# NEW KNOWLEDGE OF FOOD MICROBIOLOGY IN ASIA, VOLUME II

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# NEW KNOWLEDGE OF FOOD MICROBIOLOGY IN ASIA, VOLUME II

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# Editorial: New knowledge of food microbiology in Asia, volume II

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**food microbiology, sequencing, LAMP, antibiotic resistance, probiotic potential, Asia**

Editorial on the Research Topic
New knowledge of food microbiology in Asia, volume II

In recent years, food microbiology has received widespread attention in Asia from many different directions. Several years ago, we collaborated with the journal *Frontiers in Microbiology* to launch a Research Topic New Knowledge of Food Microbiology in Asia, and got a good response from young scientists in this field. To continue in view of new developments, we relaunched the same Research Topic in November 2021.

In this volume, 17 manuscripts were submitted and all of them were strictly peer-reviewed. Finally, 14 manuscripts were accepted for publication. In research subjects ranged from Pseudomonas fluorescens and lactic acid in milk, Lactiplantibacillus plantarum in fermented foods, Escherichia coli and Proteus mirabilis in pigs and cattle, microbial contamination risk in pork production, general microbial community composition in different foods, and further. In these studies, some modern methodologies, e.g., high-throughput sequencing, were applied.

Mahata et al. characterized Sterigmatocystin produced by Aspergillus species from the Nidulantes Section in Foeniculum vulgare. Zhang, Lai et al. screened and evaluated lactic acid bacteria with probiotic potential from raw milk. Qu et al. sequenced Proteus mirabilis isolates recovered from pig farms and identified 95 virulence factors. Zhang, Bai et al. characterized Shiga toxin-producing Escherichia coli derived from a cattle farm. Yan et al. studied the persistent effects of acute exposure to AFB1 on rat liver and identified several regulatory factors, that is, genes Lama5, Gtse1, Fabp4, and Bcl6. Tao et al. analyzed the global prevalence of foodborne pathogens exhibiting antibiotic resistance and biofilm formation using meta-analysis. Sun et al. assessed the probiotic potentials of Lactiplantibacillus plantarum strains isolated from Chinese traditional fermented food through phenotypic and genomic analyses. Yang, Qiu et al. studied the dynamical changes of bacterial communities in Manila clam (Ruditapes philippinarum) during refrigerated storage. Du et al. tracked the complex community structures and regional variations of psychrotrophic bacteria in raw milk by single-molecule real-time sequencing and traditional cultivation techniques. Bai et al. made a genotyping diarrheagenic Escherichia coli based on CRISPR loci diversity and pathogenic potential. Yang, Zhao, et al. performed a risk analysis of microbial contamination risk in pork production using the quantitative exposure assessment model. Wang et al. evaluated

chlorine tolerance and cross-resistance to antibiotics in poultry-associated Salmonella isolates in China, while Liu et al. studied the effect of bacterial resistance on Escherichia coli of in local large-scale pig farms. Bu et al. established a loop-mediated isothermal amplification (LAMP) assay for the rapid detection of Pseudomonas fluorescens in raw milk.

Taken together, we hope this volume provides some new insight into food microbiology in Asian countries.

## Author contributions

The author confirms being the sole contributor of this work and has approved it for publication.

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## Conflict of interest

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Establishment and Evaluation of a Loop-Mediated Isothermal Amplification Assay for Rapid Detection of *Pseudomonas fluorescens* in Raw Milk

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Raw milk is susceptible to microbial contamination during transportation and storage. *Pseudomonas fluorescens* producing heat-resistant enzymes have become the most common and harmful psychrophilic microorganisms in the cold chain logistics of raw milk. To rapidly detect *P. fluorescens* in raw milk, the protease gene *apr*X was selected as a detection target to construct a set of primers with strong specificity, and a loop-mediated isothermal amplification (LAMP) assay was established. The detection thresholds of the LAMP assay for pure cultured *P. fluorescens* and pasteurized milk were $2.57 \times 10^2$ and $3 \times 10^2$ CFU/mL, respectively. It had the advantages over conventional method of low detection threshold, strong specificity, rapid detection, and simple operation. This LAMP assay can be used for online monitoring and on-site detection of *P. fluorescens* in raw milk to guarantee the quality and safety of dairy products.

**Keywords:** ***P. fluorescens*, raw milk, LAMP, detection, rapid**

## INTRODUCTION

Dairy products are rich in all kinds of nutrients necessary for the human body and are easy to be digested and absorbed, so they are increasingly favored by consumers. Raw milk, the main material of dairy products, has a direct impact on the quality and safety of products. *Pseudomonas fluorescens* are types of psychrotrophic bacteria that can grow and reproduce at a low temperature. They exist widely in all links of dairy processing and production, which may cause potential hazards to raw milk (Vithanage et al., 2016). With the application of cold chain logistics in the dairy industry, *P. fluorescens* with a cold-tolerant characteristic occupy an advantage in the flora structure of raw milk and have become the main spoilage bacteria leading to the degradation of raw milk quality (Neubeck et al., 2015). In addition, some extracellular enzymes secreted by *P. fluorescens* can withstand high temperatures in the process of pasteurization and ultra-high temperature (UHT) sterilization (Alves et al., 2016). During the later storage of dairy products, the residual enzymes play a role in the hydrolysis of milk protein and fat, resulting in the reduction of nutritional value and adverse sensory changes, which shorten the shelf life (Stoeckel et al., 2016). The harm of

*P. fluorescens* and their thermostable enzymes on dairy products has been widely studied (Barbano et al., 2006; Matéos et al., 2015; Zhang et al., 2020), which is considered to be one of the negative factors restricting the development of the dairy industry. Therefore, the monitoring and control of *P. fluorescens* in raw milk are significant to ensure the safety of dairy products.

