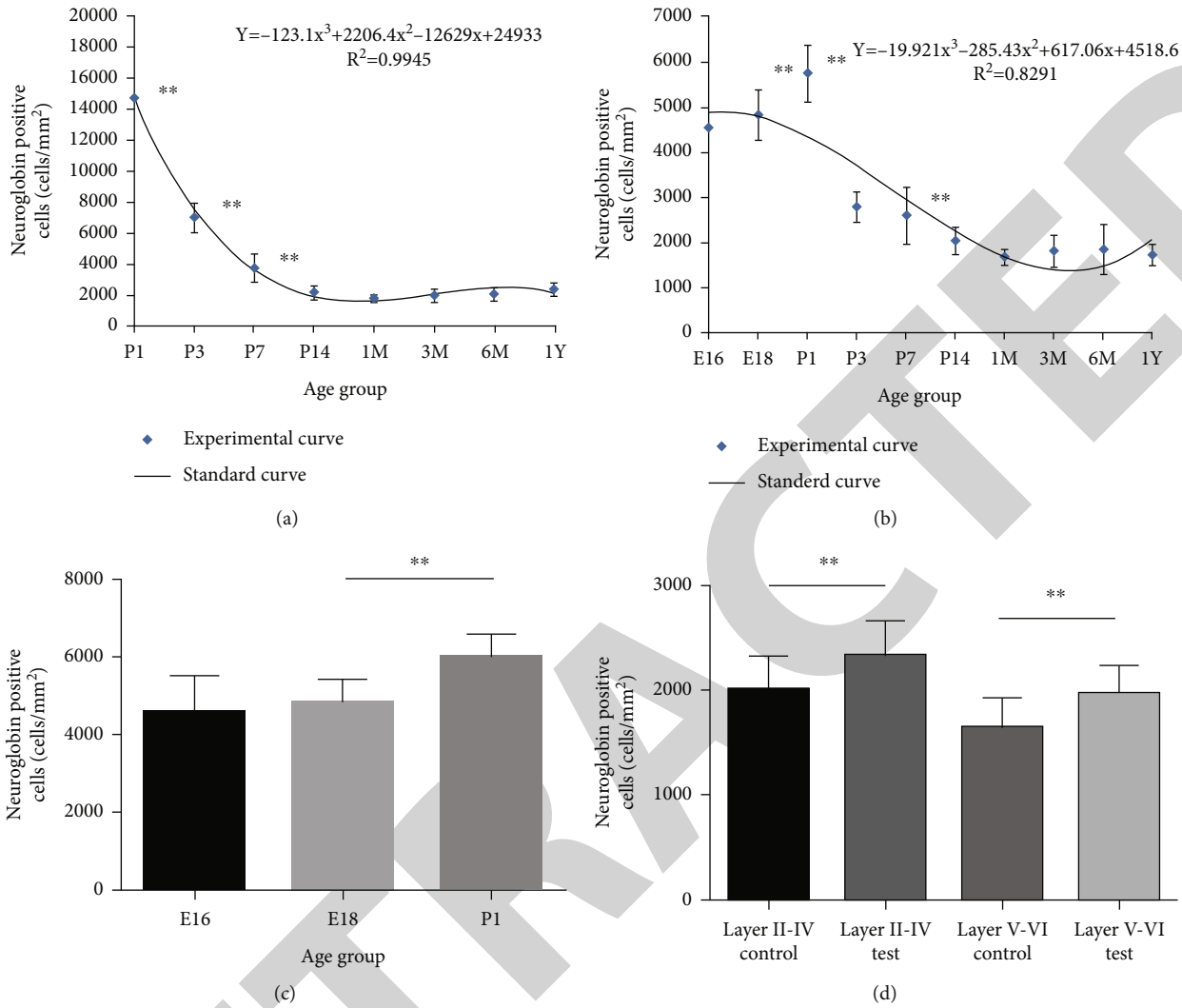
(k) (l) (m)

Figure 2: Expression of neuroglobin protein in neurons during the development of cerebral cortical. (a) Ngb (red) and NeuN (green) immunofluorescence double labeling staining indicated that the majority of Ngb-positive neurons presented in the cortex plate and subcortical plate and less expressed in the shallow cortical, IZ, and other parts of the cerebral cortex at E16. (b) Costaining of Ngb (red) with NeuN (green) demonstrating Ngb-immunoreactive neurons were detectable in all cortical layers, especially highly expressed in laminae V and VI at P1. (c) Ngb-positive neurons were observed in all layers of the neocortex, but Ngb-positive neurons showed lower expression in layer I at P3. (d) At P14, the laminated structure of the neocortex had fully formed. The cell body of Ngb-positive neurons at II to IV layers was smaller and arranged closely, while Ngb-positive neurons were larger at V to VI layers. (e) The lamination of the neocortex has entered its mature stage at 3 months, and Ngb-positive neurons distributed in all six layers. (f) At 1 year, the laminated structure of the neocortex has stabilized, and the expression of Ngb labeled neurons has not changed. (g–i) The stratification of the neocortex had basically formed at P7, and the boundaries among each stratum were easy to be identified. Strong Ngb-immunoreactive neurons were detected close to the molecular layer in layer II-IV, while neurons near the deep cortical layer showed moderate Ngb labelling. In laminar V to VI, Ngb-positive neurons strongly expressed near the shallow cortical, and weakly expressed in the VZ. (j) Costaining of Ngb (red) with Nestin (green) demonstrating similar distribution and expression of Ngb-positive neurons at P7 compared with (g–i). (k–m) The Ngb protein was generally expressed in the cytoplasm of the neurons under the oil microscope at P3. Scale bar = 25 μm in (a–j); scale bar = 5 μm in (k–m).

We also conducted double-immunofluorescence for Ngb and NeuN at P90, P180, and P360 and found that the expression of Ngb labeled neurons has not changed much even though the laminated structure of the neocortex has stabilized (Figures 2(e) and 2(f)).

3.3. Changes of the Ngb-Positive Neuron Density at Different Ages during Cortical Development Process. To get a further understanding of the changes in Ngb-positive neurons during the development of the cortical, samples from different time points at laminae II-IV (during P1 and P360) and at layer V-VI (during E16 and P360), which were easy to be distinguished, were selected to make a comparison. The number of Ngb-positive neurons per unit area in the cortical was measured by using Image-Pro Plus 6.0, while the correlation between each group was analyzed by using GraphPad Prism 6.0. Our present data revealed that, at layers II-IV, the number of Ngb-positive neurons per unit area was the highest at P1 (14665 ± 2983 cells/mm^2). Then, the number continuously dropped to 2108 ± 444 cells/mm^2 at P14, and the density fluctuation of the Ngb-positive neurons tends to a stable level (Figure 3(a)). From birth to P14, the number of Ngb-positive neurons per unit area was remarkably decreased at each time point compared with the previous time point ($P < 0.01$), and the density of Ngb-positive neurons showed no significant difference from P14 to 1 year (Figure 3(a)). Furthermore, we found that, at layers V-VI, the number of Ngb-positive neurons per unit area gradually climbed from E16 and reached the peak at P1 and then declined, till it leveled off after P14 (Figure 3(b)). To be more specific, there was an apparent increased number of the Ngb-positive neurons from E18 to P1 ($P < 0.01$). However, the density of the Ngb-positive neurons displayed a downward trend from P1 to P3 ($P < 0.01$), and the labeled cells decreased rapidly from P7 to P14 ($P < 0.01$). Finally, we found no evidence that the density of the Ngb-positive neurons changed after P14 till 1 year (Figure 3(b)). These results suggest that Ngb protein was highly expressed in neurons of the mouse cortex before birth and was gradually decreased after the birth.

3.4. The Expression of Neuroglobin Protein in Neurons under a Physiological Hypoxia Environment. Previous studies documented that hypoxia played a vital physiological role in embryonic processes [27, 28]. Compared with the partial oxygen pressure conditions after birth, the fetus has to cope with the low oxygen uterine environment during the fetal development [29]. Therefore, various strategic adaptations, such as a rise in heart rate and elevated hemoglobin concentration, were presented by fetuses to guarantee adequate oxygen supply in an oxygen-deficient environment [30]. To determine whether there were any changes of neuroglobin protein levels between prenatal and postnatal of the mice, samples at E16, E18, and P1 were observed by using Ngb and NeuN double-label immunofluorescence staining, and the variation of the Ngb-positive neurons per unit area at layers V-VI was evaluated simultaneously. Present data pointed that the Ngb-positive neurons per unit area showed no significant difference between E16 and E18 at layer V-VI, while the number of double-label cells was increased obviously from E18 to P1 ($P < 0.01$) (Figure 3(c)).



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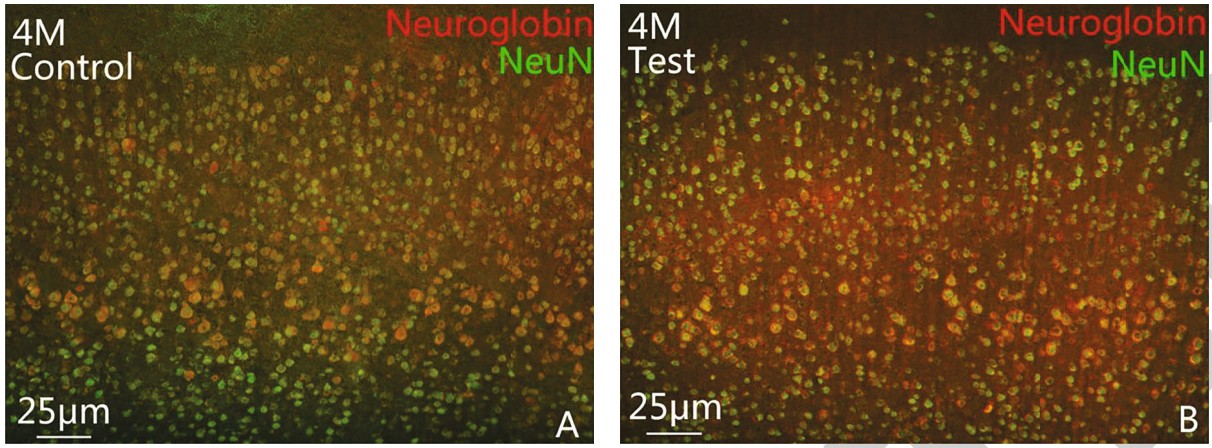

(e) (f)

Figure 3: Changes of neuroglobin positive neuron density at different ages during cortical development. (a) In the neocortex II-IV layers, fitting curve between the neuroglobin positive neuronal density (Y) and age was made; the regression equation was calculated with the following formula: $Y = -123.1X3 + 2206.4X2 - 12629X + 24933$ ($R^2 = 0.9945$). The number of Ngb-positive neurons per unit area was the highest at P1, then, the number continuously dropped at P14, and the density fluctuation of the Ngb-positive neurons tends to a stable level. From P1 to P14, the number of Ngb-positive neurons per unit area was remarkably decreased at each time point compared with the previous time point, and the density of Ngb-positive neurons showed no significant difference from P14 to 1 year ($n = 60$, $^{**}P < 0.01$). (b) In the neocortex V-VI layers, fitting curve between the neuroglobin positive neuronal density (Y) and age was made; the regression equation was calculated with the following formula: $Y = 19.921X3 - 285.43X2 + 617.06X + 4518.6$ ($R^2 = 0.8291$); at layers V-VI, the number of Ngb-positive neurons per unit area gradually climbed from E16 and reached the peak at P1 and then declined, till it leveled off after P14. Compared with E18, the density of neuroglobin positive neurons increased significantly at P1 ($n = 60$, $^{**}P < 0.01$). The density of the Ngb-positive neurons displayed a downward trend from P1 to P3, and the labeled cells decreased rapidly from P7 to P14 ($n = 60$, $^{**}P < 0.01$). Four-month-old Kunming mice were randomly assigned to the control group and hypoxic stress group. The intervention group was subjected to weight-loaded swimming exercise, while the control group was fed conventionally without any intervention. (a) Ngb (red) and NeuN (green) immunofluorescence double labeling staining in mouse neocortex in the control group. (b) The expression of Ngb-positive neurons in mouse cortex under the mild hypoxia stress. (c) The density of Ngb-positive neurons at layers II-IV and V-VI in mild hypoxic stress group presented significant increase compared with the blank group ($^{**}P < 0.01$). Scale bar $= 25\,\mu$m in both (a) and (b). $P < 0.01$.

3.5. Mild Hypoxia Stress Induced the Expression of Ngb-Positive Neurons in the Cortex. Accumulated evidence indicated that hypoxia is not only involved in various normal developmental procedures but also affected different pathological processes [31]. Nevertheless, studies have been shown that Ngb could be activated in adult rat brains in response to hypoxic preconditioning, and inhibition of Ngb expression led to a prominent reduction of neuronal survival after hypoxia [6, 17]. Here, the expression of Ngb-positive neurons in the cortex under the mild hypoxia stress was detected by using double-label immunofluorescence staining. Four-month-old Kunming mice were randomly assigned to the control group and hypoxic stress group. The intervention group was subjected to weight-loaded swimming exercise, while the control group was fed conventionally without any intervention. Our data illustrated that the number of Ngb-positive neurons in cortex showed remarkable increase in the burden swimming exercise group compared with the control group (Figures 3(e) and 3(f)). Similarly, laminae II-IV and layer V-VI were considered the target area, and the statistical difference between the two groups was measured by comparing the number of double-labeled cells per unit area in the cortex. The results demonstrated that the density of Ngb-positive neurons at layers II-IV and V-VI in mild hypoxic stress group presented a significant increase compared with the blank group ($P < 0.01$) (Figure 3(d)).

Data have been shown that both self-renewal and neurogenesis were promoted by mild hypoxic stress [32], and hypoxic preconditioning was beneficial to NSC survival and neurodifferentiation [33]. We considered that Ngb may appear to be a potential marker of neurodifferentiation, exerting neuroprotective effects under mildly hypoxic conditions by reason that it was inducible by HIF-1 and hypoxia [6, 34]. Interestingly, we found that the density of the Ngb-positive neurons under a physiological hypoxia environment (at E18) was lower than at normoxic conditions (P1), and there existed significantly difference ($P < 0.01$) (Figure 4). Similarly, Hummler et al. [35] have mentioned that cerebral Ngb mRNA levels were elevated under acute (8% O_2, 6 h) but not chronic hypoxia (10% O_2, 7 days), which supported a possible explanation that the redox homeostasis was destroyed under physiological hypoxia (similar to chronic hypoxia); then the Ngb reduced due to consumption, self-oxidation or ubiquitination.

4. Discussion

Preservation of an adequate oxygen environment in the brain is essential for the development of cerebral cortex,

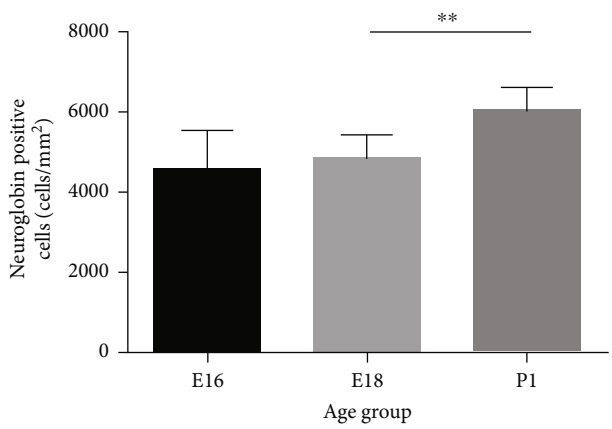

Figure 4: Changes of neuroglobin-positive neurons in cortical V-VI layers before and after birth ($\bar{x} \pm s$, $n = 60$). The Ngb-positive neurons per unit area showed no significant difference between E16 and E18 at layers V-VI, while the number of double-label cells was increased obviously from E18 to P1 ($^{**}P < 0.01$).

and oxygen deficiency is known to cause neuron injury further leading to occurrence of various nervous system diseases [36]. It is well known that neuroglobin shares similar characteristics with hemoglobin that cope with cerebral hypoxia in diving mammals by either facilitating oxygen supply or protecting from reactive oxygen species [1, 37–39]. In the present research, the expression of neuroglobin was studied in much detail on the protein level during cortical development and under mild hypoxic stress, further explaining the neuroprotective role of Ngb in both physiological and exercise hypoxia conditions in mouse corticogenesis.

