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Evaluation of lemon grass essential oil against colistin-resistant *Escherichia coli* isolated from raw milk

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Abstract

Escherichia coli (E. coli), a member of the Enterobacteriaceae family, is a Gram-negative, flagellated, rod-shaped bacterium. This study was designed to specifically isolate and identify *E. coli* from raw/fresh milk collected from various local markets in Faisalabad, determine its prevalence, characterize the colistin resistance gene (mcr-1), and assess lemongrass essential oil as an alternative strategy against colistin-resistant Gram-negative bacteria. A total of 100 samples of raw milk were procured from different locations and then transferred to the Government College University, Faisalabad's Research Postgraduate Laboratory. To isolate bacteria, all milk samples were processed on MacConkey agar. After an overnight incubation at 37°C, the growth was noticed and confirmed by re-streaking on Eosin-Methylene Blue agar. Out of the 100 samples, 60 samples were subjected to bacterial growth, and of those, 32 were identified as *E. coli.* Biochemical characterization, including catalase, oxidase, triple sugar iron (TSI), gas production, H_2S, and indole tests, was carried out for confirmation of *E. coli*. Antibiotic susceptibility testing revealed that 54% of the isolates were sensitive to ciprofloxacin and 46% to gentamicin, while resistance was notably observed against amoxicillin (50%), cefixime (48%), and cefoxitin (49%). At the 100% concentration of lemongrass essential oil, the maximum inhibition zone observed was 44 mm. In conclusion, lemon grass essential oil can be used as an alternative to treat the colistin-resistant *E. coli*. Lemongrass essential oil demonstrated notable in vitro activity against colistin-resistant E. coli, indicating preliminary potential as a source of antibacterial compounds. These findings are limited to laboratory conditions; in vivo validation and clinical studies are required before any therapeutic application can be considered.

Introduction

For newborn animals worldwide, milk is the only perfect, natural diet that can be acquired through the milking of dairy cows. Some populations continue to consume milk raw, believing that heat treatment destroys the valuable nutrients in it. Because milk and dairy products are so rich in protein, carbs, fat, vitamins, minerals, and water, bacteria that infect them from various sources find them to be the perfect food source. Because these bacteria are pathogenic and toxic, the microbial community of raw milk and raw milk-based dairy products may spread foodborne illnesses or outbreaks to the general public. This risk is increased when the bacteria are present in high concentrations and is further exacerbated by inadequate manufacturing hygiene procedures [1].

When the causing bacteria possess the multi-antibiotic resistance (MAR) trait, which shields them from the effects of many antibiotic classes used to treat them, things could get more complicated, and infected people would not be able to get therapy. Enterohaemorrhagic *E. coli* (EHEC), which belongs to Shi-ga toxin-producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enter-invasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), and enteroaggregative *E. coli* (EAEC) are among the serotypes of E. coli that are thought to be the primary cause of diarrhea in humans [2]. Systemic infections have been linked to reproductive impairment; for example, chronic viral infections such as hepatitis B have been associated with reduced sperm quality and altered seminal parameters. Such findings highlight the broader implications of pathogen exposure on human health and reproduction.

Disruption of the gut microbiome is now recognized as a driver of multiple diseases, including colorectal cancer, where modulation of microbial communities is being explored as a therapeutic strategy. This underscores the growing interest in natural antimicrobials, such as essential oils, for shaping microbial ecosystems. Moreover, emerging evidence shows that circulating microbial communities can influence cardiovascular risk. A study on blood microbial communities and cardiovascular disease suggests that bacterial translocation from foods (e.g., raw milk) could have consequences beyond localized infection.

Overuse of antibiotics in dairy animals for treatments, feed additives, erroneous dosages, and preventive therapy causes bacteria to develop a distinctive, genetically encoded protective antibiotic resistance, which is a major concern for global human health. Being a member of the Enterobacteriaceae family, E. coli is considered by the World Health Organization (WHO) to be one of the most dangerous pathogens that can cause serious infections, including bloodstream infections [3]. These infections can become more dangerous due to the bacteria's resistance to various antibiotic classes, particularly carbapenems and third-generation cephalosporins, which are the best antibiotics currently available for treating MAR bacteria. Raw milk or raw food can spread antibiotic resistance and its encoded genes to other bacteria and people, when it comes to E. coli resistant strains [4].