The traditional counting method cannot detect *P. fluorescens* in raw milk quickly and specifically, which is difficult to meet the needs of real-time and on-site detection in the process of dairy production. In recent years, a large number of studies have been devoted to applying rapid detection technologies such as polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP), and enzyme-linked immunosorbent assay (ELISA) (Hsieh et al., 2014; Hashimoto et al., 2016; Nagaraj et al., 2016; Madrid et al., 2021; Maier et al., 2021). PCR has been developed rapidly and is considered to be an accurate and reliable detection method. However, it is necessary to go through cumbersome temperature change procedures, and the results should be observed by electrophoresis in the process of PCR. As for the LAMP assay, at least four specific primers should be designed targeting six specific regions of the target gene (Niessen et al., 2013), and the amplification reaction will be completed under isothermal conditions for dozens of minutes with Bst DNA polymerase. The products of LAMP can be determined in several forms, such as agarose gel electrophoresis or turbidity measurement. In addition, a more simple and intuitive method is adding fluorescent dyes to the reaction system, and the final color difference of the system can be used to judge the occurrence of an amplification reaction directly (Wong et al., 2018). The LAMP method is superior in terms of its high specificity and low detection limit, and the equipment required for detection is simple. At present, the detection of *P. fluorescens* in raw milk based on LAMP is still in the exploratory stage. In this study, a set of specific LAMP primers was designed, and a rapid and efficient detection protocol of *P. fluorescens* in raw milk was established and evaluated, which was crucial to strengthening the quality control of dairy products.

# MATERIALS AND METHODS

## Bacterial Strains and Culture Conditions

Milk-derived protease-producing *P. fluorescens* CICC 23250, CICC 23251, CICC 23253, and PF were the target strains in detection, which were inoculated into Luria–Bertani (LB) broth (QingDao Hope Bio-Technology, Shandong, China) containing 10 g of tryptone, 5 g of yeast extract powder, and 10 g of sodium chloride and were shaking cultured at 150 rpm at 25°C for 24 h. *Staphylococcus aureus*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Pseudomonas putida*, which are common contaminative bacteria in milk, were used to evaluate the specificity of the LAMP assay. *S. aureus* and *L. monocytogenes* were cultured in brain heart infusion (BHI) medium (Qingdao Hope Bio-Technology, Shandong, China) containing 10 g of peptone, 12.5 g of brain infusion powder, 5 g of heart infusion powder, 5 g of sodium chloride, 2 g of glucose, and 2.5 g of disodium phosphate at 37°C for 24 h, and the other four strains were cultivated with shaking in LB broth at 37°C for 24 h.

## Extraction of Bacterial DNA

DNA was extracted using the Bacterial Genomic DNA Extraction Kit (Solarbio, Beijing, China), and the extracted genome was used as a template in the LAMP amplification system.

## Design and Synthesis of a Primer Set for Loop-Mediated Isothermal Amplification

The complete sequence of the protease gene *apr*X of *P. fluorescens* was submitted to Clustal Omega[1] for online multiple sequence alignment analysis. The part of the sequences with high conservatism were selected. Primer Explorer V5 software was used to design LAMP primers, including two outer primers (F3 and B3), two inner primers (FIP and BIP), and two loop primers (LF and LB). A primer set was synthesized by Shanghai Sangon Biotech. The primer sequences were listed in **Table 1**.

## Optimization of Loop-Mediated Isothermal Amplification Reaction System

The initial added amount of each component and the reaction conditions in the LAMP system were referred to previous studies (Notomi et al., 2000; Nagamine et al., 2002) and modified slightly. The volume of the system was 25 μL, containing 12.5 μL of 2 × LAMP PCR Master Mix (Sangon Biotech, Shanghai, China), 0.5 μL of each outer primer, 2 μL of each inner primer, 1 μL of each loop primer, 2 μL of DNA template, 0.5 μL of Bst DNA polymerase, and double-distilled $H_2O$ ($ddH_2O$) (Solarbio, Beijing, China). The system reacted at 65°C for 60 min. In order to achieve the optimum detection efficiency of the primer set, the LAMP detection system was optimized by taking *P. fluorescens* CICC 23250 as the representative strain. The optimization variables included the Bst DNA polymerase (Sangon Biotech, Shanghai, China) content (0.0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6 μL), $Mg^{2+}$ (New England BioLabs, Beijing, China) concentration (0.0, 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0 mmol/L), deoxynucleotide triphosphates (dNTPs) (Solarbio, Beijing, China) concentration (0.0, 0.4, 0.8, 1.2, 1.6, and 2.0

[1] www.ebi.ac.uk/clustalw

**TABLE 1 |** Sequences of the primer set for *Pseudomonas fluorescens* protease gene *apr*X.

| Primer | Sequence (5′–3′) |
|---|---|
| F3 | GTACCTGACCAACAACAGCT |
| B3 | GCTCTCGCTCCAGTAGCT |
| FIP | AGGGTATGGCCGATTTCGTGGGGAACAAGACCCCGGACCT |
| BIP | GCTCACCCTGGCGACTACAACGTGTAGCCGCGCGTGTC |
| LF | TGCCGGCCGTAGTTGTTC |
| LB | GGCAACCCGACCTACAACG |

*F3 and B3 were the outer primers, FIP and BIP were the inner primers, and LF and LB were loop primers.*

mmol/L), reaction temperature (60, 61, 62, 63, 64, and 65°C), and reaction time (20, 30, 40, 50, 60, and 70 min). An identical volume of $ddH_2O$ was used to replace the DNA template as a negative control. Bst DNA polymerase was inactivated at 80°C for 10 min to terminate the reaction. The LAMP reaction products were analyzed using 2% agarose gel electrophoresis at 100 V for 40 min, and amplification effects were judged by the gray values of electrophoretic bands with the help of Image J software.