Although Ngb presents in a wide range of vertebrates, the expression levels are quite different in various types of tissues. Evidence indicated that Ngb was expressed mainly at high oxygen consumptions sites within the retina, such as the inner segments of photoreceptor cells, and the concentration of it in the retina was much higher than in the brain due to the huge oxygen demands in the retina [40, 41]. Furthermore, Ngb transcripts could be found in all regions of the brain, but the majority of them were detected in the hypothalamic region of the mouse brain and were contrasted by the lower expressions of Ngb in the hippocampus, cerebral cortex, and cerebellum, which raised the issue of distinct Ngb functions in different expressing areas [5, 16]. Our data illustrated that the number of Ngb-positive neurons per unit area was the highest at P1 and then declined, till it leveled off after P14 at laminae II-IV and layers V-VI, and the density of the Ngb-positive neurons at layers II-IV was higher than that at layers V-VI in the early postnatal days. These findings provided critical information that the oxygen demand reached a peak at P1 during the development of cortex, then along with the descent of oxygen consumption for each layer of the cerebral cortex, the density of the labeled cells was dropped gradually. At P14, the features of six laminas presented completely, so the oxygen demand tends to a stable level, and the number of Ngb-positive neurons seems unchanged after P14. Briefly, the results revealed that Ngb potentially acted as a repository for oxygen and bonded with oxygen at a high partial pressure of oxygen (pO_2), while dissociated with oxygen at a lower level of pO_2, maintaining the normal cell functions in the process of cortex development.

Previously, some QPCR and Western blot data indirectly showed that low-level Ngb presented in early stages of mouse cerebral development, and steady ascent from E19 to P1 and further on to adults subsequently [16, 35]. Additionally, Greenberg et al. [42] have proved that Ngb is expressed early in both human embryonic stem cells (hESCs) and SVZ neuronal precursors in the course of neuronal differentiation. Here, we found that Ngb protein showed the highest expression levels in the cytoplasm of cortical NSCs at E18, and was negatively presented in cortical NSCs at P30. Meanwhile, consistent with the radial migration of the neurons, Ngb labeled cells increased in layers II to VI that at P3, supporting the idea that the Ngb levels were associated to maturational stage of the neocortex and migration of the neurons [35, 43]. Of note, our findings indicated that strong Ngb-immunoreactive neurons were detected close to the molecular layer in layers II-IV, while neurons near the deep cortical layer showed moderate Ngb labeling. Moreover, Ngb-positive neurons strongly are expressed near the shallow cortical and weakly expressed in the VZ in laminar V to VI. This phenomenon revealed that Ngb may be involved in the oxygen supplying and consuming process during the migration of neurons. Videlicet, the Ngb-immunoreactive cells were expressed abundantly in primary neurons; however, the oxygen was consumed during the migration of the neurons, leading to the downregulation of the Ngb protein in the neuronal radial migration process.

5. Conclusion

In conclusion, the increased density of Ngb-positive neurons in the cortex under mild hypoxic stress might be related to the neuroprotective functions of the Ngb in oxygen deficiency conditions. Our results suggest that neuroglobin behaves as a compensatory protein regulating oxygen provision in the process of neocortical development or under physiological hypoxia. And it is significant for us to obtain the discovery of novel therapeutic methods for neurological disorders. However, our study still has some limitations including limited data. Future works need to collect more data and make a more thorough analysis.

Abbreviation

Ngb: Neuroglobin
NSCs: Neural stem cells
IZ: Intermediate zone
SVZ: Subventricular zone
VZ: Ventricular zone
CP: Cortical plate.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding authors on reasonable request.

Conflicts of Interest

All authors claim that there are no conflicts of interest.

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Research Article

The Role of Methyl-(Z)-11-tetradecenoate Acid from the Bacterial Membrane Lipid Composition in *Escherichia coli* Antibiotic Resistance

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Background. The bacterial membrane plays a critical role in the survival of bacteria and the effectiveness of antimicrobial peptides in protecting the host. The lipid constituents of the bacterial membrane are not evenly distributed, and they could be affected by clustering anionic lipids with cationic peptides with multiple positive charges. That could be harmful to bacteria because it prevents lipids from interacting with other molecular components of the cell membrane, disrupts existing natural domains, or creates phase boundary defects between the clustered lipids and the bulk of the membrane. This preliminary quantitative study is aimed at assembling a correlation between antibiotic resistance and bacterial lipid composition in *E. coli*, based on the function and arrangement of the bilipid coating of the bacterial cell, intimately associated with the path of antimicrobials through membranes. *Methods*. Fifteen multiresistant *E. coli* samples are collected from swine with enterocolitis tested for resistance levels using the disc diffusimetric method (Kirby-Bauer disc diffusion). Pathogen identification completed using the API 20E multitest system revealed the *E. coli* presence in 11 samples. In these samples, bacterial membrane detection of fatty acid methyl esters (FAME) operating a 240 MS Ion Trap (Varian) GC/MS (Agilent Technologies, Santa Clara, CA, USA) was performed, using the MIDI Sherlock recognition software model. *Results*. Interpreting the descriptive statistical method, the correlation matrix, and regression curves and after ANOVA analysis, we ascertained that the studied *E. coli* population statistically confirmed different degrees of resistance in most of the samples analyzed in this test. *Conclusions*. In one case, the methyl-(Z)-11-tetradecenoate acid was observed to have a relationship with the susceptibility evaluation by using the disc diffusimetric method, which has revealed the lowest rate of antimicrobial resistance, so it has importance in further resistance evaluation studies.

1. Introduction

Antimicrobial resistance (AMR) is the capability of microorganisms to adapt to antimicrobials, mainly antibiotics. Exaggerated and inappropriate use of antimicrobials and inefficient infection management approaches resulted in AMR being a serious global public health menace. Databases and supervision systems from both human and veterinary sectors are becoming increasingly abundant in data since. As of today, resistance is registered for almost all antibiotics [1, 2].

Though antimicrobials have revised medical practices today, this benefit is a distinct threat due to the intense or inappropriate antibiotic administration. The irresponsible use of antibiotics has expanded the occurrence and spread of multidrug-resistant bacteria driving the optimization of veterinary antimicrobial treatment as a crucial concern. Along with antibiotics employed in human therapy, their

use to cure infections or for prophylactic treatments in animals has led to pressure on the emergence and quick spread of resistant bacterial strains [3]. Recent studies described that faulty antimicrobial use in slaughterhouse animals could influence the health of farmworkers and employees from meat processing units or the final consumer health. Why is resistance spreading so quickly, and what are their intimate/specific resistance mechanisms? They are not fully established yet [3].

Animals can operate as intermediates, pools, and disseminators of resistant bacterial strains or genes. The alarming augment in reports about multidrug-resistant (MDR) bacteria, combined with the decline in new drug approvals, is indisputable that this reduces the supply of clinical treatment options [4–7]. In these conditions, attempts to manage bacterial infections remain challenging, spoiling the efficacy of infectious diseases' treatments. As a result, the need for renewed infection control strategies is becoming crucial [6, 7].

The bacterial membrane plays a critical role in the survival of bacteria and the effectiveness of antimicrobial peptides in protecting the host, and Gram-negative bacteria's outer membrane has an additional layer of protection [8, 9].

The outer leaflet of these bacteria's outer membrane is composed mainly of lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria localized in the outer layer of the membrane. Phosphatidylglycerol (PG), phosphatidylethanolamine (PE), and cardiolipin are the main lipid elements of the inner monolayer of Gram-negative bacteria's outer membrane, as well as both monolayers of their cell membranes (CL). The bacterial species vary considerably in the proportions of these three lipids [10–13]. Also, certain bacterial species include significant quantities of glycosyl-diglycerides and a mixture of other minor lipid components [10–13]. Numerous antimicrobial agents exert their activity by interfering with the synthesis of mature LPS at different stages. This action enhances the susceptibility of these bacteria to destruction by making the outer membrane more permeable, driving this additional protective layer inefficient. Surprisingly, there are some examples where the antimicrobial agents avert solutes from passing through the outer membrane of Gram-negative bacteria, resulting in their lethality [14]. The lipid components of biological membranes are not distributed equally throughout the membrane but supplemented in specific domains, and this dispersal is altered by clustering anionic lipids with cationic peptides with multiple positive charges [15–17]. This could be harmful to bacteria, since it prevents lipids from interacting with other molecular components of the cell membrane, disrupts existing natural domains, or creates phase boundary defects between the clustered lipids and the bulk of the membrane [18].

The lipid composition of bacterial membranes differs significantly between species [19]. Among these unknowns, the fatty acid function in Gram-negative bacteria membrane is not completely understood yet, though the studies demonstrate that these lipid configurations may impact the penetration or binding of antimicrobials. For example, tetracycline [20, 21] is dependent on membrane permeability, penicillin, and quinolones on high lipid content [22–24]. Furthermore, the viability of Gram-negative bacteria is associated with the integrity of the cell membrane, and lipids could play a role in their structure and function due to the quantitative differences in fatty acids. For this reason, researchers have developed comprehensive investigations in this direction [25, 26].

Table 1: Sample preparation and reagents used.

Phase	Substances used
Saponification	Reagent 1: (i) 45 g sodium hydroxide (ii) 150 mL methanol (iii) 150 mL distilled water
Methylation	Reagent 2: (i) 325 mL clorhidric acid 6.0 N (ii) 275 mL methanol
Extraction	Reagent 3: (i) 200 mL hexane (ii) 200 mL methyl tert-butyl-ether (MTBE)
Rinse	Reagent 4: (i) 10.8 g sodium hydroxide (ii) 900 mL distilled water

Table 2: The antibiotic susceptibility results according to CLSI 2018 [29].

Crt. No.	Sample no./ antibiotic tested	1	2	3	4	5	6	7	8	9	10	11
1.	Amoxicillin	R	R	R	R	R	R	R	R	R	R	R
2.	Gentamycin	S	S	S	S	S	S	S	S	S	S	S
3.	Florfenicol	R	S	R	S	R	S	R	R	R	R	R
4.	Oxacillin	S	R	R	R	R	S	S	S	S	S	S
5.	Cephalothin	R	R	R	R	R	R	R	R	R	R	R
6.	Spectinomycin	R	R	R	R	R	S	R	R	R	R	S
7.	Norfloxacin	R	S	S	S	S	S	R	R	S	R	S
8.	Tiamulin	S	S	S	S	S	S	S	S	R	S	S
9.	Ciprofloxacin	S	S	R	R	R	S	R	S	R	S	R
10.	Penicillin	R	S	R	R	R	S	S	R	R	R	S
11.	Tetracycline	R	R	R	R	R	S	S	R	S	R	R

Note: R: resistant; S: sensitive.

Scientific reports have presented diverse techniques for differentiating fatty acids with a length of 9-20 carbons. Fatty acids with a short chain were found in Gram-negative bacteria, and those with a branched chain were found in Gram-positive bacteria. These techniques help identify and classify them. The microorganisms are isolated and cultured on selective media, in temperature and humidity optimal conditions, and subjected to tests to highlight fatty acids in their bacterial membrane [27].

The present study tries to assemble an initial qualitative correlation between antibiotic resistance and bacterial lipid composition in *E. coli*. The function and structure of the bilipid coating of the bacterial cell are intimately related to the passage of antimicrobials through membranes. To reach the proposed goals, this was followed:

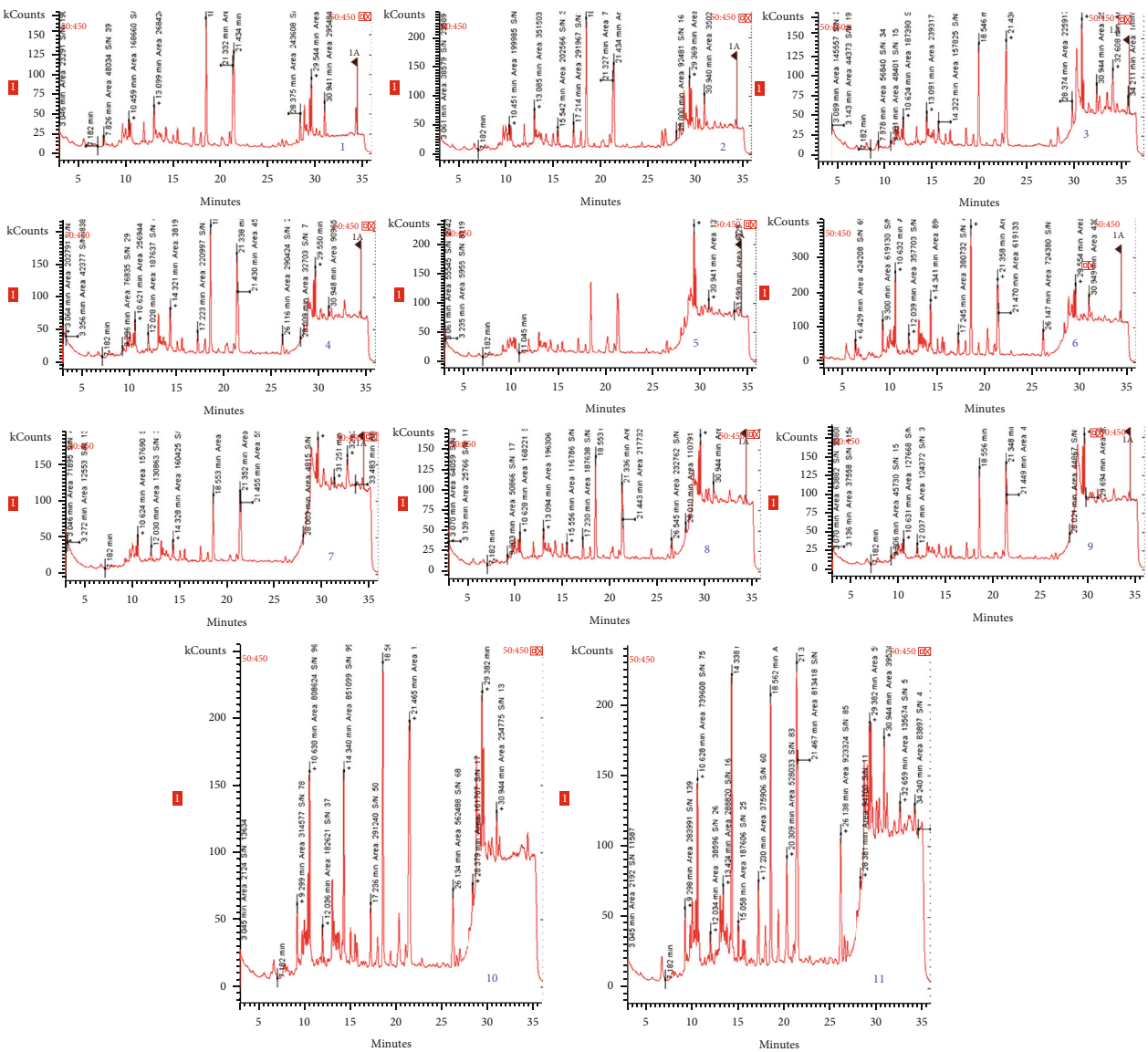

Figure 1: The chromatograms of the identified fatty acids.