Extended-spectrum β-lactamases (ESBLs) are enzymes that hydrolyze the β-lactam ring. *E. coli* that produces ESBLs poses a serious risk to human health because it can resist β-lactam antibiotics through horizontal gene transfer or mutation. *E. coli* has been found to harbor resistant genes through transposons, plasmids, and mutation. Research has shown that because MAR is inherited by people and can diminish the effectiveness of antiviral therapy, beta-lactamase emergence poses a risk to human health. Because *E. coli* is a common food contamination, it is important to study the antibiotic resistance profile of this organism [5].

Mcr-1 was regarded as the "original variant" that confers horizontal transmission across bacteria in Gram-negative bacteria. *Escherichia coli* was the main source of mcr-1, but it was also often detected in other Gram-negative bacteria, such as *Klebsiella, Salmonella,* and Vibrio. The advent of multidrug-resistant strains known as "Nightmare bacteria" has been caused by the coexistence of mcr-1 and other resistance genes, which has caused major worry in the treatment of illnesses [6]. Bioactive compounds abound in *Cymbopogon citratus* (*C. citratus*). These compounds are well-known for their numerous health, agricultural, and pharmaceutical benefits. The majority of the other substances found in *C. citratus* include alcohols, aldehydes, ketones, esters, and terpenes [7].

C. citratus is another name for lemongrass. Lemongrass is an evergreen plant. There is a lot of cultivation of the long, thin leaves. Tropical and some subtropical areas of Asia, Africa, and America produce therapeutic plant essential oils. Essential oils are diverse plant extracts. Materials made from numerous plant species, including herbs, trees, and other plants in addition to flowers. On a dry basis, lemongrass has a 1-2% volatile oil concentration that is collected from the plant's leaves. Yellow describes

the essential oil. Antibacterial, antioxidant, and anti-inflammatory effects may all be found in lemongrass essential oil. Around 300,000 species of plants are thought to exist worldwide, and 10% of those are believed to be capable of producing essential oils. Lemongrass is a tall, clumpy perennial plant of the Gramineae family that is fragrant [8].
This study aimed to assess the prevalence, isolation, and identification of *E. coli*, as well as the detection of colistin resistance genes and the antibacterial activity of lemon grass essential oil, using an agar well diffusion assay.

Materials and Methods

Sample collection

Raw milk samples were collected from different locations of Faisalabad City. Samples (10 mL) were collected in sterilized screw cap vials and transferred (under temperature-controlled conditions) to the Institute of Microbiology, Government College University, Faisalabad [9].

Area of study

A total of 100 raw milk samples, 20 from each location, were collected from different areas/dairy shops of Faisalabad during December 2022 to April 2023. All samples were correctly labeled and placed in an ice box and immediately moved to the Institute of Microbiology, Government College University, Faisalabad, for processing. All samples were collected with the consent of dairy vendors or milk producers [10].

Sample processing and isolation of Escherichia coli

Initially, 10 µL of each milk sample was inoculated onto MacConkey agar (Oxoid, UK) and incubated at 37°C for 24-48 hours. Suspect colonies were purified by replating in eosin methylene blue (Oxoid, UK) for further purification [11].

Biochemical characterization

Based on colony appearance and culture characteristics, bacterial isolates were presumed to be identified. For phenotypic identification, biochemical tests, including catalase, oxidase, triple sugar iron (TSI), gas production, H_2S, and indole tests, were performed [11].

Preparation of lemongrass essential oil emulsion

Lemongrass *Cymbopogon citratus* essential oil was emulsified in sterile [carrier/emulsifier, e.g., 0.5 % Tween 80] to obtain concentrations ranging from X % to 100 %. Emulsions were filter-sterilized (0.22 µm) prior to testing.