## Specificity Evaluation of Loop-Mediated Isothermal Amplification Assay

Under the optimized reaction conditions, four strains of *P. fluorescens* and six strains of common pollutant bacteria were amplified with the designed primer set. The specificity of the LAMP assay system was analyzed by visualization and electrophoresis. One microliter of SYBR Green I (Solarbio, Beijing, China) was added into the LAMP reaction system, and the color change was observed directly to determine *P. fluorescens*. Another 5 μL of the reaction product was detected by 2% agarose gel electrophoresis at 100 V for 40 min to judge the specificity according to the generation of the characteristic map. *P. fluorescens* CICC 23250 was used as a positive control and $ddH_2O$ acted as a negative control.

## Detection Limit Measurement of the Loop-Mediated Isothermal Amplification Assay

Pure culture and artificially contaminated pasteurized milk with *P. fluorescens* CICC 23250 were taken for 10-fold gradient dilution. The DNA of *P. fluorescens* with different concentrations was extracted, and the concentration of bacteria was calculated by plate counting. Amplification was performed in the optimized LAMP system, and the detection limit of the LAMP system was determined by 2% agarose gel electrophoresis.

# RESULTS

## Optimization of the Loop-Mediated Isothermal Amplification Reaction System

The electrophoretic bands of products under different LAMP amplification conditions were shown in **Figure 1**. When Bst DNA polymerase was absent in the reaction system, there was no band present, while the band reached maximum brightness with Bst DNA polymerase content of 0.6 μL (**Figure 1A**). The results in **Figure 1B** revealed that, when the $Mg^{2+}$ concentration was 4.0 mmol/L, an electrophoretic strip was detectable, which was brightest at a concentration of 6.0 mmol/L. dNTPs are the main materials for DNA synthesis determining the reaction degree of amplification. The brightest amplification band was observed at dNTPs concentration of 0.8 mmol/L (**Figure 1C**). It could be seen from **Figure 1D** that the brightness of the amplification band in lane 4 was more intense than that in other lanes, so the best reaction temperature was 63°C. For the reaction time, the band was visible at 20 min, but amplification was still incomplete. With the extension of the reaction time, there were no significant differences in the light intensity of the strips, indicating that the amplification was basically completed at 30 min. Therefore, 30 min was selected as the best reaction time to shorten the detection time (**Figure 1E**). In view of the above observations, the optimal LAMP assay system parameters were determined as 0.6 μL Bst DNA polymerase, 6.0 mmol/L $Mg^{2+}$, 0.8 mmol/L dNTPs, and reaction temperature and time of 63°C and 30 min, respectively.

## Specificity of the Loop-Mediated Isothermal Amplification Assay

The strains of *P. fluorescens* and common polluting bacteria (*S. aureus*, *E. coli*, *L. monocytogenes*, *S. typhimurium*, *P. aeruginosa*, and *P. putida*) in milk were amplified in the optimum LAMP reaction system. The results of the visualization and electrophoresis analysis were shown in **Figures 2**, **3**, respectively. It was found that the amplification systems of the LAMP primers for the four *P. fluorescens* strains were positive (green), while presented negative (orange) for other contaminated strains, which suggested that the LAMP assay system would not be interfered by non-target strains (**Figure 2**). In **Figure 3**, the specific bands were observed for the four *P. fluorescens* strains, but no bands were found for the non-target strains. This indicated that the LAMP primer set had strong specificity for *P. fluorescens*, which was consistent with the results of visualization.

## Detection Limits of the Loop-Mediated Isothermal Amplification Assay

LAMP amplification was performed in pure culture and contaminated pasteurized milk with different concentrations of *P. fluorescens*. The amplified products were subjected to 2% agarose gel electrophoresis. For *P. fluorescens* in pure culture, the concentrations ranging from $2.57 \times 10^7$ to $2.57 \times 10^2$ colony-forming unit (CFU)/mL could be amplified by the LAMP assay system. When the concentration of the reaction template was lower than $2.57 \times 10^2$ CFU/mL, no band was detected, which suggested that the LAMP detection limit for pure cultured *P. fluorescens* was $2.57 \times 10^2$ CFU/mL (**Figure 4A**). As shown in **Figure 4B**, the target amplification bands were observed in lanes 2–7, but no band was presented in lane 8, which demonstrated that the detection limit of the LAMP system for *P. fluorescens* in pasteurized milk was $3 \times 10^2$ CFU/mL.