(i) Simple identification of the pathogen using the API 20E multitest system

(ii) Setting the level of resistance by the disc diffusimetric method

(iii) Ascertaining the fatty acids in the bacterial membrane by gas chromatography detection of fatty acid methyl esters (GC-FAME)

(iv) Assembling all possible statistical correlations

To our knowledge, this is a prime initial study made in Romania on *Enterobacteriaceae*.

2. Materials and Methods

All procedures used in this study by providing clinical samples and written informed consent have the approval of the Ethical Committee of USAMVB Timișoara (Ethical Committee Approval no. 164/05.12.2019).

2.1. Location and Sample Collection for AST. For this research, large capacity swine exploiting units from Arad County, Western Romania, have been included, where clinical cases were diverse, and the identified incidence of colibacillary infections was high. The examination was completed on biological material from pure *E. coli* cultures (of maximum 20 mg), gathered directly from the fresh intestinal contents of swine.

2.2. Bacterial Isolation and Microbial Testing. During a two-year period, a total of 15 *E. coli* strains from the level of the small intestine of pigs having acute diarrheal diseases were recovered. The bacteria isolation was made, following the standard protocol within the bacterial determinations. MacConkey (MAC; Merck, Darmstadt, Germany) agar specific

Table 3: Quantification of results and highlighting of fatty acids in the bacterial membrane.

Retention time (min.)	Identified compound	No. of collection dial (chromatogram)										
		1	2	3	4	5	6	7	8	9	10	11
8.2	Decanoic acid (methyl ester)	−	+	−	−	+	−	−	−	−	−	−
10.3	4-Octadecenal	−	−	−	−	−	+	−	−	−	−	−
10.6	Methyl-10-methyl undecanoate	−	+	+	+	+	+	+	+	+	+	+
13.8	Methyl-(Z)-11-tetradecenoate	−	−	−	−	−	+	−	−	−	−	−
14.3	Methyl-iso-myristate	+	+	+	+	+	+	+	+	+	−	+
15.7	Methyl-(Z)-11-tetradecenoate	−	−	−	−	−	−	−	−	−	+	+
17.2	I-propyl-tetradecanoate	+	+	+	+	+	+	+	+	+	+	+
20.1	Methyl-palmitoleate	+	+	+	+	+	−	−	−	+	+	−
20.2	Methyl-hexadec-9-enoate	−	+	−	−	−	+	+	−	−	−	+
21.3	Methyl-14-methyl-pentadecanoate	−	−	−	+	−	+	+	+	−	−	+
21.4	Methyl-3-(3.5-di-tert-butil-4-hidroxy-phenyl) propionate	+	−	−	−	−	−	−	−	−	−	−
26.0	Methyl-8-heptadecenoate	−	+	−	−	−	−	−	−	+	−	−
26.1	Methyl-9,10-metylene-hexadecanoate	−	−	+	−	−	+	+	−	−	+	+
26.1	Cis-10-heptadecenoic-acid (methyl ester)	+	−	−	+	+	−	−	−	−	−	−
29.0	Methyl-(10E)-10-octadecenoate	−	−	−	+	−	−	−	−	−	−	−
29.0	Methyl-11-octadecenoate	+	−	−	−	+	+	−	−	−	−	−
29.0	Methyl-trans-8-octadecenoate	−	+	−	−	−	−	−	−	−	−	−
29.1	Methyl-13-octadecenoate	−	−	−	−	−	−	−	−	−	−	+
29.5	Methyl-16-methyl-heptadecanoate	−	−	+	+	+	−	+	−	+	−	−
29.5	Methyl-14-methyl-heptadecanoate	−	+	−	−	−	+	−	−	−	+	+
30.4	Decanoic acid (decyl ester)	+	+	−	−	−	+	−	+	−	+	+
31.2	Methyl-dihydrosterculate	−	−	−	−	−	−	−	−	−	+	−
31.2	Methyl-9,10-metilene-octadecanoate	−	−	−	−	−	−	−	−	−	−	+

was used to develop *E. coli* strains. The lactose-fermenting (pink) *E. coli* colonies were subsequently selected and cultured on triple sugar iron (TSI; Oxoid, Basingstoke, UK) slopes and tryptone soy agar (TSA, Oxoid, Basingstoke, UK). In addition, the presumptive *E. coli* colonies were Gram-stained and cultured on TSA agar to be identified based on their biochemical properties with the API 20E typing system (BioMérieux, Marcy L'Etoile, France).

The ATCC 25922 *E. coli* was used as a control strain, with whole bacterial strains maintained on nutrient agar. From the collected samples, the bacterial resistance of the 15 isolates was initially tested. The susceptibility was tested through the Kirby-Bauer disc diffusion standardized technique, completed by measuring the diameter of the growth inhibition zone. The strains were classified as resistant or sensitive to the drug, according to the current interpretation standards presented in the Clinical Laboratory Standards Institute (CLSI), Performance Standards for Antimicrobial Disc Susceptibility Tests [28–30].

2.3. Analytical Profile Index (API). For the biochemical characterization of *E. coli* was used the API 20E kit (BioMérieux, Marcy L'Etoile, France). Twenty plastic tubes (cups) containing dehydrated reagents comprise the test strip. After 16-24 hours of culture with a bacterial solution, the bacterial metabolites change the color of the domes, allowing the identification of different bacterial species. The six reagents included in this test are glucose (GLU), D-mannitol (MAN), inositol (INO), D-sorbitol (SOR), D-sucrose (SAC), and amygdalin (AMY), and each contains a pH indicator. We have used these six reagents since the *Enterobacteriaceae* have a common biochemical metabolic pathway known as mixed acid fermentation, specific for the metabolism and breakdown of sugars, such as glucose, and the production of various acidic products that can cause the reagents to change color due to the acid-base indicator [4, 31].

Compared to the classical methods, the use of multitest identification systems has benefits: receiving rapid results, in some circumstances even within 5 hours; uniformity of results; precision/safety of results; simplicity of work procedures; and lowest consumption of materials and culture media [4, 31]. The final evaluation involved the registration of positive (+) or negative (-) results and the species identification completed by consulting the Index supplied by the manufacturer. Of the 15 samples investigated, in 11 cases, *Escherichia coli* were present, and consecutive confirmation of the results was identified in resistant and sensitive species to antibiotics investigated. Specimens were tested to determine the fatty acids in their bacterial membranes, using the GC-MS technique with numerous methyl ester compounds identified.

Table 4: Descriptive statistic correlation between the evolution of resistance and the fatty acid presence/absence in the bacterial membrane of the studied samples.

(a)

Sample no.	Sensitive no.	Resistant no.	Fatty acid no.
1	4	7	7
2	6	5	10
3	3	8	6
4	4	7	8
5	3	8	8
6	9	2	11
7	5	6	7
8	4	7	5
9	4	7	6
10	4	7	8
11	6	5	11
Mean	4.727272727	6.272727273	7.909090909
Standard error	0.523813101	0.523813101	0.609836721
Median	4	7	8
Mode	4	7	8
Standard deviation	1.737291518	1.737291518	2.022599587
Sample variance	3.018181818	3.018181818	4.090909091
Kurtosis	3.033156723	3.033156723	-0.866962963
Skewness	1.629735293	-1.629735293	0.416039579
Range	6	6	6
Minimum	3	2	5
Maximum	9	8	11
Sum	52	69	87
Count	11	11	11
Confidence level (95.0%)	1.167128323	1.167128323	1.358800892

(b)

Sensitive		Resistant		Fatty acids	
Lower	Higher	Lower	Higher	Lower	Higher
4.7273-1.1671	17273 + 1.1671	6.2727 − 1.1671	6.2727 + 1.1671	7.9091 − 1.3588	7.9091 + 1.3588
3.560144	5.894401	5.105599	7.439856	6.55029	9.267892

2.4. GC-MS Methodology and the Study of Bacterial Membrane Fatty Acids. Short-chain fatty acids typically identify the anaerobic bacteria, especially the acids having between 9 and 20 carbon bonds [27]. With gas chromatography, the fatty acid methyl esters (GC-FAME) could be determined. The richness of information contained in these compounds was estimated, considering the presence/absence of each acid and the acid fluctuation existing associated with the development of antimicrobial resistance [27, 32].

Bacterial growth conditions can influence the accumulation of fatty acids in membranes. Quantitative and qualitative fluctuations were reported, depending on the incubation temperature and the growth media used. For these variables minimization, using a TSA medium (Trypticase Soy Agar, Sigma-Aldrich, Darmstadt, Germany) incubated at 28°C or a TSBA (Trypticase Soy Blood Agar, Sigma-Aldrich, Darmstadt, Germany) at 35°C for 24 hours is recommended.

The mass spectrometry (MS) used a 240 MS Ion Trap (Varian, Santa Clara, CA, USA) mass spectrometer, with the ionization conditions set as follows: ion trap temperature at 170°C and the transfer line temperature at 230°C using helium as mobile phase. The EI mode at 70 eV was used for ionization, and the compounds' nominal mass recording used the full spectra. Each standard was injected separately to check if the fragmentation data matched the database information. Fragmentation patterns were extracted from

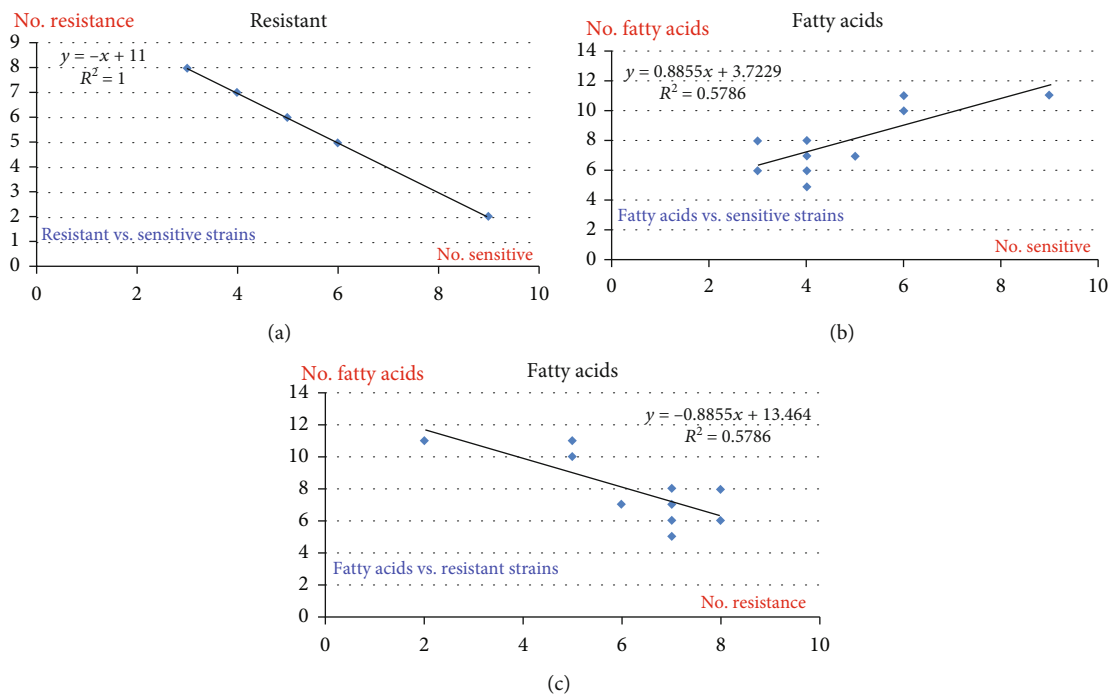

Figure 2: The descriptive statistic correlation between (a) the fatty acids vs. sensitive strains and (c) fatty acids vs. resistant strain presence/absence in the bacterial membrane, where a high significance correlation was registered for (b, c).

the identified peaks and referred to the spectral database (Wiley Registry/NIST 2020 Library/12th Edition) for the confirmation of the identity of the separated compounds.

Chromatography was performed on 450 GC Varian (Varian, Santa Clara, CA, USA) using hydrogen as carrier gas at a constant flow rate of 1.5 mL/minute and a linear velocity of 50.1 cm/sec. For qualitative analysis, a column BR-5ms (30 m × 0.25 mmID, 0.25 μm film) (Brucker Daltonics, MA, USA) was maintained at a temperature of 100°C, 1 min, raised to 300°C with a rate of 10°C/min, held for 2 min. The set injection pool's temperature was 230°C. Samples were injected at a volume of 1 μL using a split ratio for 5 min, followed by splitless mode for 10 min [27, 33, 34].

As the GC-FAME analysis is used to identify bacterial species, the fatty acid profile is a certain indication of these species. This is why we designed this study observing the fatty acid profile composition of *E. coli* in strains that are and are not resistant to the same antibiotic considering that any registered modifications are associated with the resistance presence/absence [27, 33, 34].

For these determinations, four preliminary operations were made as shown in Table 1.

3. Results

The investigation of swine samples, confirming the failure of antibiotic therapy, started with the pathogen's identification using the API methodology (API kit 20E, Biomerieux, Marcy L'Etoile, France). The disc diffusimetric method followed the CLSI 2018 instructions to test bacterial susceptibility [28, 29].

Table 2 presents the interpretation of antibiotic susceptibility.

Gas chromatography identified fatty acids extracted with the help of methanol. The obtained chromatograms are presented in Figure 1.

Results of the 11 chromatograms (collection dials) qualification and the highlighting of the fatty acids in the bacterial membrane of *E. coli* depending on the retention time are presented in Table 3.

3.1. Statistic Assays and Correlation

3.1.1. Descriptive Statistics. Analyzing after the descriptive statistic correlation between matrix and regression curves had shown that the *E. coli* population studied statistically confirmed different degrees of resistance in most of the samples analyzed (Table 4 and Figure 2).

Figure 2 presents the descriptive statistic correlation between (a) fatty acids vs. sensitive strains and, respectively, (c) fatty acids vs. resistant strain presence/absence in the bacterial membrane, where a high significance correlation has been registered for b and c.

3.1.2. ANOVA. In addition, to ensure the accuracy of the results, they were statistically analyzed also by the bidirectional ANOVA method, using the GraphPad Prism 6.0 program for Windows (GraphPad Software, San Diego, USA). Statistical values were expressed as the mean ± SEM (standard error), where $*$ means $0.01 \leq p < 0.05$, significant; $**$ means $0.001 \leq p < 0.01$, very significant; and $***$ means $p < 0.001$, extremely significant (Table 5).

Table 5: The ANOVA statistic results.

(a)

ANOVA	SS	dF	MS	F (DFn.DFd)	p value/significance
Treatment (between columns)	55.70	2	27.85	$F(2.30) = 8.25$	
Residual (within columns)	101.30	30	3.37		$p = 0.0014$
Total	157	32	31.22		
ANOVA summary					
F					8.25
p value					0.0014
p value summary					**
Are differences among means statistically significant? ($p < 0.05$)					Yes
R square					0.3548

(b)

Parameter	Fatty acids vs. sensitive	Fatty acids vs. resistant
Pearson r		
r	0.7606	-0.7606
95% confidence interval	0.2955 to 0.9343	-0.9343 to -0.2955
R square	0.5786	0.5786
p value		
p (two-tailed)	0.0066	0.0066
p value summary	**	**
Significant? (alpha = 0.05)	Yes	Yes
Number of XY pairs	11	11

(c)

Summary Groups	Count	Sum	Average	Variance
Sensitive	11	52	4.727272727	3.018182
Resistant	11	69	6.272727273	3.018182
Fatty acids	11	87	7.909090909	4.090909

(d) The matrix of statistical correlation

Category	Sensitive	Resistant	Fatty acids
Sensitive	1	-1	0.760627515
Resistant	-1	1	-0.760627515
Fatty acids	0.760627515	-0.760627515	1

The obtained statistical values were highly significant, and the matrix of statistical correlation for fatty acids vs. sensitive strains' and fatty acids vs. resistant strains' presence/absence in the bacterial membrane confirmed the descriptive statistic correlation made for b and c.