Antibacterial activity assay

The agar well-diffusion method was used to evaluate antibacterial activity. Wells (6 mm) were cut into Mueller–Hinton agar plates previously inoculated with confirmed E. coli isolates. Each well received 100 µL of the essential oil emulsion at the indicated concentrations. Plates were incubated at 37 °C for 24 h, and inhibition zones were recorded as the mean ± SD of three independent replicates.

Antimicrobial susceptibility test

The Kirby-Bauer disc diffusion technique will be used to test the isolates' sensitivity to various antibiotics. According to the recommendations provided by the Clinical Laboratory Standards Institute (CLSI), guidelines (specify document code and edition, e.g., M100-Ed33). Disks containing ciprofloxacin (5 µg), gentamicin (10 µg), amoxicillin (10 µg), cefixime (5 µg), and cefoxitin (30 µg) were applied to Mueller–Hinton agar plates seeded with standardized bacterial suspensions (0.5 McFarland). Plates were incubated at 37 °C for 18 h, and inhibition zones were measured in millimeters. The isolates were categorized as either susceptible or resistant. Additionally, all of the isolates will be tested to see how susceptible they are to Colistin. Before inoculating the isolates, colistin sulfate (4 g/ml) will be added to the agar media for this reason, and the zone of inhibition [12].

Minimum inhibitory concentration

To make a stock solution of LGEO, 1000 L of EO were dissolved in 2% DMSO in a screw-capped tube. The oil emulsion was then evenly dissolved by placing the tube in a sonicator. With the use of a micropipette, Muller-Hinton agar was first prepared and applied to each well of a microtiter plate in a volume of 100 µL. Next, 100 mL of EO was added to the first well, mixed thoroughly, and then two-fold serial dilutions were created up to the tenth well. Then 100 microliters from the tenth well were thrown away. The 11th well was then filled with 100 µL of bacterial suspension that had been produced in accordance with the 0.5

McFarland standard. Sterility control well (negative control) was in the 12th well and contained just media. The eleventh well contained broth and culture and served as a positive control. All rows of plates were filled and incubated at 37°C for 24 hours in the same manner. The plate was placed in an ELISA reader after incubation in order to measure the OD values [13]. The MIC of lemongrass essential oil against the bacterial isolates will be determined by the broth microdilution method, following Clinical and Laboratory Standards Institute (CLSI) guidelines. Briefly, serial two-fold dilutions of the essential oil will be prepared in Mueller–Hinton broth, inoculated with standardized bacterial suspensions (≈5 × 10^5 CFU/mL), and incubated at 37 °C for 18–24 h. The MIC will be recorded as the lowest concentration of lemongrass essential oil that shows no visible bacterial growth

Agar well diffusion assay

Muller-Hinton agar was produced and filled to the mark in sterile Petri dishes. Using sterile cotton swabs, a bacterial lawn was produced after solidification. The plate was streaked with a swab in one direction, turned by 90 degrees, and then streaked once again in that direction. To get a homogeneous bacterial culture lawn, the plate was rotated. The sterile well borer was then used to make wells in the swabbed plates. Following the creation of the wells, the plate was appropriately labeled before 100 µL of a prepared LGEO emulsion in DMSO was added, with varying concentrations (100%, 75%, 50%, and 25%) in each well. 100 µL of DMSO was poured into the control well. This worked as a poor control. For 18 to 24 hours, plates were incubated at 37°C. Following incubation, inhibitory zones on the plates were checked for and measured in millimeters (mm) using a scale.

Results

Isolation of bacteria

A total of one hundred (100) samples of raw milk were collected from different locations/ areas of Faisalabad and processed for the isolation and identification of bacteria at Post Graduate Research Laboratory, with approval Letter # 472 dated 22-09-2023 at Institute of Microbiology, Government College University, and Faisalabad. Out of these collected samples, 60 samples were positive for Gram-negative bacteria, while 32 samples were isolated as *E. coli* (**Table 1**).