# DISCUSSION

With the development and application of low-temperature preservation in the food industry, psychrophilic bacteria have become the main microorganisms leading to the corruption and deterioration of food. As the most common and hazardous cryophiles in raw milk, *P. fluorescens* can secrete extracellular enzymes with strong heat-resistant properties, which may reduce the nutritional value of food and cause foodborne diseases, affecting the quality and shelf life of dairy products seriously. At present, studies on the harmful effects of *P. fluorescens* and their

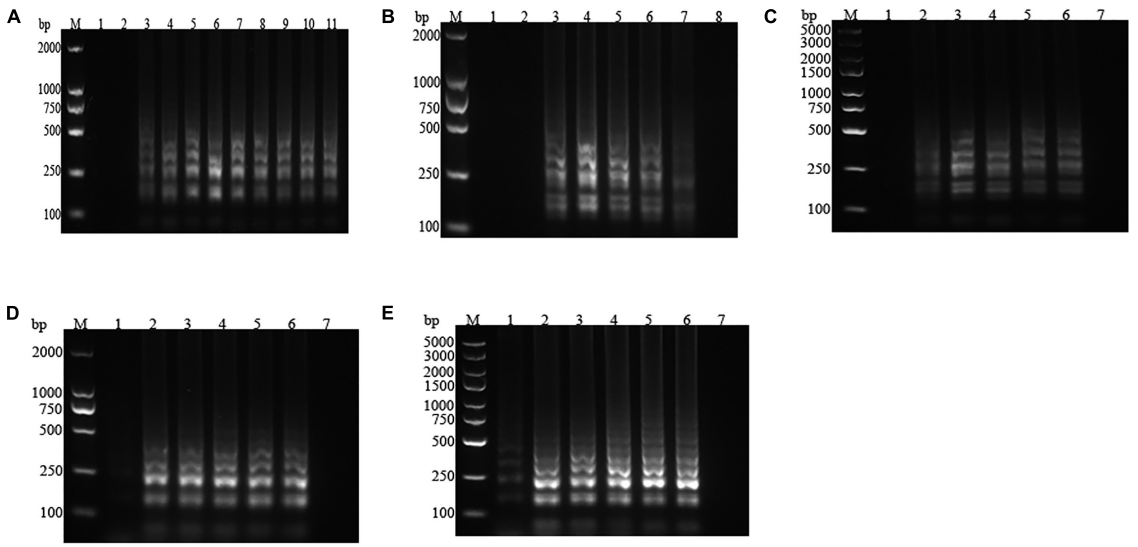


**FIGURE 1 |** Effects of different Bst DNA polymerase contents, $Mg^{2+}$ concentrations, deoxynucleotide triphosphates (dNTPs) concentrations, reaction temperatures, and reaction times on the electrophoretic bands of the loop-mediated isothermal amplification (LAMP) products. **(A)** Bst DNA polymerase content. *1*: negative control; *2–11*: 0.0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6 μL. **(B)** $Mg^{2+}$ concentration. *1–7*: 0.0, 2.0, 4.0, 6.0, 8.0, 10.0, and 12.0 mmol/L; *8*: negative control. **(C)** dNTPs concentration. *1–6*: 0.0, 0.4, 0.8, 1.2, 1.6, and 2.0 mmol/L; *7*: negative control. **(D)** Reaction temperature. *1–6*: 60, 61, 62, 63, 64, and 65°C; *7*: negative control. **(E)** Reaction time. *1–6*: 20, 30, 40, 50, 60, and 70 min; *7*: negative control. *M* represents the D2000 plus DNA Ladder (Solarbio, Beijing, China).

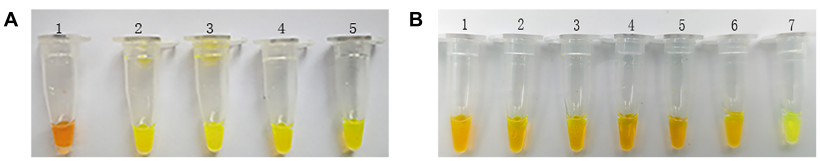


**FIGURE 2 |** Visualization results of the loop-mediated isothermal amplification (LAMP) assay for *Pseudomonas fluorescens* and common contaminating bacteria in raw milk. **(A)** *P. fluorescens*. *1*: negative control; *2–5*: *P. fluorescens* CICC 23250, CICC 23251, CICC 23253, and PF. **(B)** Common contaminating bacteria. *1–6*: *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhimurium*; *7*: positive control.

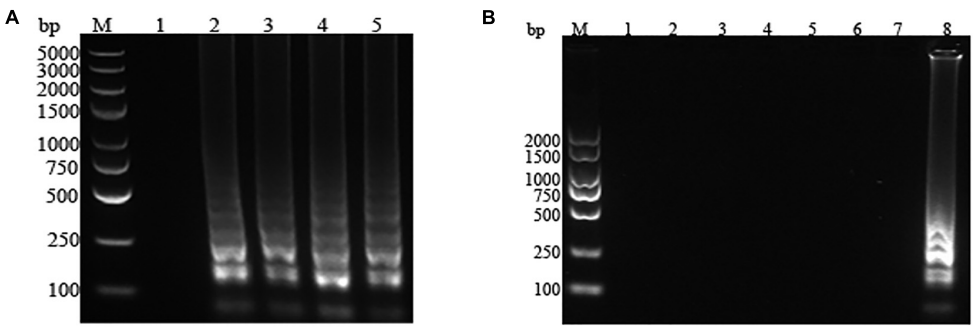


**FIGURE 3 |** Electrophoresis map of the loop-mediated isothermal amplification (LAMP) assay for *Pseudomonas fluorescens* and common contaminating bacteria in raw milk. **(A)** *P. fluorescens*. *1*: negative control; *2–5*: *P. fluorescens* CICC 23250, CICC 23251, CICC 23253, and PF. **(B)** Common contaminating bacteria. *1*: negative control; *2–7*: *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli*, and *Salmonella typhimurium*; *8*: positive control. *M* represents the D2000 DNA Ladder (Solarbio, Beijing, China).

thermostable enzymes to dairy products are mainly focused on pasteurized milk and UHT milk. Aging gels, protein hydrolysis, fat floating, and bitterness are common quality problems in UHT milk (Liu et al., 2007; Baglinière et al., 2013; Martin et al., 2018) and restrict the progress of the dairy industry. Therefore, it is essential to realize rapid detection of *P. fluorescens* in raw milk in order to ensure quality and safety in the dairy industry.