4. Discussion

The last decade had shown that there is an urgent need for new antibiotics against microbial infections. The incidence of microbial resistance to traditional antibiotics has advanced continuously [16, 35], and in this case, the bacterial cell membrane is one of the possible targets of new antibiotics that may be effective against resistant bacteria [36, 37].

A recent ample analysis highlighted the antimicrobial peptides' characteristics that interact with bacterial membranes [38, 39]. The assessment of the portion of the fatty acids, compared to the sensitivity rate of the antibiotics used in present study, was higher in phenotype S, compared to phenotype R, a fact also found by other researchers. They identified significant differences in terms of the quantitative

fatty acid composition of sensitive versus resistant strains [25, 36, 37].

Gram-positive bacteria membranes are fundamentally different in their molecular composition and morphology from Gram-negative bacteria membranes. Gram-negative bacteria are encased in two membranes: the cytoplasmic and outer membranes. The outer monolayer of the membrane is primarily composed of LPS, a lipid species found only in Gram-negative bacteria [40, 41].

The most notable fact in the present research was that it confirmed the proportional reductions in acids in the resistant strains, compared to the sensitive strains of *E. coli*. It is known that antibiotics from the β-lactam category are targeting penicillin-binding membrane proteins in the cell wall. They are hydrophilic and cross the outer membrane with the help of porins through a process mediated by the physicochemical properties of the molecule, and quinolones focus on cytoplasmic enzymes (DNA gyrase and topoisomerase IV), so these substances must cross the cell wall to be effective [3, 25, 42]. For these antimicrobials, the resistance is correlated with the outer membrane diffusion channel descent through the porins and oversizing of the efflux pumps [23, 24].

Like other authors, our results fall in that fatty acids can influence the permeability of antibiotic molecules by acting directly on transmembrane proteins or collectively by impacting the fluidity, size of the bilipidic layer, and the shape they can give to the membrane [3, 26, 42].

The activity of the used antibiotics was not directly correlated to the metabolism of fatty acids but may indirectly affect the membrane permeability, contributing to the development of antibiotic resistance [43, 44].

It is acknowledged that, at high levels of saturated fatty acids, the fluidity of the membrane decreases. This decrease has been reported by authors in the case of exposure to subinhibitory concentrations of quinolones and cephalosporins in *E. coli* [45–47].

Variation in cyclopropane fatty acids is a response to antibiotic exposure of tetracycline-resistant strains of *E. coli* so that the amount of cyclopropane fatty acids decreases and the concentration of unsaturated fatty acids increases compared to susceptible phenotypes [22]. Although changes in the composition of fatty acids in the bacterial membrane reflect the differences in phenotype in terms of antimicrobial susceptibility, their interactions can be hard to interpret, and relatively, little is known yet about these interferences [9].

In this respect, even though this study is an initial and a qualitative investigation, it brings new data and can generate the following in what means multifaceted analysis of qualitative interrelations between the bacterial membrane's fatty acid presence/absence vs. sensitive/resistant *E. coli* strains.

5. Conclusions

The presence of methyl-(Z)-11-tetradecenoate acid ascertained was in positive connection (where $p < 0.01$) with the susceptibility of *E coli*; this fatty acid was not found in the resistant *E. coli* strains identified. This allows us to affirm that the bacterial fatty acid amount and composition could reflect the differences in antimicrobial susceptibility.

In order to elucidate all interactions, more research would be opportune to continue this initial study, by observing the fatty acids' structure variation in the bacterial membranes and the link with the antibiotics' efficiency, with valuable outcomes in the combat against antibioresistance.

Data Availability

All data is contained within the article.

Disclosure

This paper is in the frame of COST Action CA18217-European Network for Optimization of Veterinary Antimicrobial Treatment.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Conceptualization was performed by R.T.C. and A.O.D.; methodology was performed by D.C.V., R.P., A.C., D.C.B., and J.D.; software was prepared by M.B., E.D., and F.M.; validation was performed by R.T.C.; formal analysis was performed by M.B., E.D., and K.I.; investigation was performed by J.D., R.P., A.C., and D.C.B.; resources were prepared by R.T.C. and I.K.; data curation was performed by R.T.C., E.D., D.C.V., and A.O.D.; writing (original draft preparation) was performed by R.T.C. and A.O.D.; writing (review and editing) was performed by R.T.C. and J.D.; visualization and supervision were performed by R.T.C. and F.M.; project administration and funding acquisition were performed by R.T.C. All authors have read and agreed to the published version of the manuscript.

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Review Article

Klebsiella pneumonia and Its Antibiotic Resistance: A Bibliometric Analysis

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The rapid development of antibiotic resistance in K. pneumonia has led to a major concern. In order to analyze the hotspots and develop trends in this field through visual the analysis, this study used CiteSpace software to summarize the available data in the literature to provide insights. A total of 9366 research articles were retrieved from the Web of Science Core Collection, and the number of published papers is increasing year by year. The country with the most articles was the USA, followed by China and India. The institution with the highest number of publications was LERU. The author with the highest number of articles was Li. The journal with the highest citation rate was Antimicrobial Agents and Chemotherapy. In addition, based on keyword coword analysis and cited literature prominence analysis by CiteSpace, the current research focus in the field was therapy, CRKP, and resistance genes. This paper provides a new quantitative visualization way for the development of the field in the recent ten years. The results show global trends that researchers can use to determine future directions.

1. Introduction

Klebsiella pneumonia, belonging to the family *Enterobacteriaceae*, is a natural inhabitant of the gastrointestinal tract microbiome of healthy humans and animals [1]. It has a variety of antibiotic resistance mechanisms and is a common pathogen causing hospital-acquired surgical wound infections, digestive tract infections, and community-onset infections, which can cause outbreaks of nosocomial infection [2]. The global drug resistance rate of *K. pneumonia* has reached as high as 70%, and the infection-related fatality rate has also reached 40%~70% [3]. In recent years, multiple-drug resistance (MDR) *K. pneumonia* and *carbapenem-resistant Klebsiella pneumonia* (CRKP) have emerged as a major global public health problem.

In this study, we reviewed the literature on antibiotic resistance of *K. pneumonia* by bibliometrics [4]. This study is aimed at analyzing the trend of antibiotic resistance research of *K. pneumonia* in recent 10 years. According to data from the Web of Science Core Collection (WoSCC), the CiteSpace5.8.R3 software system was employed to display the detailed analysis based on visualization of the cooperative network of the countries, coauthors, and co-occurring keywords [5]. The method and results of this paper may provide potential prospects for the study of antibiotic resistance of *K. pneumonia* in the future.

2. Data Collection and Methodology

The data from 2012 to 2021 (10 years) were from WoSCC on April 13, 2022, to reduce bias incurred by database updating. Search terms were used as follows: "(((((((TS=(Antibiotic resistance)) OR TS=(Antibiotic resistant)) OR TS=(Antimicrobial Resistance)) OR TS=(Antimicrobial Resistant)) OR TS=(Drug Resistance)) OR TS=(Drug Resistant)) AND ((TS=(Klebsiella pneumonia)) OR TS=(K. pneumonia))

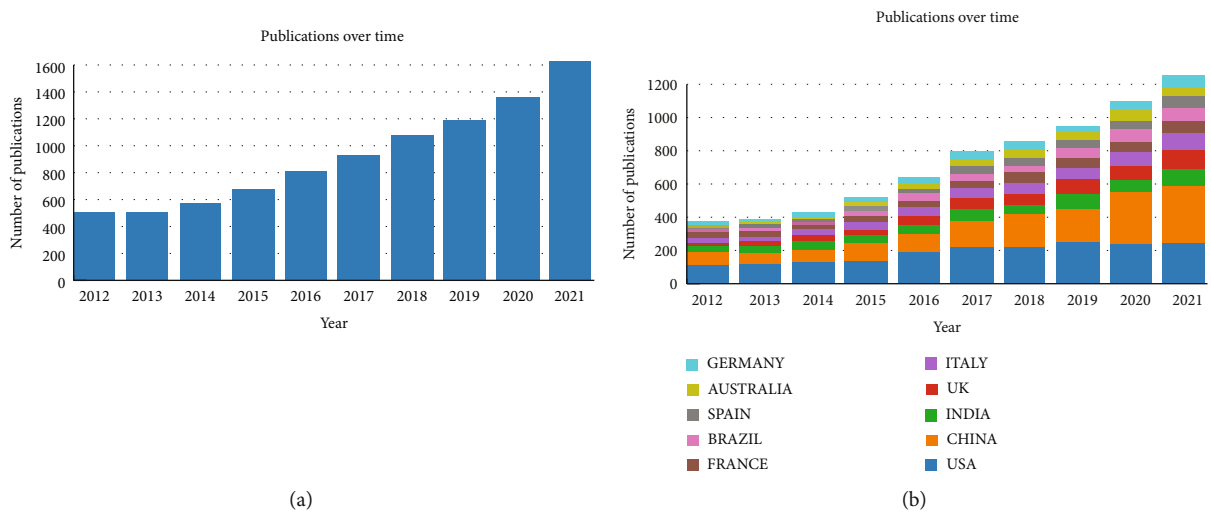


Figure 1: (a) Publication volume and growth trends in each year on antibiotic resistance research of K. pneumonia from 2012 to 2021. (b) Publication volume and growth trends of the top 10 countries/regions on antibiotic resistance research of K. pneumonia from 2012 to 2021. Bar chart reflects number of publications per year.

Table 1: Top 10 most productive and influential countries.

Rank	Countries/regions	Publications	Citations	Average citation per item	*H*-index
1	USA	1908	65125	34.13	109
2	China	1416	24047	16.98	58
3	India	664	12636	19.03	50
4	Italy	550	16284	29.61	58
5	UK	530	21504	40.57	64
6	France	511	17375	34	61
7	Brazil	424	6720	15.85	38
8	Spain	397	11076	27.9	49
9	Australia	366	14155	38.67	60
10	Germany	360	9481	26.34	50

AND (EDN==("WOS.SCI") AND DT==("ARTICLE" OR "REVIEW") AND LA==("ENGLISH")))" in Advanced Search. In addition to data collection, the CiteSpace 5.8.R3 (64-bit) software system was also used to show the detailed data analysis. All records such as titles, abstracts, and references are exported to CiteSpace for subsequent analysis.

3. Results and Discussion

3.1. Publication Characteristics. The Web of Science Core Collection (WoSCC) database shows 9366 publications that use our search terms, with 8398 articles and 968 reviews. In general, the number of papers published in recent years shows an upward trend, which indicates that the number of papers published in antibiotic resistance research in *K. pneumonia* is receiving more and more attention from researchers. Especially in the past three years, the number of publications has increased sharply, accounting for almost half of the total number of publications. Therefore, the analysis period of this study is from 2012 to 2021, and the research period is divided into one year.

3.2. Countries/Regions and Institutions Co-Operation Analysis. The distribution of studies on antibiotic resistance research in *K. pneumonia* in different countries can be understood by analyzing the country information according to the affiliation of the authors. Many institutions or universities from different countries around the world have contributed to this research field. Research papers from 178 countries have been published, and we have listed the top 10 most productive and influential countries and institutions in *K. pneumonia* antibiotic resistance research field, based on the total publications, citations, and *H*-index during 2012–2021 (Figure 1). As shown in Table 1, the USA and China ranked first and second, with 1,908 and 1,416 publications, accounting for 20.37% and 15.12% of the total, respectively, ahead of India (664 publications and 7.09%), which ranked third. In regard to institutions (Table 2), League of European Research Universities (LERU) is the most

Table 2: Top 10 most productive and influential institutions.

Rank	Institutions	Publications	Citations	Average citation per item	H-index
1	League of European Research Universities (LERU)	521	18990	36.45	65
2	Udice French Research Universities (UFRU)	261	12201	46.75	51
3	Egyptian Knowledge Bank (EKB)	225	3633	16.15	30
4	Institut National de la Sante et de la Recherche Medicale (INSERM)	218	8951	41.06	41
5	Zhejiang University	197	7449	37.81	34
6	Assistance Publique Hopitaux Paris	171	8307	48.58	42
7	Universite de Paris	145	4532	31.26	33
8	US Department of Veterans Affairs	143	7162	50.08	44
9	Veterans Health Administration (VHA)	142	7059	49.71	44
10	Monash University	133	3825	28.76	33

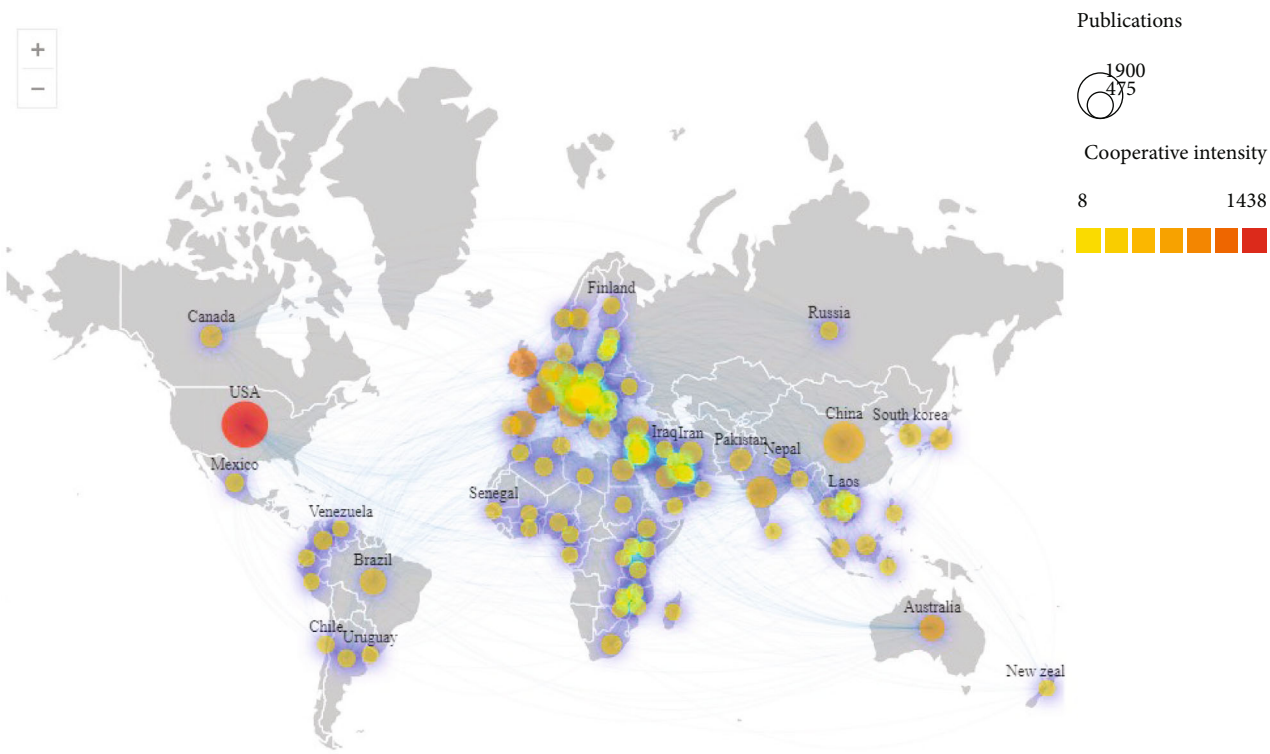


Figure 2: The cooperation network of productive countries.

productive institution in the field of "antibiotic resistance research in *K. pneumonia*" contributing 521 papers and 18990 total citations. Udice French Research Universities (UFRU) and Egyptian Knowledge Bank (EKB) are ranked second and third in terms of the number of papers.