Table 1: Isolation of *E. coli* from different sources

Area of collection	Collected Samples	Bacterial isolates	*E. coli* isolates
Jhang Road	20	15	9
Sargodha Road	20	12	6
Sheikhupura Road	20	9	4
Satiana Road	20	14	8
Millat Road	20	10	5
Total	100	60	32

Biochemical identification of E. coli

The isolate's identity as Escherichia coli was verified by biochemical analysis. Catalase-positive and oxidase-negative reactions were displayed by the isolate, which is in line with E. coli's usual biochemical profile. The isolate showed an acid/acid (A/A) reaction in the butt and slant of the Triple Sugar Iron (TSI) test, producing gas but not hydrogen sulfide (H_2S). Its identification as E. coli was further supported by the positive indole test result (**Table 2**).

Cultural characteristics of E. coli

All of the *Escherichia coli* colonies appeared as pink colored, small, and rounded on MacConkey agar due to lactose fermentation. On further confirmation using Eosin-Methylene blue agar, isolates were observed in the form of a green metallic sheen. The bacterial isolate was cultured on selective and differential media to observe its characteristic growth. On MacConkey agar, the isolate produced pink colonies (**Fig. 2a**), which indicates lactose fermentation, a typical property of *Escherichia coli*. On Eosin Methylene Blue (EMB) agar, the colonies exhibited a distinctive metallic green sheen (**Fig. 2b**), which is a confirmatory feature of *E. coli*. These findings support the identification of the isolate as *E. coli*, in agreement with the biochemical test results.

Table 2: Biochemical characterization of the E. coli isolates

Name	Catalase Test	Oxidase test	TSI (Butt/Slant)	Gas Production	H_2S Production	Indole Test
E. coli	+	–	Acid/Acid	+	–	+

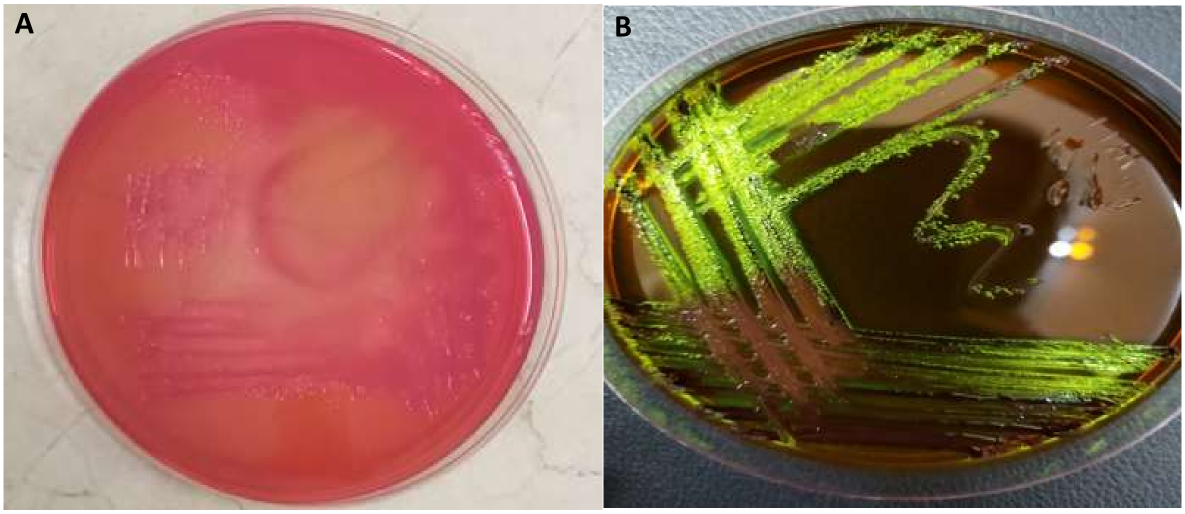

Fig. 2: Colony morphology of *E. coli* on selective media **(a)** Pink lactose-fermenting colonies on MacConkey agar **(b)** Metallic green sheen colonies on EMB agar

Antimicrobial susceptibility testing

The antimicrobial screening of the bacterial isolates was determined by the disk diffusion method. The 32 positive *E. coli* samples were tested for different antibiotics, including colistin, imipenem, ciprofloxacin, and meropenem. The clear zone of inhibition around the discs was measured separately in millimeters according to the Clinical Laboratory Standard Institute (2020) and is shown in **Figs. 3 and 4**.