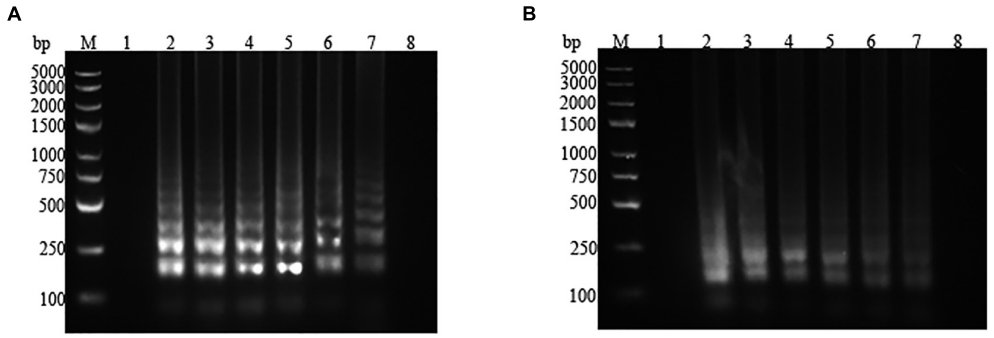


**FIGURE 4 |** Electrophoresis map of the loop-mediated isothermal amplification (LAMP) assay for pure culture and artificially contaminated pasteurized milk with different concentrations of *Pseudomonas fluorescens.* **(A)** Pure culture. *1*: negative control ($ddH_2O$); *2–8*: $2.57 \times 10^7$, $2.57 \times 10^6$, $2.57 \times 10^5$, $2.57 \times 10^4$, $2.57 \times 10^3$, $2.57 \times 10^2$, and $2.57 \times 10^1$ CFU/mL. **(B)** Pasteurized milk. *1*: negative control ($ddH_2O$); *2–8*: $3 \times 10^7$, $3 \times 10^6$, $3 \times 10^5$, $3 \times 10^4$, $3 \times 10^3$, $3 \times 10^2$, and $3 \times 10^1$ CFU/mL. *M* represents the D2000 plus DNA Ladder.

The traditional microbial counting method cannot meet the actual needs of the dairy enterprise due to its time-consuming process and inaccuracy. With the development of molecular biotechnology, an increasing number of rapid detection methods have been widely used for food microbe detection. LAMP assay relies on at least four especially designed primers and Bst DNA polymerase with strand-displacing activity to complete the amplification process at a constant temperature (Mori et al., 2013), which has a particular advantage over other methods. In the aspect of equipment, the reaction is carried out under thermostatic temperature, and a simple water bath can meet the requirement of temperature control without the need for expensive and sophisticated instruments. Moreover, a stabilized temperature reduces the time of temperature change, which greatly shortens the whole reaction process. With regard to the determination of the reaction products, it can be achieved using agarose gel electrophoresis and judged by the amount of flocculent turbidity or obvious color difference in the reaction system, which is more intuitive and reliable than the traditional plate counting method.

Owing to the short detection time, strong specificity, and simple operation (Notomi et al., 2015), the LAMP assay has the potential for on-site and real-time detection and has been widely used in microbial detection and identification (Wang et al., 2010; Duarte-Guevara et al., 2016). The LAMP assay or new detection approaches on the basis of LAMP have been reported to realize the rapid detection of microorganisms in food. Wang et al. (2013) established an *in situ* LAMP assay to detect *Vibrio parahaemolyticus* from seafood. It was found that the detection rates of the *in situ* LAMP, regular LAMP, and PCR were 100, 93.8, and 70.8%, respectively (Wang et al., 2013). In order to detect *L. monocytogenes* in food, primers targeting the genes *hly*A and *iap* were designed to construct the double LAMP (dLAMP) method. The results illustrated that the detection sensitivity was 10 fg DNA of *L. monocytogenes* per tube with reaction at 63°C for 15 min. At the same time, mineral oil and GoldView II dye were added into the system to avoid aerosol pollution and realize the visualization analysis (Wu et al., 2014). Kim et al. (2021) designed three LAMP primer sets for the detection of *Bifidobacterium longum* subspecies in probiotic products. The detection level was about $10^2$ CFU/mL, which could detect the target subspecies within 45 min specifically and sensitively (Kim et al., 2021). Restriction fragment length polymorphism (RFLP) combined with multiplex LAMP (mLAMP) was used to detect *Salmonella* spp. and *Shigella* spp. in milk. It was shown that the overall detection process could be completed in approximately 20 min. The sensitivity of mLAMP-RFLP for *Salmonella* and *Shigella* strains was up to 100 fg DNA per tube, and the detection limit in artificially contaminated milk was 5 CFU/10 mL (Shao et al., 2011).