Besides the total number of publications, the H-index, which represents the cited influence of papers, was given in the tables, too. Sometimes, a high total number of publications or citation number alone does not ensure a high H-index. Therefore, some scientists prefer to use the H-index to rank top countries (institutions), because this bibliometric indicator can more fully evaluate the impact of research [4].

In our case, in terms of "most productive countries," the USA topped the H-index by a wide margin. However, in regard to prolific institutions, LERU is the most prolific institution with an H-index value of 65. We also analyzed the results using the CtieSpace 5.8.R3 to visually show the most productive countries (institutions) and their connections to each other (Figures 2 and 3). Meanwhile, to highlight the attention of core countries (regions) and relevant academic institutions in this field, the VOSviewer 1.6.18 and Scimago Graphica 1.0.17 software were used for analysis [6].

3.3. Authors' Coanalysis. Among the top 10 authors with publications, four are from China, three from the USA, two from Australia, and one from France, respectively, as shown in Table 3. Li of Monash University, Australia, tops

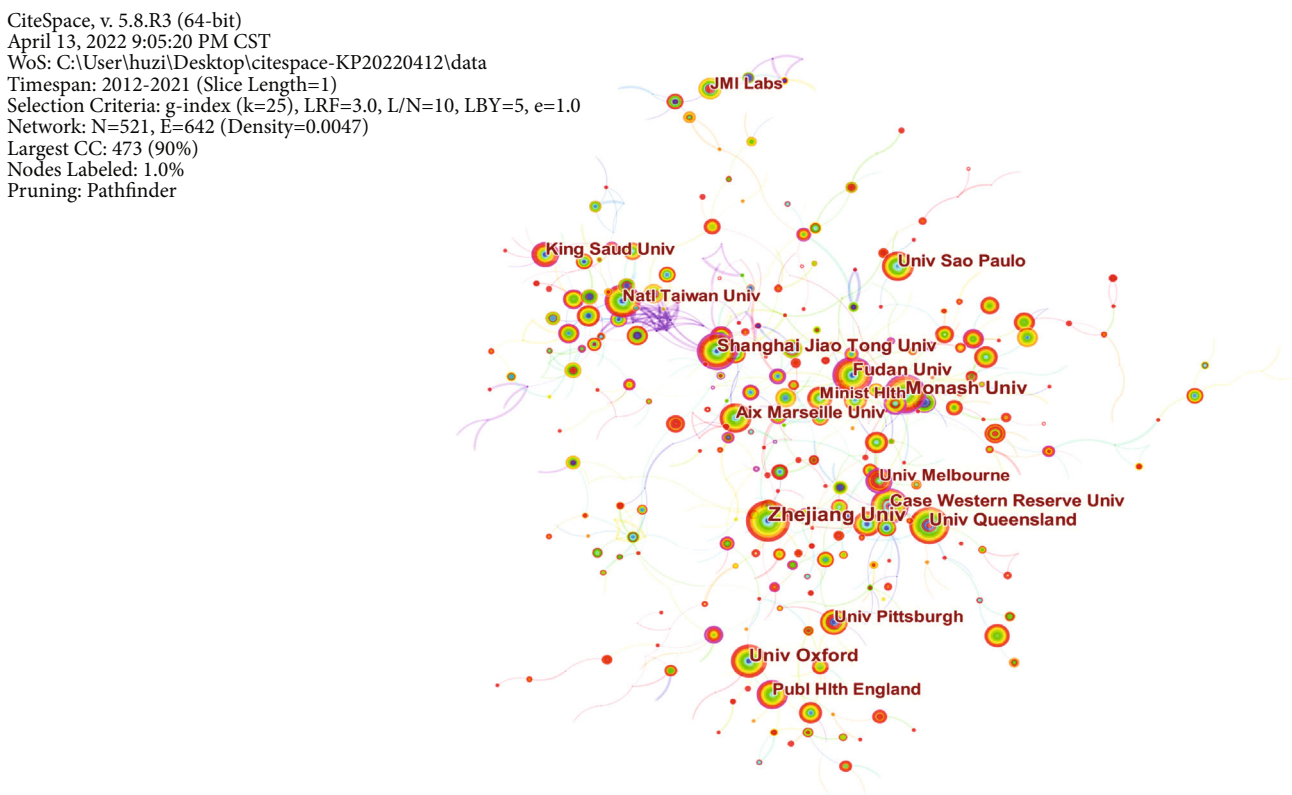


Figure 3: The cooperation network of prolific institutions.

Table 3: Top 10 authors in terms of publications.

Rank	Author	Publications	Citations	Average citation per item	*H*-index
1	Li (Australia)	87	2593	29.8	28
2	Bonomo (USA)	84	4852	57.76	34
3	Wang (China)	81	4608	56.89	21
4	Rolain (France)	75	2763	36.84	22
5	Chen (USA)	56	2077	37.09	24
6	Hsueh (China)	55	1642	29.85	23
7	Paterson (Australia)	55	4173	75.87	29
8	Liu (China)	53	562	10.6	13
9	Wang (China)	49	1045	21.33	16
10	Kreiswirth (USA)	47	2094	44.55	24

the list with 87 publications. Bonomo from the USA and Wang from China ranked second and third, with 84 and 81 publications, respectively. On *H*-index, Bonomo (34) and Paterson (29) rank the top two, showing the absolute advantages and influence of these authors on antibiotic resistance research in K. pneumonia.

Generating a productive author collaboration network is shown in Figure 4. The size of the circle indicates the number of papers the author has published, and the lines between the circles indicate the connections between the authors. The group of primary core authors is the most representative and provides centralized information. From the centrality analysis, Paterson (0.25) ranked the highest followed by Hsueh (0.15), Badal (0.09), and Bonomo (0.09), which reflect their emphasis on this field.

3.4. Journal Analysis. From 2012 to 2021, a total of 1301 journals worldwide published academic papers on antibiotic resistance research in *K. pneumonia*. Among them, the number of publications of the top 10 journals is more than 140, accounting for 28.25% of the total number of publications, as shown in Table 4. Antimicrobial Agents and Chemotherapy ranked first in terms of published research with 426 articles, accounting for 4.54% of total articles, followed by Frontiers in Microbiology and Journal of Antimicrobial Chemotherapy, and 364 and 303 papers were published,

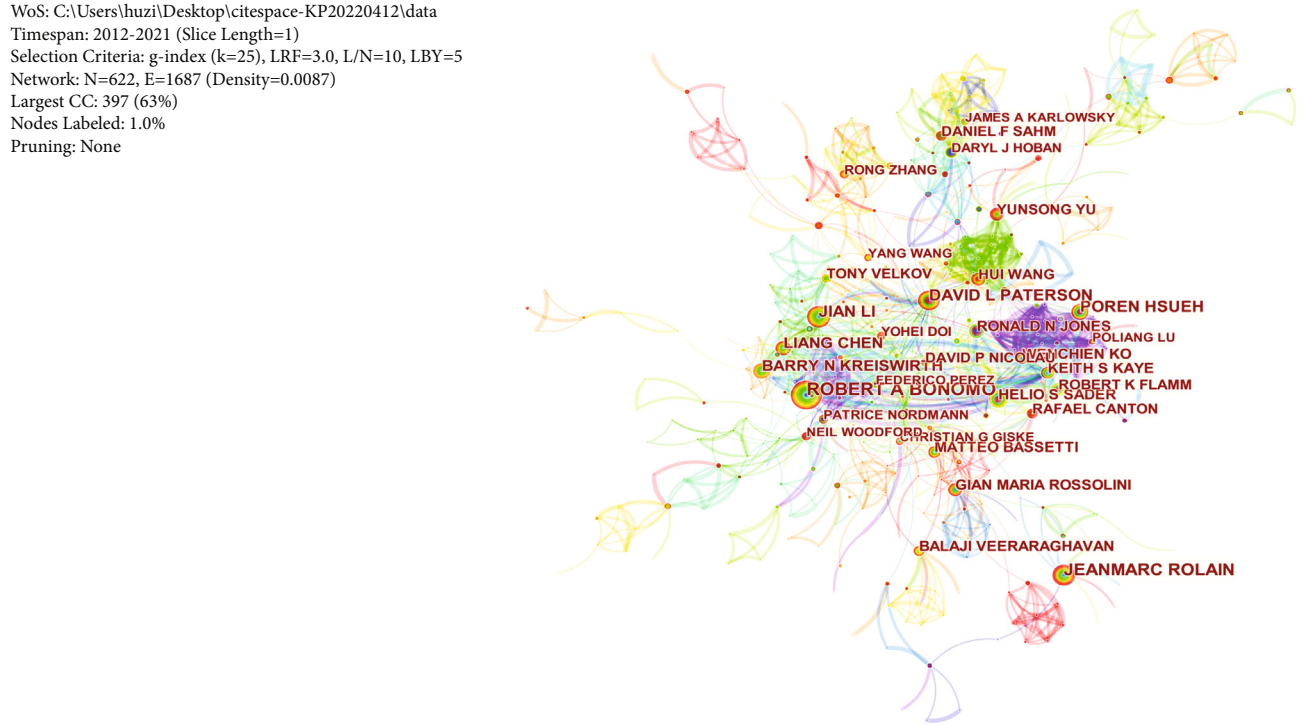


Figure 4: Collaborative network of productive authors.

Table 4: Top 10 journals in terms of publication number.

Rank	Journal	Publications	Citations	Average citation per item	*H*-index	IF	JCR
1	Antimicrobial Agents and Chemotherapy	426	16731	39.27	69	5.191	Q2
2	Frontiers in Microbiology	364	8462	23.25	41	5.640	Q2
3	Journal of Antimicrobial Chemotherapy	303	8926	29.46	50	5.790	Q1
4	PLOS ONE	287	7119	24.8	46	3.240	Q2
5	Microbial Drug Resistance	250	2754	11.02	24	3.430	Q3
6	Infection and Drug Resistance	235	1869	7.95	21	4.003	Q2
7	Journal of Global Antimicrobial Resistance	225	1912	8.5	21	4.035	Q2
8	International Journal of Antimicrobial gents	222	5724	25.78	39	5.283	Q2
9	Antibiotics Basel	189	1226	6.49	17	4.639	Q2
10	BMC Infectious Diseases	145	2500	17.24	29	3.090	Q3

Table 5: Top 10 high-frequency keywords related to antibiotic resistance research in *K. pneumonia*.

Number	Freq.	Keyword
1	4345	Klebsiella pneumonia
2	4105	Antibiotic resistance
3	2791	Escherichia coli
4	1526	Enterobacteriaceae
5	1295	Infection
6	1107	Epidemiology
7	995	Pseudomonas aeruginosa
8	895	Prevalence
9	886	Risk factor
10	864	Emergence

respectively, accounting for 3.88% and 3.23% of the total publications. These top two journals also ranked first and second in average citation per article and *H*-index, respectively. Among the 10 journals, one is in Q1 subregion, and eight are in Q2 subregion with impact factors ranging from 3.0 to 5.7, among which, Journal of Antimicrobial Chemotherapy has the highest impact factor.

3.5. Keyword Analysis

3.5.1. Coword Analysis. Through keyword coword analysis, research topics and hotspots can be analyzed, and the transition of research frontiers in a certain knowledge field can be monitored [7]. We used Citespace software to identify keywords from selected papers. At this stage, keywords with