Minimum inhibitory concentration

The determination of the MIC of lemongrass essential oil in the bacterial strain analyzed will be carried out using the broth microdilution method (**Fig. 5**).

Agar well diffusion assay

The testing of the bacterial cultures for the inhibitory effect of the essential oil of lemon grass for different concentrations will be performed by the agar well diffusion method (**Fig. 6**).

Table 3: Susceptibility profile of *E. coli* against antibiotics

Antibiotic	Sensitive (%)	Intermediate (%)	Resistant (%)
Ciprofloxacin	54	17	52
Gentamycin	46	25	51
Amoxicillin	38	14	50
Cefixime	41	12	48
Tetracycline	36	25	46
Cefoxitin	44	15	49

Discussion

Among these, *E. coli* is the most significant reservoir because it colonizes the gastrointestinal tracts of people and animals and is the organism that is the subject of the most research globally [14]. Raw milk is the main cause of *Escherichia coli* infections. Raw milk is a possible source of several food-borne diseases, while also containing critical elements

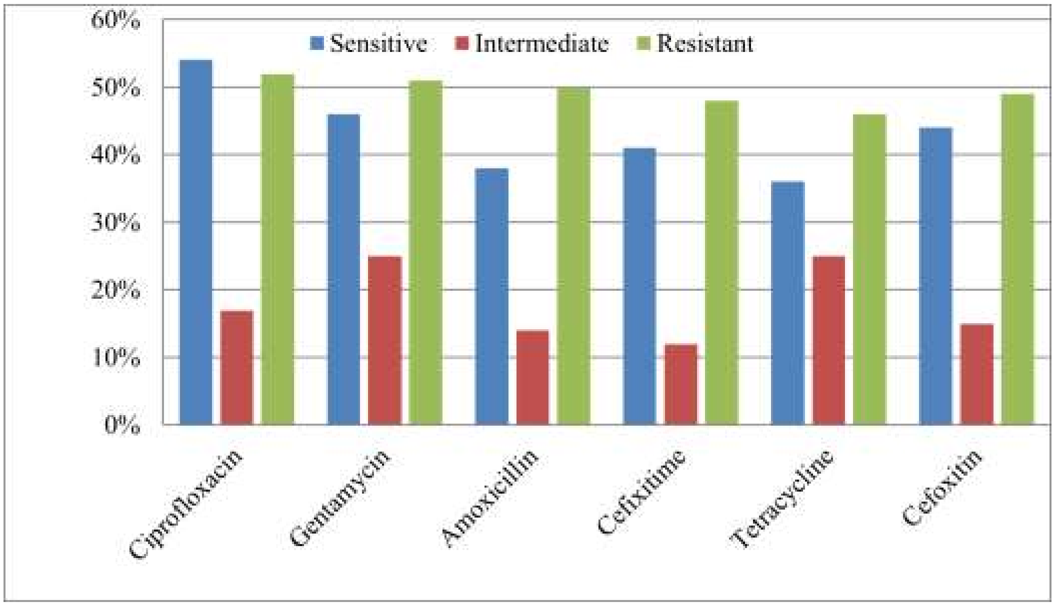

Fig. 3: Antibiotic susceptibility testing of *Escherichia coli* isolates

Fig. 4: Growth of colistin-resistant *Escherichia coli* isolates on colistin-containing media