The use of LAMP assay for detection in various food products has been reported (Sun et al., 2015; Spielmann et al., 2019; Liu et al., 2021). However, applying LAMP to detect *P. fluorescens* in raw milk is rare. The exploitation of a specific LAMP assay for detecting *P. fluorescens* in raw milk has attracted much attention. In our previous study, a LAMP assay system was established based on the conserved gene sequence of *P. fluorescens* 38 lipases, and we evaluated its application in raw milk (Xin et al., 2017). Although the reaction time of this LAMP system was 50 min, the dairy enterprise expected a faster detection speed. In this study, the primers were designed targeting the *P. fluorescens* protease gene, and the LAMP detection system was established and optimized. The results of the visual analysis and electrophoresis confirmed that the primer set had high specificity for *P. fluorescens*. For *P. fluorescens* in pure culture and pasteurized milk, the detection limits of the LAMP assay were $2.57 \times 10^2$ and $3 \times 10^2$ CFU/mL, respectively, which were applicable for the detection of *P. fluorescens* in raw milk during the actual production process. It was worth mentioning that, compared with the previous study (Xin et al., 2017), the addition of loop primers shortened the detection time to 30 min and improved the efficiency significantly.

## CONCLUSION

A LAMP assay system for rapid detection of *P. fluorescens* in raw milk was established and evaluated. The optimal reaction

conditions were 0.6 μL Bst DNA polymerase, 6.0 mmol/L $Mg^{2+}$, 0.8 mmol/L dNTPs, and 63°C reaction temperature. The LAMP detection system had strong specificity and low detection limit. Compared with the existing detection methods, the detection process of this LAMP assay could be completed in 30 min, which was significant in strengthening the quality control of dairy products.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## AUTHOR CONTRIBUTIONS

YB and WQ conducted the experiments. YB wrote the manuscript. ZZ, TL, and PG supervised the experiments. LZ, YH, and HY revised the manuscript. All authors contributed to the article and approved the submitted version.

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## REFERENCES

Alves, M. P., Salgado, R. L., Eller, M. R., Vidigal, P. M. P., and Carvalho, A. F. (2016). Characterization of a heat-resistant extracellular protease from *Pseudomonas fluorescens* 07A shows that low temperature treatments are more effective in deactivating its proteolytic activity. *J. Dairy Sci.* 99, 7842–7851. doi: 10.3168/jds.2016-11236

Baglinière, F., Matéos, A., Tanguy, G., Jardin, J., Briard-Bion, V., Rousseau, F., et al. (2013). Proteolysis of ultra high temperature-treated casein micelles by AprX enzyme from *Pseudomonas fluorescens* F induces their destabilisation. *Int. Dairy J.* 31, 55–61. doi: 10.1016/j.idairyj.2013.02.011

Barbano, D. M., Ma, Y., and Santos, M. V. (2006). Influence of raw milk quality on fluid milk shelf life. *J. Dairy Sci.* 89, E15–E19. doi: 10.3168/jds.S0022-0302(06)72360-8

Duarte-Guevara, P., Duarte-Guevara, C., Ornob, A., and Bashir, R. (2016). On-chip PMA labeling of foodborne pathogenic bacteria for viable qPCR and qLAMP detection. *Microfluid Nanofluid* 20:114. doi: 10.1007/s10404-016-1778-2

Hashimoto, K., Nakamura, N., and Ito, K. (2016). Rapid, highly sensitive and highly specific gene detection by combining enzymatic amplification and DNA chip detection simultaneously. *Sen. Biosen. Res.* 8, 27–30. doi: 10.1016/j.sbsr.2016.03.004

Hsieh, K., Mage, P. L., Csordas, A. T., Eisenstein, M., and Soh, H. T. (2014). Simultaneous elimination of carryover contamination and detection of DNA with uracil-DNA-glycosylase-supplemented loop-mediated isothermal amplification (UDG-LAMP). *Chem. Commun.* 50, 3747–3749. doi: 10.1039/c4cc00540f

Kim, M. J., Shin, S. W., Kim, H. B., Kim, E., and Kim, H. Y. (2021). Direct loop-mediated isothermal amplification (LAMP) assay for rapid on-site detection of *Bifidobacterium longum* subspecies *longum*, *infantis*, and *suis* in probiotic products. *Food Chem.* 346:128887. doi: 10.1016/j.foodchem.2020.128887

Liu, M., Wang, H., and Griffiths, M. W. (2007). Regulation of alkaline metalloprotease promoter by *N*-acyl homoserine lactone quorum sensing in *Pseudomonas fluorescens*. *J. Appl. Microbiol.* 103, 2174–2184. doi: 10.1111/j.1365-2672.2007.03488.x

Liu, R., Zhang, Y., Ali, S., Haruna, S. A., He, P., Li, H., et al. (2021). Development of a fluorescence aptasensor for rapid and sensitive detection of *Listeria monocytogenes* in food. *Food Control.* 122:107808. doi: 10.1016/j.foodcont.2020.107808

Madrid, R., García-García, A., Cabrera, P., González, I., Martín, R., and García, T. (2021). Survey of commercial food products for detection of walnut (*Juglans regia*) by two ELISA methods and real time PCR. *Foods.* 10:440. doi: 10.3390/foods10020440

Maier, C., Hofmann, K., Huptas, C., Scherer, S., Wenning, M., and Lücking, G. (2021). Simultaneous quantification of the most common and proteolytic *Pseudomonas* species in raw milk by multiplex qPCR. *Appl. Microbiol. Biotechnol.* 105, 1693–1708. doi: 10.1007/s00253-021-11109-0