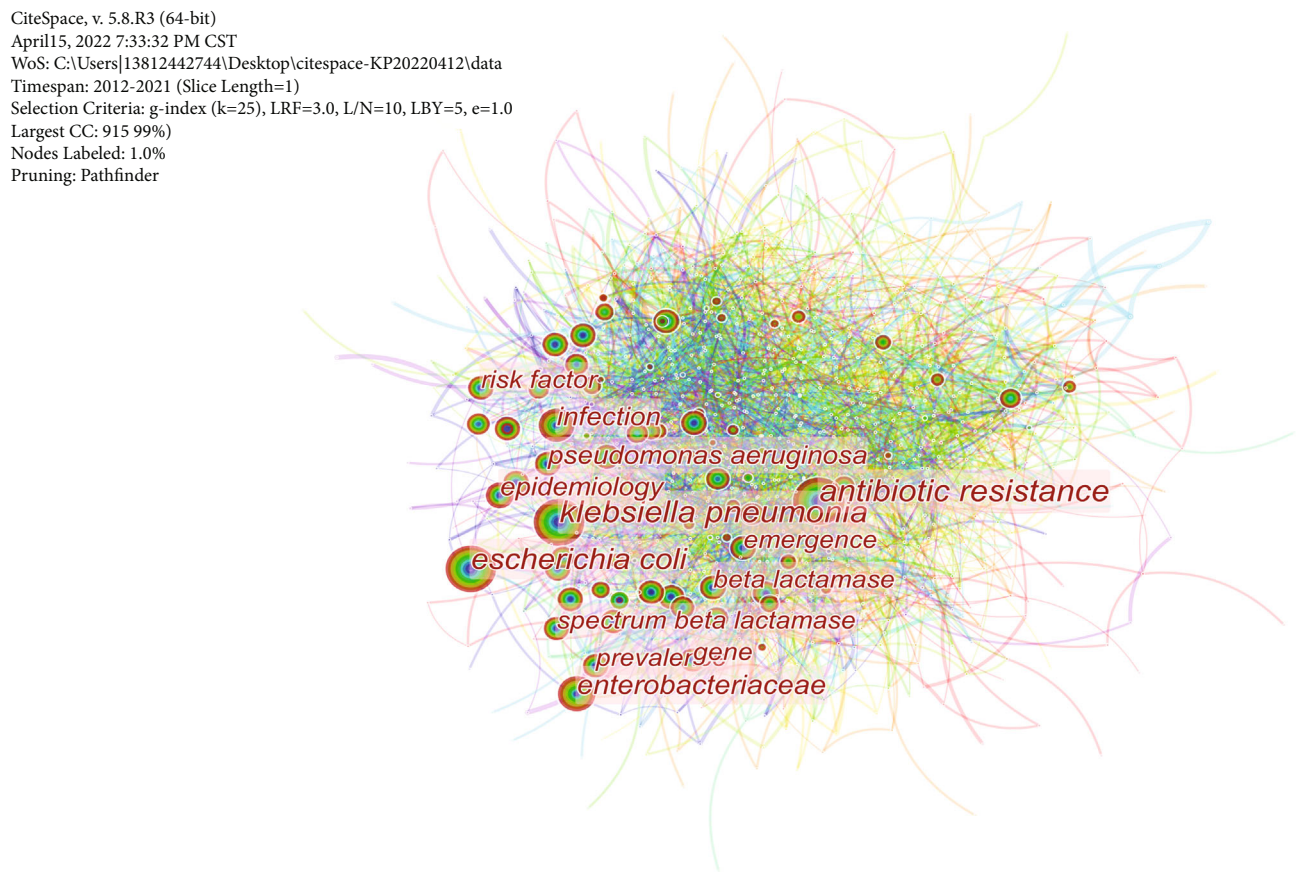


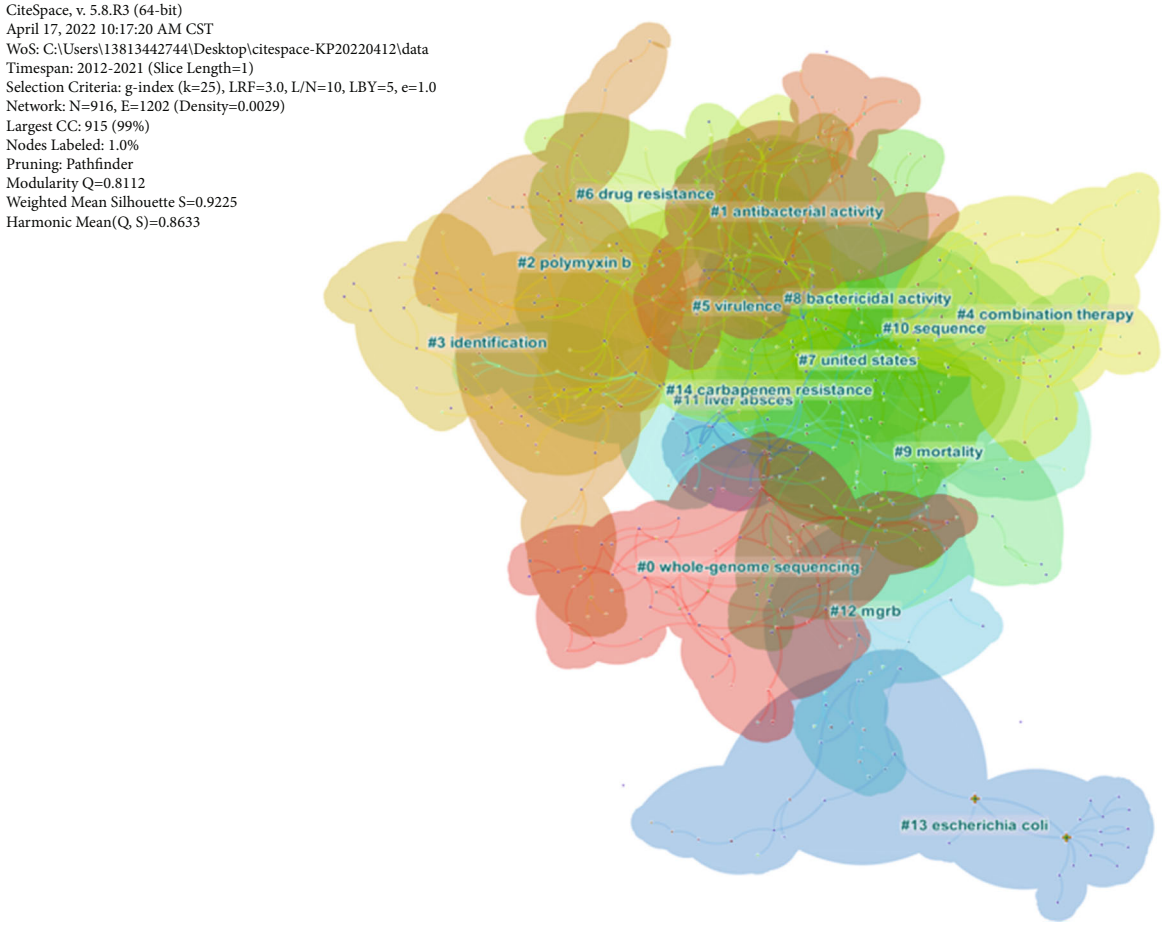
Figure 5: Co-occurrence network of keywords in research papers.

similar meanings were combined, such as "antibiotic resistance," "antimicrobial resistance," and "drug resistance." Table 5 shows the top 10 keywords for frequency and centrality in this field over the past 10 years. We also developed networks for the analysis in Figure 5. It can be seen that the top five keywords of co-occurrence were *Klebsiella pneumonia*, antibiotic resistance, *Escherichia coli*, *Enterobacteriaceae*, and infection. In order of centrality, the top five keywords were biological activity (0.17), synergy (0.14), penetration (0.14), antimicrobial peptide (0.12), and high prevalence (0.12). Centrality refers to the ratio of the shortest path that passes through a certain point and connects the two points to the total number of shortest path lines between the two points, which is used to describe the importance of a node. The larger the value is, the more influential the node is in the key position in the network [8].

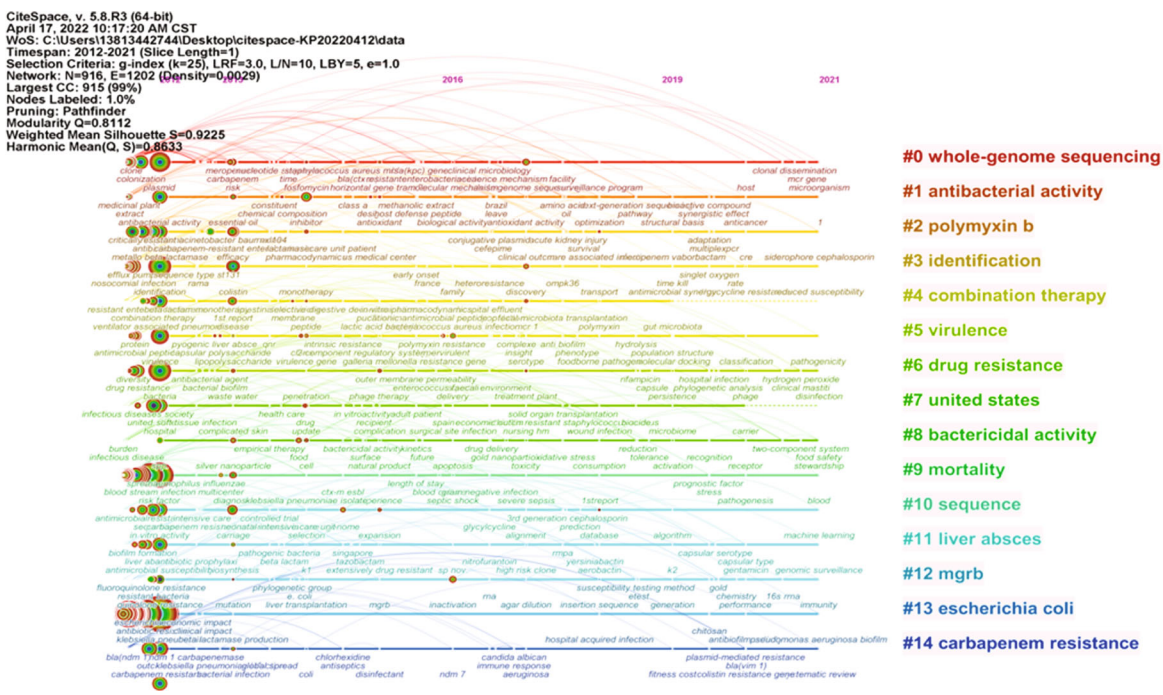
3.5.2. Clustering Analysis. Keyword clustering analysis can cluster similar points together and identify clusters that represent relevant research areas. We use CiteSpace to summarize the clustering of the keyword timeline map. Cluster size is the number of terms contained in each cluster. CiteSpace gives cluster ID 0 to the largest cluster, cluster ID 1 to the second largest, and so on. As shown in Figure 6, the main research directions can be classified as follows: #0 whole genome sequencing, #1 antibacterial activity, #2polymyxin b, #3 identification, #4 combination therapy, #5 virulence, #6 drug resistance, #7 United States, #8 bactericidal activity, #9 mortality, #10 sequence, #11 liver abscess, #12 mgrb, #13 Escherichia coli, and # 14 carbapenem resistance: these clusters can summarize the development of antibiotic resistance research in *K. pneumonia* and reveal the current research hotspots.

3.6. Keyword and Reference Burst Detection. Keyword burst detection refers to a significant increase in the frequency of keywords in a short period of time. If we understand the studies with high attention in this period, we can judge the hot topics and frontiers of research in the field accordingly. Keyword burst detection of antibiotic resistance research in 10 years in *K. pneumonia* is shown in Figure 7(a). Sorted by emergence time, it can be seen that the research frontier is whole genome sequencing, activation, phage therapy, gut microbiota, and care associated infection. To some extent, this result represents the trend of the future research.

According to Figure 7(b), it can be seen that in the past 10 years that the research hotspot changes and advances the process of antibiotic resistance research in *K. pneumonia*. Depending on the burst strength, the first *t* papers are reviewed from two high-level journals: Lancet Infections Diseases and Clinical Microbiology Reviews [9–12]. The

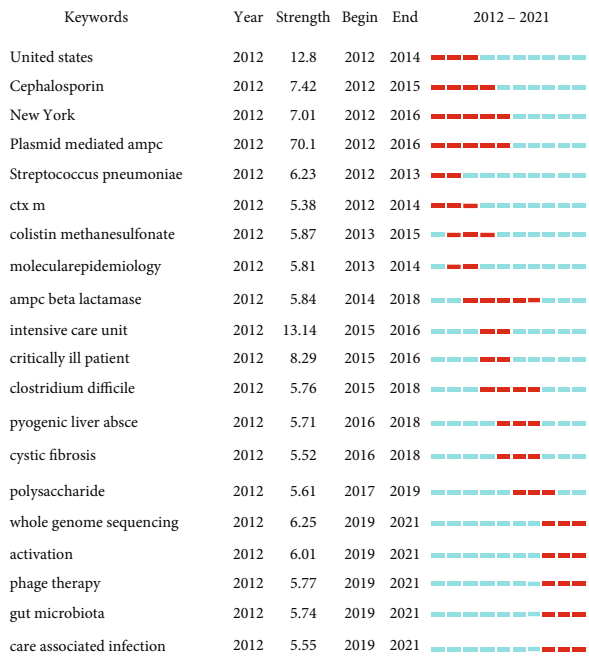


(a)



(b)

Figure 6: (a) Clustered networks of keywords in research papers via CiteSpace. The top 15 largest clusters are shown. (b) Timeline view of the top 15 largest clusters. The horizontal axis represents the evolution time, and #0-#14 represent keywords.



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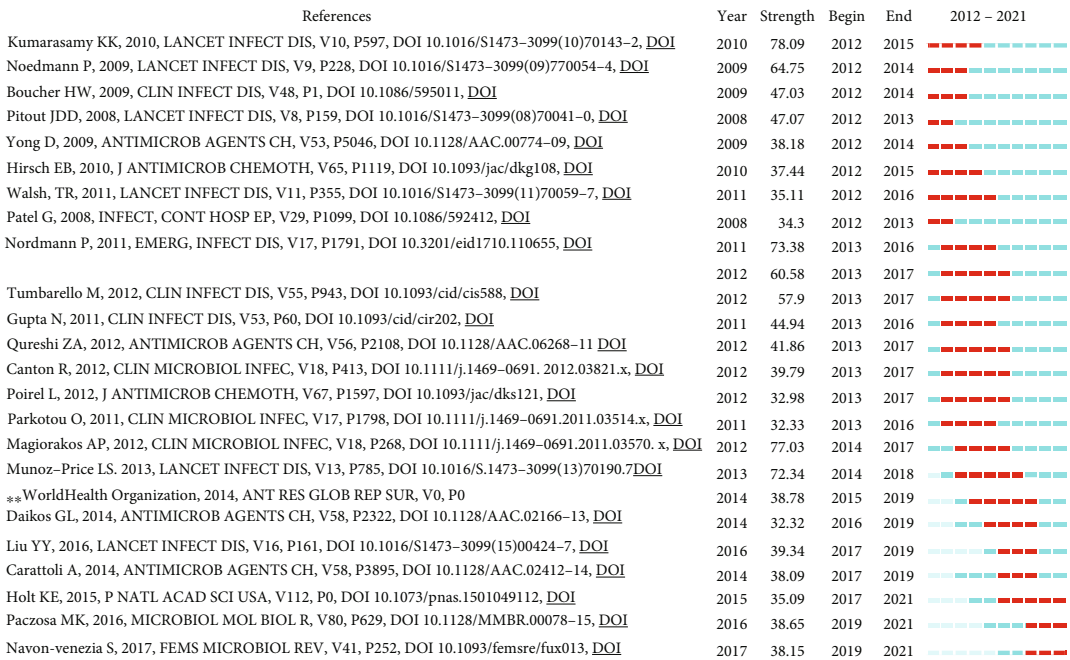

(b)

Figure 7: (a) Keywords with the strongest burst strength. Keywords marked in red indicate that the keyword usage frequency increases suddenly during this period. Blue represents a period of relative unpopularity. (b) References with the strongest burst strength. References marked in red indicate that the keyword usage frequency increases suddenly during this period. Blue represents a period of relative unpopularity.

article with the strongest burst strength was published in Lancet Infect Dis in 2010, which reported a new antibiotic resistance mechanism of a new type of carbapenem resistance gene designated blaNDM-1 [13]. Another three papers show that the heat continues to this day. Two of them are reviews about *K. pneumonia* [14–17]. Another article was published in PNAS in 2015 which sequenced the genomes of 300 diverse *K. pneumonia* and performed a pan-genome-wide association study (PGWAS) to look for associations between gene profiles associated with virulence and antibiotic resistance and the differing disease outcomes seen for *K. pneumonia* [18].

4. Conclusion

This paper provides a visual and comprehensive literature review of antibiotic resistance research in *K. pneumonia*. We examined the characteristics of publications, collaboration between countries, institutions, and authors, and co-occurrence analysis of journals and keywords. In the past 10 years, there have been 9366 journal articles related to this field, and the number of publications increased rapidly, which showed that scientists are paying increasing attention to this area. Antibiotic resistance in *K. pneumonia* has emerged as a significant threat to global public health problems [19].

We find several interesting keywords with high co-occurrence frequency through co-word analysis. According to recent research, carbapenem-resistant Klebsiella pneumonia (CRKP) has been paid much attention. Carbapenems have strong antibacterial activity and a wide antibacterial spectrum. Carbapenems are the preferred drugs for the treatment of serious Enterobacteriaceae bacterial infections [20]. However, the emergence and prevalence of CRKP pose a serious threat to patients with low immune function and have become an independent risk factor leading to the death of patients with nosocomial infections [21].

Drug efflux, biofilm formation, enzymatic inactivation of the drug, alteration of drug targets, and reduced permeability due to porin loss or modification are the major mechanisms conferring antibiotic resistance to *K. pneumonia* [22]. For combating antibiotic resistance in *K. pneumonia* infections, several therapies are currently being developed. Phage therapy, phytotherapy, photodynamic therapy, antimicrobial peptides, and nanoantibiotics are the potential of some alternatives.

Phages have low natural toxicity and high strain specificity. Due to the specificity of phages, their action is limited to the site of infection and can prevent the destruction of the inherent microbiome [23]. This reduces the development cost of phage therapy compared to antibiotics [24].

Additionally, we highlight the highly cited papers and reveal the research hotspots on antibiotic resistance research in *K. pneumonia*. This paper uses CiteSpace software to perform bibliometrics and visualization analysis on antibiotic resistance research in *K. pneumonia* data from the Web of Science database. This work presents direct and specific ways to describe existing information in different perspectives to the reader and can provide reference and reference for the relevant scientific research workers in the topic selection and development direction of the research.