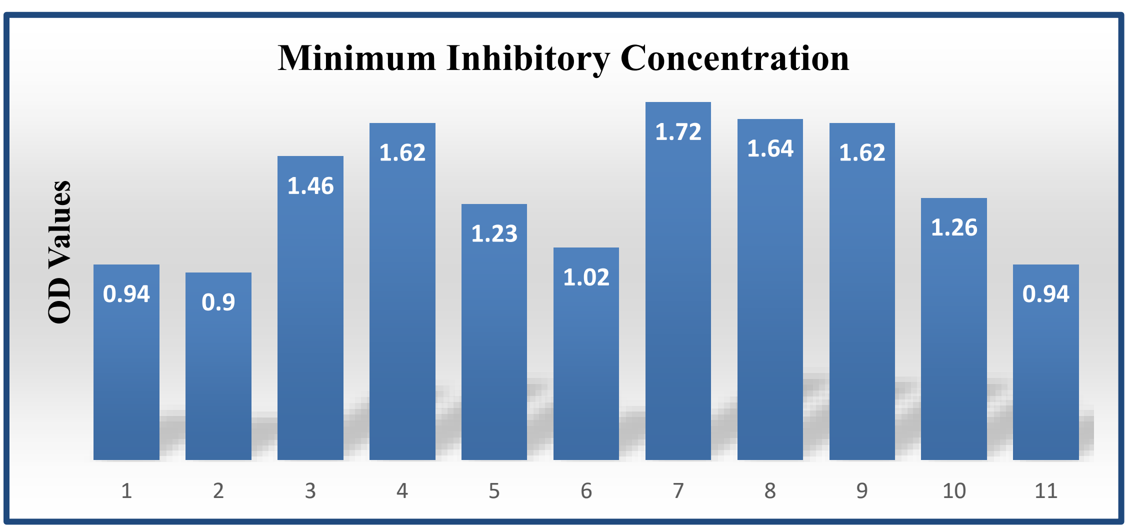

Fig. 5: Determination of minimum inhibitory concentration (MIC) of *Escherichia coli* isolates