Martin, N. H., Boor, K. J., and Wiedmann, M. (2018). Symposium review: effect of post-pasteurization contamination on fluid milk quality. *J. Dairy Sci.* 101, 861–870. doi: 10.3168/jds.2017-13339

Matéos, A., Guyard-Nicodème, M., Baglinière, F., Jardin, J., Gaucheron, F., Dary, A., et al. (2015). Proteolysis of milk proteins by AprX, an extracellular protease identified in *Pseudomonas* LBSA1 isolated from bulk raw milk, and implications for the stability of UHT milk. *Int. Dairy J.* 49, 78–88. doi: 10.1016/j.idairyj.2015.04.008

Mori, Y., Kanda, H., and Notomi, T. (2013). Loop-mediated isothermal amplification (LAMP): recent progress in research and development. *J. Infect. Chemother.* 19, 404–411. doi: 10.1007/s10156-013-0590-0

Nagamine, K., Hase, T., and Notomi, T. J. M. C. P. (2002). Accelerated reaction by loop-mediated isothermal amplification using loop primers. *Mol. Cell. Probes* 16, 223–229. doi: 10.1006/mcpr.2002.0415

Nagaraj, S., Ramlal, S., Kingston, J., and Batra, H. V. (2016). Development of IgY based sandwich ELISA for the detection of staphylococcal enterotoxin G (SEG), an *egc* toxin. *Int. J. Food Microbiol.* 237, 136–141. doi: 10.1016/j.ijfoodmicro.2016.08.009

Neubeck, M. V., Baur, C., Krewinkel, M., Stoeckel, M., Kranz, B., Stressler, T., et al. (2015). Biodiversity of refrigerated raw milk microbiota and their enzymatic spoilage potential. *Int. J. Food Microbiol.* 211, 57–65. doi: 10.1016/j.ijfoodmicro.2015.07.001

Niessen, L., Luo, J., Denschlag, C., and Vogel, R. F. (2013). The application of loop-mediated isothermal amplification (LAMP) in food testing for bacterial pathogens and fungal contaminants. *Food Microbiol.* 36, 191–206. doi: 10.1016/j.fm.2013.04.017

Notomi, T., Mori, Y., Tomita, N., and Kanda, H. (2015). Loop-mediated isothermal amplification (LAMP): principle, features, and future prospects. *J. Microbiol.* 53, 1–5. doi: 10.1007/s12275-015-4656-9

Notomi, T., Okayama, H., Masubuchi, H., Yonekawa, T., Watanabe, K., Amino, N., et al. (2000). Loop-mediated isothermal amplification of DNA. *Nucleic Acids Res.* 28:e63. doi: 10.1093/nar/28.12.e63

Shao, Y., Zhu, S., Jin, C., and Chen, F. (2011). Development of multiplex loop-mediated isothermal amplification-RFLP (mLAMP-RFLP) to detect *Salmonella* spp. and *Shigella* spp. in milk. *Int. J. Food Microbiol.* 148, 75–79. doi: 10.1016/j.ijfoodmicro.2011.05.004

Spielmann, G., Ziegler, S., Haszprunar, G., Busch, U., Huber, I., and Pavlovic, M. (2019). Using loop-mediated isothermal amplification for fast species delimitation in eels (genus *Anguilla*), with special reference to the European eel (*Anguilla anguilla*). *Food Control* 101, 156–162. doi: 10.1016/j.foodcont.2019.02.022

Stoeckel, M., Lidolt, M., Achberger, V., Glück, C., Krewinkel, M., Stressler, T., et al. (2016). Growth of *Pseudomonas* weihenstephanensis. *Pseudomonas proteolytica* and *Pseudomonas* sp. in raw milk: impact of residual heat-stable enzyme activity on stability of UHT milk during shelf-life. *Int. Dairy J.* 59, 20–28. doi: 10.1016/j.idairyj.2016.02.045

Sun, M., Gao, H., Xiao, X., Chen, J., Liu, C., and Feng, L. (2015). A novel loop-mediated isothermal amplification method for detection of the carrot materials in foods. *Eur. Food Res. Technol.* 241, 295–302. doi: 10.1007/s00217-015-2459-1

Vithanage, N. R., Dissanayake, M., Bolge, G., Palombo, E. A., Yeager, T. R., and Datta, N. (2016). Biodiversity of culturable psychrotrophic microbiota in raw

milk attributable to refrigeration conditions, seasonality and their spoilage potential. *Int. Dairy J.* 57, 80–90. doi: 10.1016/j.idairyj.2016.02.042

Wang, D., Huo, G., Ren, D., and Li, Y. (2010). Development and evaluation of a loop-mediated isothermal amplification (LAMP) method for detecting *Listeria monocytogenes* in raw milk. *J. Food Saf.* 30, 251–262.