Data Availability

All data generated or analyzed during this study are included in this published article. Other data may be requested through the corresponding author.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Research Article

Potentiation of the Activity of Antibiotics against ATCC and MDR Bacterial Strains with (+)-α-Pinene and (-)-Borneol

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The increasing rates of antimicrobial resistance have demanded the development of new drugs as conventional antibiotics have become significantly less effective. Evidence has identified a variety of phytocompounds with the potential to be used in the combat of infections caused by multidrug-resistant (MDR) bacteria. Considering the verification that terpenes are promising antibacterial compounds, the present research aimed to evaluate the antibacterial and antibiotic-modulating activity of (+)-α-pinene and (-)-borneol against MDR bacterial strains. The broth microdilution method was used to determine the minimum inhibitory concentration (MIC) of the compounds and antibiotics and further evaluate the intrinsic and associated antibiotic activity. These analyses revealed that (+)-α-pinene showed significant antibacterial activity only against *E. coli* (MIC = 512 μg.mL^{-1}), while no significant inhibition of *S. aureus* and *P. aeruginosa* growth was observed (MIC ≥ 1024 μg mL^{-1}). However, when combined with antibiotics, this compound induced a significant improvement in the activity of conventional antibiotics, as observed for ciprofloxacin, amikacin, and gentamicin against *Staphylococcus aureus*, as well as for amikacin and gentamicin against *Escherichia coli*, and amikacin against *Pseudomonas aeruginosa*. On the other hand, (-)-borneol was found to inhibit the growth of *E. coli* and enhance the antibiotic activity of ciprofloxacin and gentamicin against *S. aureus*. The present findings indicate that (+)-α-pinene and (-)-borneol are phytocompounds with the potential to be used in the combat of antibacterial resistance.

1. Introduction

In the last decades, the treatment of infections has been threatened by the emergence and spread of an increasing variety of pathogens developing resistance mechanisms against antimicrobial drugs. Antimicrobial resistance occurs when microorganisms such as bacteria, viruses, fungi, and parasites present modifications to evade the action of antimicrobial drugs, resulting in increased rates of transmission, morbidity, and mortality [1].

Consistent evidence has indicated that the antibiotic resistance process has been accelerated in recent years due to the inadequate and uncontrolled use of these drugs, which represents an issue of significant concern for future generations [2].

In order to reduce the irrational use of antimicrobials, the Brazilian Health Regulatory Agency (ANVISA) has published a resolution [3] to regulate and control the use of antimicrobials drugs so that, either alone or in association, they should be sold exclusively under prescription.

In response to the bacterial resistance threat, researchers have made significant efforts to isolate and identify new compounds with antibacterial properties [4], as conventional antibiotics have become significantly less effective [5]. In this context, the development of studies using plant-derived natural products has been pointed as a promising strategy to accelerate and cheapen the production of novel antibacterial compounds [6].

In fact, the use of medicinal plants for therapeutic purposes is an ancient practice. Currently, it is well-established that the therapeutic properties of medicinal species are due to the presence of secondary metabolites [7] which, besides playing critical physiological roles, can interfere with pharmacological targets in human beings and many other species. Therefore, medicinal plants are relevant sources of new molecules with the potential to be used in drug development [8, 9].

However, the development of new antibiotics is limited by the high cost of the process and the restrictions on profits compared to other drugs. In addition, the clinical benefits of antibiotics can decrease over time, so their use needs to be restricted to prevent antibacterial resistance [10].

Terpenes are a class of lipophilic hydrocarbon compounds composed of isoprene units. Such physicochemical characteristic favors their interaction with the lipid bilayer of cell membranes. Consequently, terpenoids can induce significant changes in the structure of membrane components in different microorganisms [11–13].

Pinene ($C_{10}H_{16}$) is a bicyclic, double-bonded terpenoid hydrocarbon compound [14]. The compound α-pinene is found in nature in essential oils (EO) approximately 40 different essential oils. It is among the best-known representatives of an extensive family of monoterpenes. This compound has two enantiomers (+) and (-), which are commercially available and have proven pharmacological activities, among which their antimicrobial properties stand out [15, 16]. Additionally, these isomers present a number of applications, especially in the composition of flavors and fragrances [17] and in the composition of medicines for the treatment of renal and hepatic diseases [18]. Importantly, it has been postulated that the antibacterial properties of α- and β-pinene are due to their toxic effects on the cell membrane [19].

Borneol is a monoterpene identified in the essential oils of several medicinal plants. It is classified as a bicyclic monoterpenoid alcohol that exists as the D and L enantiomers. This compound has been used in the treatment of gastrointestinal diseases in traditional medicine in China and India [9]. A large number of borneol derivatives have been both designed and synthesized, demonstrating significant activity against *Streptococcus sanguinis*, *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans*. Among them, the bornyl 3′,4′-dimethoxybenzoate derivative stood out for its strong activity against several pathogens [20].

Recent research identified a number of pharmacological activities for borneol including neuroprotective [21], analgesic [22, 23], muscle relaxing [24], anti-inflammatory [25], antitumor [26], antiasthmatic [27], and anxiolytic [28]. In addition, due to its antiadhesive antimicrobial properties, borneol has potential applications in multifunctional textiles and healthcare [29].

Consistent evidence has indicated that the effectiveness of combined drugs against microorganisms can be greater than that of isolated antibiotics, which has long been observed from studies analyzing the synergism between natural products and conventional antibiotics [30].

Therefore, considering the verification that terpenes are promising antibacterial compounds, the present research aimed to evaluate the antibacterial and antibiotic-modulating activity of (+)-α-pinene and (-)-borneol against MDR bacterial strains.

2. Materials and Methods

2.1. Bacterial Cultures. The standard bacteria used in the tests were obtained from the American Type Culture Collection, clinical isolates were obtained from the University Hospital of the Federal University of Paraíba, and both were stored under refrigeration (8°C) in slanted test tubes containing heart infusion agar (Heart Infusion Agar-HIA, Difco, USA). The standard bacterial strains *Escherichia coli* ATCC 2592, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 9027, and multiresistant isolates of *E. coli* 06, *S. aureus* 10, and *P. aeruginosa* 24 were used in the antibacterial tests. All experimental protocols were carried out at the Laboratory of Microbiology and Molecular Biology (LMMB) of the Regional University of Cariri (URCA). Antibiotic susceptibility testing (Table 1) was performed by Kirby-Bauer's disk diffusion method on Muller-Hinton agar (Hi Media, Mumbai, India) in accordance with the standards of the Clinical Laboratory Standards Institute (CLSI) [31].

2.2. Drugs and Reagents. The compounds (+)-α-pinene and (-)-borneol were weighed and 10 mg of each substance was diluted in 1 mL of dimethyl sulfoxide (DMSO, purity = 99.9%) and sterile distilled water until reaching a concentration of 1,024 μg/mL. Resazurin, sodium salt (Sigma-Aldrich, St. Louis, MO, USA) was used as a colorimetric indicator of bacterial growth through the oxidation-reduction method [32, 33].

The test substances were prepared as previously described in the literature [34, 35]. The antibiotics, trimethoprim/sulfamethoxazole, metronidazole, ciprofloxacin, clindamycin, amikacin, and gentamicin, were dissolved and diluted in sterile water to 1,024 μg/mL.

Table 1: Resistant profile of the strains.

Bacteria	Origin	Resistance profile
Staphylococcus aureus 10	Rectal swab	Amc, Amox, Amp, Asb, Azi, Ca, Cef, Cf, Cip, Cla, Clin, Eri, Lev, Mox, Oxa, Pen
Pseudomonas aeruginosa 24	Nasal discharge	Ami, Cip, Cpm, Ctz, Imi, Lev, Mer, Ptz
Escherichia coli 06	Urine culture	Asb, Ca, Cef, Cfo, Cmp, Cro

Subtitle: Amc: amoxicillin + clavulanic acid (20/10 μg); Ami: amikacin (30 μg); Amox: amoxicillin (20 μg); Amp: ampicillin (10 μg); Asb: ampicillin + sulbactam (10/10 μg); Azi: azithromycin (15 μg); Ca: cefadroxil (30 μg); Cef: cephalexin (30 μg); Cfo: cefoxitin (30 μg); Cip: ciprofloxacin (5 μg); Cla: clarithromycin (15 μg); Clin: clindamycin (2 μg); Cmp: cefepime (30 μg); Cro: ceftriaxone (30 μg); Ctz: ceftazidime (30 μg); Eri: erythromycin (15 μg); Imi: imipenem (10 μg); Lev: levofloxacin (5 μg); Mer: meropenem (10 μg); Mox: moxifloxacin (5 μg); Oxa: oxacillin (1 μg); Pen: penicillin (30 μg); Ptz: piperacillin (100 μg) [40].

2.3. Strains. Bacterial culture samples were seeded in Petri dishes containing solid heart infusion agar (HIA) medium and stored at 37°C for growth for 24 h. Then, an aliquot of the microbial culture was removed with an inoculation loop and transferred to test tubes containing sterile saline solution (0.9%). The turbidity of the inoculum was compared to the McFarland scale corresponding to 1×10^8 CFU. This test was carried out in triplicate.

2.4. Determination of Minimum Inhibitory Concentration (MIC). The minimum inhibitory concentration (MIC) was defined as the lowest concentration capable of preventing bacterial growth in the microdilution plate wells as detected macroscopically [31]. The MIC was determined using standard nonresistant bacterial strains. To this end, each strain was cultured in three Petri dishes containing HIA. After 24 h, an aliquot of each plate was collected to obtain an inoculum with a final concentration of 10^5 CFU. Test tubes were filled with 1350 μL of 10% brain heart infusion (BHI) + 150 μL of inoculum. Then, 100 μL of this solution was distributed in each well of 96-well plates. Then, 100 μL of each monoterpene was added to the first well, and a serial dilution was performed in each column of the plate to achieve concentrations ranging from 512 μg/ml to 0.5 μg/ml. The plates were then placed in an incubator for 24 h at 37°C, followed by the addition of 20 μl of resazurin to each well. After 1 h, the reading was carried out by ocular observation of the solution color, so that a change from blue to red or purple was used as an indication of bacterial growth. Of note, according to Houghton et al. [36], a natural product with an effective concentration higher than 1 mg/mL cannot be considered clinically relevant due to the impossibility of achieving adequate plasma concentrations.

2.5. Modulation Antibiotic Activity by Direct Contact. The method proposed by Coutinho et al. [37] was used in the analysis of antibiotic activity modulation against MDR isolates. Briefly, the bacterial inoculum was prepared in BHI as described above, and the compounds were added at a subinhibitory concentration (equivalent to its MIC÷8). The wells in a 96-well plate were filled with 100 μL of this solution, followed by the addition of 100 μL of each antibiotic at concentrations ranging from 512 to 0.5 μg/mL. The MIC of each drug in the presence or absence of the natural product was determined, and the occurrence of synergism was interpreted as increased antibiotic activity. Experimental controls and readings were performed as previously described.

2.6. Statistical Analysis. The data were analyzed through the statistical program GraphPad Prism version 7.0. The analysis was performed by two-way ANOVA, using the geometric average of the triplicates as the central data and the standard deviation of the average. A Bonferroni post hoc test was then performed, and a $p < 0.05$ was considered significant.

3. Results

As shown in Table 2, both (+)-α-pinene and (-)-borneol presented a MIC of 512 μg/mL against the ATCC strain of *E. coli*, while MICs $\geq$ 1024 μg/mL were obtained against *S. aureus* and *P. aeruginosa*. Therefore, both compounds were found to present significant antibacterial effects only against *E. coli*.

To evaluate the potentiation of antibiotic activity, we investigated the ability of the natural products to reduce the antibiotic MIC. Figure 1 shows that the MIC of ciprofloxacin against *S. aureus* was reduced from 101.5 μg/mL to 80.6 μg/mL when associated with (+)-α-pinene. The results were even more expressive with the drugs amikacin and gentamicin whose MIC was reduced by 90% and 92%, respectively, against the same strain. On the other hand, the association of (+)-α-pinene with the trimethoprim/sulfamethoxazole, metronidazole, and clindamycin had no significant impact on their MIC, indicating an absence of antibiotic activity modulation.

The analysis of antibiotic-enhancing activity of (-)-borneol in *S. aureus* cultures (Figure 2) demonstrated that its association with ciprofloxacin caused a reduction of 37% in the antibiotic MIC. In addition, the association with gentamicin, reduced the antibiotic MIC by 75%, changing from 32 μg/mL to 8 μg/mL, indicating potentiated antibiotic activity.

The analysis of antibiotic resistance modulation by (+)-α-pinene against *E. coli* is shown in Figure 3. Among the antibiotics, only amikacin and gentamicin had their MIC changed by the compound. While the MIC of amikacin was reduced from 50.8 μg/mL to 40.3 μg/mL, the MIC of gentamicin was reduced from 20.1 μg/mL to 16 μg/mL.

On the other hand, under the same conditions described above, an antagonistic activity was observed from the combination of (-)-borneol with trimethoprim/sulfamethoxazole against *E. coli*, since the antibiotic MIC increased from 512 μg/mL to $\geq$1024 μg/mL (Figure 4). However, no significant modulation of antibiotic activity was observed from the association of (-)-borneol with the other drugs.

Table 2: Minimum inhibitory concentration of (+)-α-pinene and (-)-borneol.

Microorganisms	MIC	
	(+)-α-Pinene	(-)-Borneol
E. coli	512 μg/mL	512 μg/mL
S. aureus	≥1024 μg/mL	≥1024 μg/mL
P. aeruginosa	≥1024 μg/mL	≥1024 μg/mL

P. aeruginosa was found to present significant resistance to antibiotics, whose MIC was poorly affected by the association with (+)-α-pinene. Nevertheless, it is worth mentioning that this monoterpene caused a reduction of 37% in the MIC of amikacin in comparison with the control (Figure 5).

Under the same conditions described above, (-)-borneol failed to modulate the activity of all antibiotics against *P. aeruginosa*, as no significant MIC change was observed (Figure 6).

Discussion

The use of these natural compounds in the treatment of infections is considered a traditional alternative to the use of synthetic drugs [33]. Studies have demonstrated that monoterpenes can improve the activity of antimicrobial drugs, increasing their effectiveness against resistant pathogens, which can accelerate the healing, as well as hinder the microbial adaptability. Resistance to aminoglycosides and other antibacterial drugs has been a major threat to public health. Aminoglycosides inhibit protein synthesis by altering the conformation of the bacterial ribosome [34, 38] presenting enzymatic inactivation and efflux pump expression as major resistance mechanisms [37].

A significant body of research has demonstrated that antibiotic resistance can be reversed using natural products such as extracts, fractions, essential oils, and isolated phytocompounds, as well as their synthetic and semisynthetic derivatives [33, 34, 37, 38]. While the molecular mechanism underlying this phenomenon remains mostly unknown, it has been suggested that it involves interactions between the natural product structure and constituents of the bacterial cell membrane, such as transmembrane proteins [37, 38].

Yousefzadi et al. [39] isolated α-pinene from the essential oil of *Salvia chloroleuca* and evaluated its antibacterial activity, demonstrating moderate and strong inhibitory activities against *S. aureus* and *E. coli*, respectively. However, no activity was found against *P. aeruginosa*, which can be explained due to differences in the structure of the cell membrane of Gram-positive and Gram-negative bacteria, in particular, the lipid bilayer [38, 40].

Da Silva et al. [17] evaluated the antimicrobial activity of pinenes, demonstrating that (-)-α-pinene and (-)-β-pinene had no significant antimicrobial activity at concentrations below 20 mg/mL, while the positive enantiomers showed inhibitory activities against methicillin-resistant *S. aureus* strains with MIC values ranging from 117 μg/mL to 6,250 μg/mL. Accordingly, the studies of Da Silva et al. [17] and Dhar et al. [41] found that (+)-α-pinene presented antibacterial activity against *S. aureus* strains. Moreover, De Sousa Eduardo et al. [42] and Freitas et al. [43] showed that α-pinene has promising effects against *S. aureus*, demonstrating a potential to be used in the combat of antibacterial resistance.