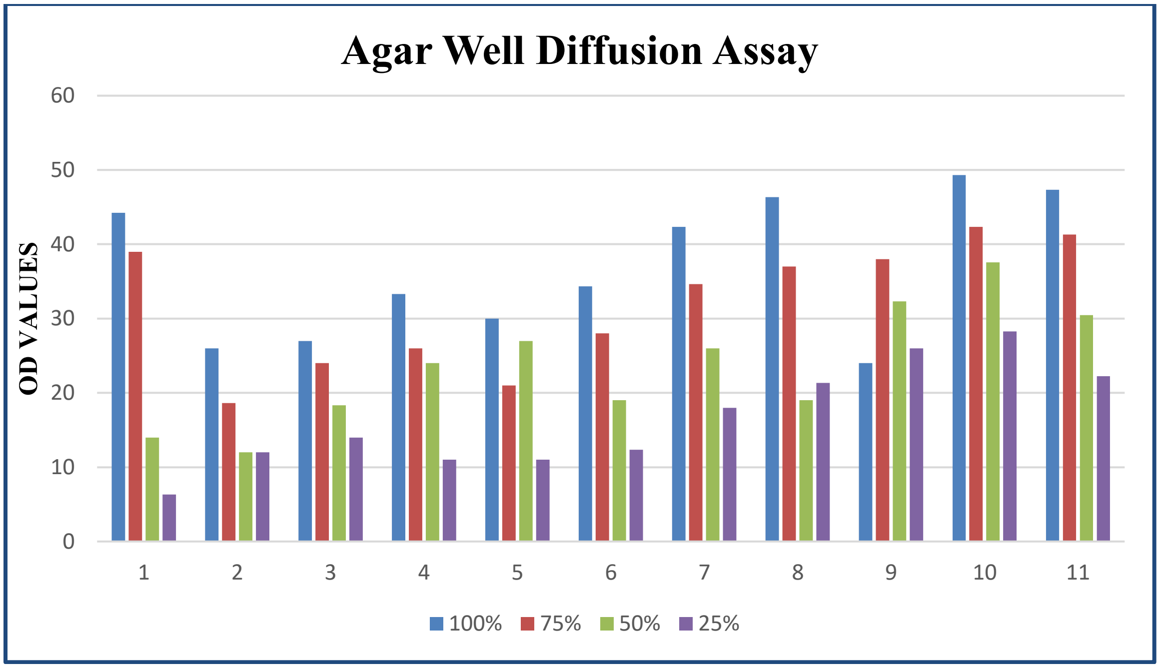

Fig. 6: Agar well diffusion assay of *Escherichia coli* isolates

including calcium, proteins, and vitamins that strengthen the local immune response [15]. Several milk- and food-borne infections, such as brucellosis, TB, and typhoid fever, have been reported in industrialized nations, particularly in underdeveloped ones characterized by raw milk and its byproducts [16].

A study in Pakistan (Sindh, Karachi) assessed 270 raw-milk samples for microbiological contamination (not necessarily E. coli prevalence alone) as part of a safety assessment. In Iran, a study of raw milk found E. coli in 42 % of raw milk samples. In a global or regional context, a study from Ethiopia found E. coli prevalence in raw milk chain samples of ~15.3 % (collectors) and 4.3 % (farm level) in one value-chain analysis. In Pakistan's Peshawar region, an investigation into raw/unpasteurized dairy milk reported prevalence and resistance profiles of multidrug-resistant E. coli.

The focus of current research is on the colistin resistance problem, which is becoming more and more of a problem worldwide, particularly with *E. coli,* which affects people. Therefore, the present study was conducted for the isolation and molecular characterization of *Escherichia coli* that harbored the colistin resistance gene (MCR-1) from raw milk. In this current study, 100 samples of raw milk were collected from milk vending shops in different areas of Faisalabad. For the isolation of *E. coli,* the samples of raw milk were inoculated on MacConkey agar plates and incubated overnight at 37 ºC. After 24 hours of incubation, the bacterial growth was checked, and the culture was re-streaked on EMB agar for pure culture. Following Gram's staining and a microscopic examination, the morphological and cultural properties of the *E. coli* were used to identify them [17].

According to Mondal, Khare [18] and Tahmasebi, Dehbashi [19] the biochemical analysis was carried out by doing the catalase test, oxidase test, TSI test, and Indole test. Furthermore, molecular characterization and genomic amplification using the PCR technique were used to find the colistin resistance gene mcr-1. In the current study, *Escherichia coli* was detected in 60 out of the 100 samples. On MacConkey agar, the colonies were discovered pink, while on EMB agar, they showed a metallic green sheen. *Escherichia coli* isolation from raw/unpasteurized milk was discussed in several prior works [20]. In their research, they calculated the high incidence of *E. coli* from raw milk at 33.9%, 58%, and 63% compared to all other Gram-negative bacteria.

Lemongrass *(Cymbopogon citratus)* essential oil demonstrated pronounced in vitro inhibition of E. coli, with maximum zones of 44 mm—greater than those reported in several previous investigations. The antibacterial effect is likely attributable to major constituents such as citral (a mixture of the isomers geranial and neral) and related terpenoids. These lipophilic molecules are known to integrate into the bacterial lipid bilayer, disrupting membrane integrity, increasing permeability, and causing leakage of ions and intracellular proteins. Such disruption can impair proton-motive force, enzyme activity, and ultimately lead to cell death.

Numerous research articles have extensively investigated the antibacterial properties of lemongrass essential oil against colistin-resistant *E. coli* strains isolated from raw milk, employing agar well diffusion assays. These studies offer valuable insights into the potential of lemongrass essential oil as an antimicrobial agent against antibiotic-resistant pathogens [21].

The antimicrobial effect of lemongrass essential oil is promising; however, the study relies on very high concentrations (up to 100%). Whether comparable concentrations can be achieved in food matrices, topical formulations, or clinical settings without compromising sensory qualities, toxicity limits, or regulatory thresholds.