The results of the present work corroborate those presented by Da Silva et al. [17] who evaluated the combination of (+)-α-pinene and (+)-β-pinene with ciprofloxacin, showing synergistic activity against methicillin-resistant *S. aureus* (MRSA).

An antibiotic-potentiating effect was also obtained by Do Amaral et al. [44] who showed that the association of α-pinene with ceftazidime, amoxicillin, cefepime, cefoxitin, and amikacin resulted in enhanced antibiotic activity against *E. coli*.

This enhanced antibiotic effect may result from different mechanisms such as alteration in membrane permeability; inhibition of efflux pumps activity, or alteration in the expression of genes that codify proteins that mediate these mechanisms [45].

S. aureus can become resistant to antibiotics through genetic mutations that alter the target DNA gyrase or reduce outer membrane proteins, thus reducing drug accumulation [46, 47]. Martin et al. [48] reported a marked increase in resistance to trimethoprim-sulfamethoxazole in clinical isolates of *Staphylococcus aureus* and 7 genera of Enterobacteriaceae, including *E. coli*, from 1988-1995 at a hospital in California.

E. coli is naturally susceptible to almost all clinically relevant antimicrobial agents, in addition to being capable of accumulating resistance genes, mainly through horizontal gene transfer [49].

Studies performed by Breidenstein et al. [50] showed that *Pseudomonas aeruginosa* presents a high level of intrinsic resistance to most antibiotics, which can be explained by the restricted permeability of its outer membrane, in addition to the expression of efflux systems and antibiotic-inactivating enzymes such as β-lactamases.

Corroborating the results of this study, Ali et al. [51] evaluated *P. aeruginosa* isolates and found significant resistance to amikacin, while resistance to trimethoprim-sulfamethoxazole was described by Bayraktar et al. [52].

Barbosa [53] evaluated the essential oil of *Chamaemelum nobile*, which has α-pinene and β-pinene as major components. The oil strongly modulated the activity of amikacin activity against *P. aeruginosa* PA01, causing a 128-fold reduction in the MIC of this antibiotic, corroborating the results of this study.

Nitroimidazole prodrugs such as metronidazole are activated by the reduction of the nitro group, which occurs at low oxygen rates, since oxygen can inhibit metronidazole uptake. Thus, the effective use of nitroimidazoles is limited to anaerobic bacteria, protozoa, and microaerophiles [54–57], corroborating the resistance profile observed in this study.

It is known lipophilic substances like beta-caryophyllene can induce significant changes in the membrane structure, resulting in morpho-physiological damage, such as reduced membrane potential, cytochrome C/protein and radical loss, proton pump collapse, and ATP depletion, among other

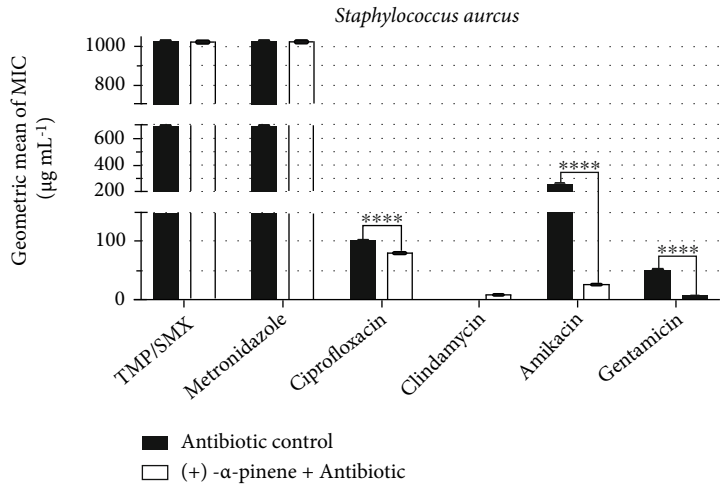

Figure 1: Antibiotic-modulating activity of (+)-α-pinene associated with antibiotics against *S. aureus*. TMP/SMX: trimethoprim/sulfamethoxazole. $p < 0.0001 = ****$.

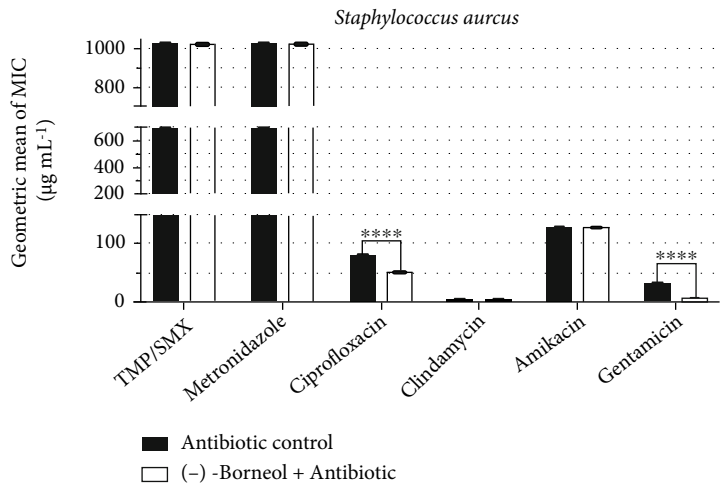

Figure 2: Antibiotic-modulating activity of (-)-borneol associated with antibiotics against *S. aureus*. TMP/SMX: trimethoprim/sulfamethoxazole. $p < 0.0001 = ****$.

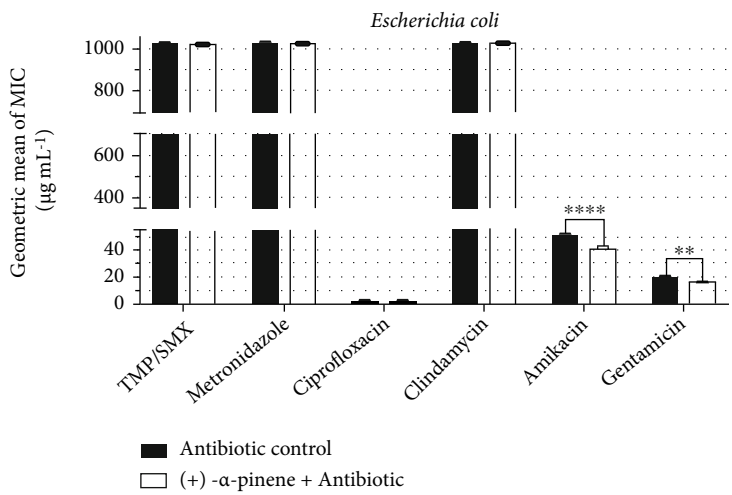

Figure 3: Antibiotic-modulating activity of (+)-α-pinene associated with antibiotics against *E. coli*. TMP/SMX: trimethoprim/sulfamethoxazole. $p < 0.0001 = ****$.

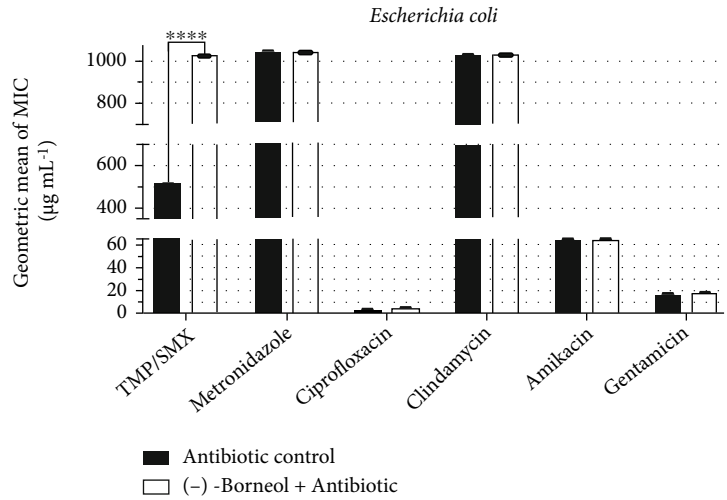

Figure 4: Antibiotic-modulating activity of (-)-borneol associated with antibiotics against *E. coli*. TMP/SMX: trimethoprim/sulfamethoxazole. $p < 0.0001 = ****$.

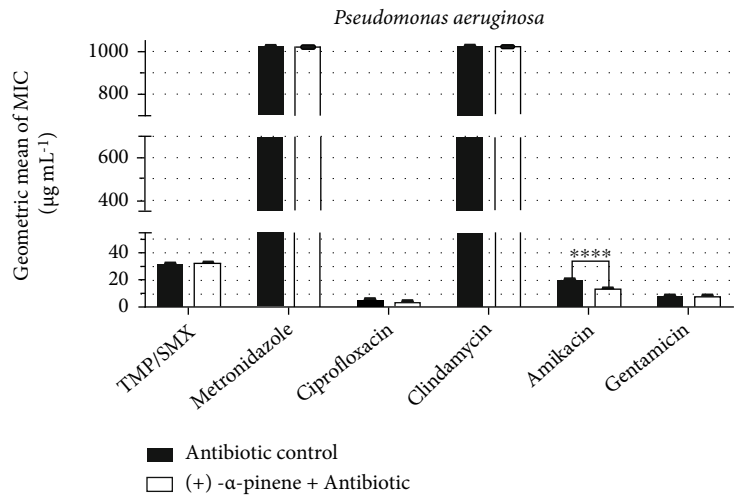

Figure 5: Antibiotic-modulating activity of (+)-α-pinene associated with antibiotics against *P. aeruginosa*. TMP/SMX: trimethoprim/sulfamethoxazole. $p < 0.0001 = ****$.

toxic effects [30, 58–63]. Accordingly, studies by Andrews et al. [63], Harrewijn et al. [64], and Singh et al. [65] state that the mechanism of action by α-pinene is associated with cell membrane damage.

Kovač et al. [66] evaluated the antibacterial activity of the negative enantiomer α-pinene at the concentration of 125 mg/L, showing that this compound increased the membrane permeability, in addition to inducing an intracellular accumulation of antibiotics due to the inhibition of antimicrobial efflux systems, providing further inhibition of antimicrobial resistance. A number of studies [63–66] have also demonstrated that pinene compounds have caused damage to the membrane, which may also explain its effects on antibiotic activity potentiation, as demonstrated in this research.

Badawy et al. [67] described the antimicrobial effects of various monoterpenes, among which thymol and α-terpineol had the most potent activity against *E. coli* and *S. aureus*. De Souza et al. [68] evidenced the efficacy of the association between carvone and penicillin against MRSA, as well as demonstrated the potentiating effects of eugenol and thymol associated with penicillin against beta-lactam-resistant *E. coli*. In corroboration, the work of De Souza et al. [68] found that d-limonene had a synergistic effect when associated with gentamicin against *S. aureus* and *E. coli* while the monoterpene geraniol enhanced the activity of kanamycin against the bacterial strain 358 of *S. aureus* [69].

Sill with regard to effects of monoterpenes against resistant *S. aureus* strains, Freitas et al. [43] also stated that α-pinene potentiated the effect of tetracycline against the *S. aureus* IS-58 strain, while studies with *E. coli* conducted by Pereira et al. [70] showed that the complex (+)-β-citronellol (βCT)/β-cyclodextrin (β-CD) in combination with gentamicin showed a synergistic effect against *E. coli*.

Research by Gachkar et al. [71] has attributed the antimicrobial activity of some essential oils to the presence

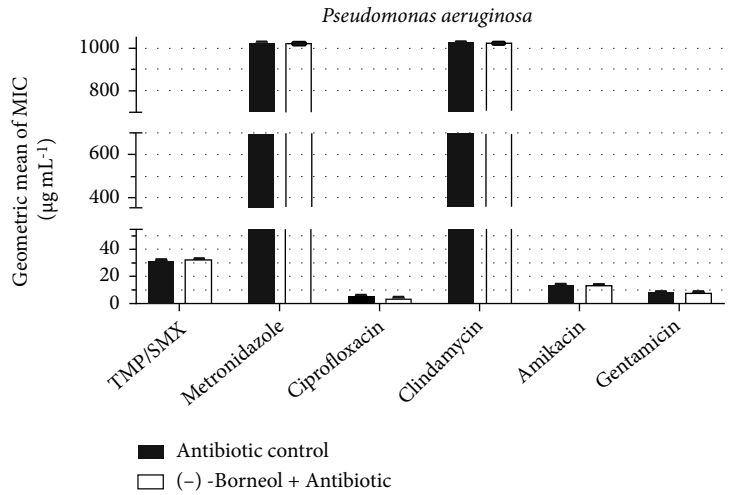

Figure 6: Antibiotic-modulating activity of (-)-borneol associated with antibiotics against *P. aeruginosa*. TMP/SMX: trimethoprim/sulfamethoxazole. $p < 0.0001 = ****$.

borneol, which has been identified as a major constituent of the essential oils obtained from the flowers, leaves, and stem of *Rhynchanthus beesianus* [72], which presented significant antibacterial activity against *Bacillus subtilis*, *Enterococcus faecalis*, *S. aureus*, *Proteus vulgaris*, *P. aeruginosa*, and *E. coli*.

Studies on the antibacterial mechanism of action of terpenes [73–75] have indicated that the antimicrobial activities of thymol and carvacrol are associated with their ability to cause changes in membrane permeability. According to Breidenstein et al. [50], the restricted permeability of the outer membrane, as well as the presence of efflux systems, and the production of antibiotic-inactivating enzymes, such as β-lactamases, collaborate to the high level of antibiotic resistance observed in *Pseudomonas aeruginosa*, which could justify the lack of significant modulation of the antibiotic activity by the compounds evaluated in the present research. Finally, the present findings corroborate those obtained by Siddique et al. [76] who showed that borneol did not exhibit antibacterial activity against MDR clinical isolates of *S. aureus* and MRSA.

While specific mechanisms involved in the antimicrobial action of monoterpenes remain poorly characterized, studies by Sikkema et al. [61] and Sikkema et al. [11] have suggested that due to their lipophilic character, monoterpenes will preferentially divide from an aqueous phase into membrane structures, thus causing structural and functional damage, which has been used to explain the antimicrobial action of oils and their monoterpenoid components in most works. According to Trombetta et al. [77], the action of these compounds on the bacterial membrane leads to expansion, increased fluidity and permeability, disturbance of protein function, and inhibition of ion transport. Thus, the existence of certain epistatic interactions that result in variable responses in studies addressing different species and antibiotics cannot be ruled out.

Besides the antibacterial activity, research has demonstrated that borneol has analgesic, anti-inflammatory, antioxidant, healing, and antifungal activities [78, 79].

Yang et al. [79] reported that (+)-borneol (BNL1) and (-)-borneol (BNL2) can induce drug accumulation in cells due to its interference with P-glycoprotein (Pgp), an efflux protein that contributes to multidrug resistance to antibiotics and anticancer drugs, which could potentially explain the synergistic effects observed from the association between monoterpenes and antibiotics, as demonstrated in this work.

Of note, to date, no study evaluating the antibiotic-enhancing activity of (-)-borneol has been found in the literature, highlighting the pioneering aspect of the present research.

4. Conclusions

The results presented in this work suggest that (+)-α-pinene and (-)-borneol are promising compounds in the inhibition of antibiotic resistance, although further research is required to investigate both the safety and effectiveness of this combined treatment in the management of infections caused by *S. aureus*, *E. coli*, and *P. aeruginosa*.

The results of this work can contribute to the development of new antibacterial therapies using lower doses of monoterpenes and antibiotics, increasing the effectiveness and reducing the side effects resulting from antibiotic therapy.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare no conflict of interest.

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