For instance, documented Noenchat, Srithong [22] a mean inhibition zone of 18.5 ± 2.1 mm when evaluating the susceptibility of colistin-resistant E. coli isolates to lemongrass essential oil via agar well diffusion. Similarly, Milanović, Sabbatini [23] observed inhibition zones ranging from 16.2 to 21.8 mm, with an average of 19.4 mm, against colistin-resistant E. coli strains.

These findings align with Phuadraksa, Wichit [24] results, indicating a mean inhibition zone of 20.1 ± 1.5 mm for lemongrass essential oil against colistin-resistant E. coli isolates. Additionally, Yasir, Nawaz [25] highlighted the significant antibacterial effect of lemongrass essential oil, with a mean inhibition zone of 17.8 ± 1.9 mm, underscoring its potential in combating antibiotic-resistant E. coli strains.

Nevertheless, variations in lemongrass essential oil potency have been observed across studies. For instance, reported Kocsis, Gulyás [26] a slightly lower mean inhibition zone of 15.6 ± 1.3 mm against colistin-resistant *E. coli* isolates. These discrepancies may stem from differences in oil composition, assay conditions, and bacterial strains utilized in the investigations.

While the agar-well diffusion assay demonstrates the antimicrobial effect of lemongrass essential oil, the study would be significantly strengthened by including mechanistic evidence, such as membrane-integrity assays (e.g., propidium-iodide uptake) or electron microscopy to confirm bacterial cell-wall or membrane damage.

Future studies should focus on evaluating the synergistic potential of lemongrass essential oil in combination with conventional antibiotics, as such interactions could lower effective dosages and help mitigate the development of antimicrobial resistance.

In summary, findings from agar well diffusion assays across diverse research articles collectively support the significant antibacterial activity of lemongrass essential oil against colistin-resistant *E. coli* strains isolated from raw milk. Future research should delve into elucidating its mechanisms of action and optimizing its application as an alternative therapeutic approach against antibiotic resistance in foodborne pathogens.

Recently, it was shown that plasmid-encoded genes drive a new colistin mechanism known as mobilized colistin resistance genes (*mcr* genes). However, the Enterobacteriaceae MCR-1 gene was discovered to be the first plasmid-mediated colistin resistance gene in China's livestock, raw foods, and people in November 2015. In contrast to Amábile-Cuevas [27] work, where *Escherichia coli,* which had the MCR-1 gene, was discovered to be resistant to the antibiotic colistin, our investigation revealed the MCR-1 gene to be present in 4 of the 60 bacterial isolates. 8.6% of the isolates from the 35 *Escherichia coli* strains that had been identified in a different investigation were found to be MCR-1 resistant [28]

Escherichia coli containing the colistin resistance gene (MCR-1) has been found in people, animals, and the environment. The presence of bacteria with the mcr-1 gene in the environment has made it feasible for Enterobacteriaceae containing the MCR-1 gene to enter people through food. It's important to keep an eye on how quickly this resistance is developing because of the rising levels of resistance. To determine if MCR-1 was present, this study set out to evaluate the *E. coli* isolates. Detection of the mcr-1 gene was planned but not completed because the PCR equipment was unavailable during the study period. Consequently, no molecular confirmation of mcr-1 was obtained. The presence of colistin resistance in these isolates should therefore be considered presumptive and requires verification in future work

Conclusion

The frightening discovery of *mcr-1*-harboring *E. coli* from raw milk presents a risk to public health. The presence of numerous resistance genes in MDR Gram-negative bacteria from dairy cattle signals the entry of antibiotics used as a last resort and shows how the bacteria migrated from the veterinary to the human population. Therefore, in order to combat MDR bacteria, antimicrobial monitoring plans and the sensible use of medicines in both humans and animals are urgently needed. Lemon Grass essential oil is used to treat the colistin-resistant *E. coli* in future prospects.

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Conflict of interest

The authors declare no conflict of interest.

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