Wang, L., Shi, L., Su, J., Ye, Y., and Zhong, Q. (2013). Detection of *Vibrio parahaemolyticus* in food samples using in situ loop-mediated isothermal amplification method. *Gene* 515, 421–425. doi: 10.1016/j.gene.2012.12.039

Wong, Y. P., Othman, S., Lau, Y. L., Radu, S., and Chee, H. Y. (2018). Loop-mediated isothermal amplification (LAMP): a versatile technique for detection of micro-organisms. *J. Appl. Microbiol.* 124, 626–643. doi: 10.1111/jam.13647

Wu, R., Liu, X., Guo, B., Chen, F., and Wang, X. (2014). Development of double loop-mediated isothermal amplification to detect *Listeria monocytogenes* in food. *Curr. Microbiol.* 69, 839–845. doi: 10.1007/s00284-014-0661-1

Xin, L., Zhang, L., Meng, Z., Lin, K., Zhang, S., Han, X., et al. (2017). Development of a novel loop-mediated isothermal amplification assay for the detection of lipolytic *Pseudomonas fluorescens* in raw cow milk from North China. *J. Dairy Sci.* 100, 7802–7811. doi: 10.3168/jds.2017-12740

Zhang, C., Bijl, E., Muis, K. E., and Hettinga, K. (2020). Stability of fat globules in UHT milk during proteolysis by the AprX protease from *Pseudomonas fluorescens* and by plasmin. *J. Dairy Sci.* 103, 179–190. doi: 10.3168/jds.2019-17150

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# Chlorine Tolerance and Cross-Resistance to Antibiotics in Poultry-Associated *Salmonella* Isolates in China

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Chlorine disinfectants have been widely used in the poultry supply chain but this exposure can also result in the development of bacterial tolerance to chlorine and this is often linked to antibiotic cross-resistance. The objectives of this study were to investigate sodium hypochlorite (NaClO) tolerance of *Salmonella* isolated from poultry supply chains and evaluate cross-resistance. We collected 172 *Salmonella* isolates from poultry farms, slaughter houses and retail markets in China during 2019–2020. We found that *S.* Enteritidis, *S.* Kentucky, and *S.* Typhimurium constituted > 80% of our *Salmonella* isolates. Overall, 68% of *Salmonella* isolates were resistant to > 3 antibiotics and *S.* Kentucky displayed a significantly ($p > 0.05$) higher frequency (93.2%) of multidrug resistance than the other serovars. Tolerance to chlorine at MIC > 256 mg/L was detected in 93.6% of isolates (161/172) and tolerant isolates displayed higher decimal reduction times (*D* value) and less ultrastructural damage than did the suspectable strains under chlorine stress. Spearman analysis indicated significant positive correlations between chlorine tolerance (evaluated by the OD method) and antibiotic resistance ($p < 0.05$) to ceftiofur, tetracycline, ciprofloxacin and florfenicol and this was most likely due to efflux pump over-expression. The most frequently detected chlorine resistance gene was *qacEΔ1* (83.1%, $n = 143$) and we found a positive correlation between its presence and MIC levels ($r = 0.66$, $p < 0.0001$). Besides, we found weak correlations between chlorine-tolerance and antibiotic resistance genes. Our study indicated that chlorine disinfectants most likely played an important role in the emergence of chlorine tolerance and spread of antibiotic resistance and therefore does not completely control the risk of food-borne disease. The issue of disinfectant resistance should be examined in more detail at the level of the poultry production chain.

**Keywords: poultry, bacterial resistance, sodium hypochlorite (NaClO), *qacEΔ1*, efflux pump**

## INTRODUCTION

*Salmonella* is a frequent cause of gastroenteritis in humans and is an important public health concern worldwide and in China causes 9.87 M gastroenteritis cases annually (Xiao et al., 2019; Yang et al., 2020a). More than 2600 *Salmonella enterica* serovars have been identified and *S.* Enteritidis, *S.* Typhimurium, *S.* Kentucky, and *S.* Indiana are the most frequent causes of human salmonellosis in China (Andoh et al., 2016; Elnekave et al., 2018; Zeng et al., 2021). Poultry products are the most common vehicles for *Salmonella* transmission and a recent study in China found that 37.5% of poultry samples were contaminated with *Salmonella* (Yang et al., 2020b). Contamination sources include the animals when introduced into the poultry house environment and direct or cross-contamination between poultry carcasses. All these events can result in foodborne salmonellosis when improperly cooked or handled products are consumed (Xiao et al., 2019).

Chlorine disinfection is one of the most common disinfection technologies for controlling the risks of microorganisms in industrial processing (Jia et al., 2020). In this process, bacteria can survive and even reproduce in residual chlorine and are designated chlorine tolerant (Langsrud et al., 2003; Luo et al., 2020). In particular, *Salmonell*a possesses a high level of regeneration capacity in reclaimed water after chlorine disinfection (Li et al., 2013). Recent studies have focused on chlorine tolerance of bacteria in water while few studies addressed contamination on the processed poultry products (Roy and Ghosh, 2017; Bhojani et al., 2018; Wang et al., 2019; Luo et al., 2020). In China, 50–100 mg/L of sodium hypochlorite (NaClO) is commonly used in poultry processing and environmental disinfection while the inactivation of *Salmonella* was limited ($< 1$ Log) and could be due to chlorine tolerance (Jun et al., 2013; Lee et al., 2014; Xiao et al., 2019).

Current studies have also demonstrated that long-term antibiotic usage during animal breeding has led to a marked increase in the levels of antibiotic resistance (Zeng et al., 2021). Antibiotic susceptibility testing of the 318 poultry-associated *Salmonella* revealed that only 5 (1.6%) were susceptible to all 22 tested antibiotics, while 191 (60.1%) exhibited multidrug resistance. Poultry frequently harbor antibiotic-resistant *Salmonella* isolates that can be transmitted to humans via the food chain (Yang et al., 2020a). The emergence of quinolone, tetracycline and extended-spectrum β-lactam-resistant *Salmonella* is a serious public health concern (Mechesso et al., 2020). Cross-resistance between disinfectants and antibiotics is also becoming widespread for bacterial pathogens (Carey and McNamara, 2015; Puangseree et al., 2021). For example, a higher level of chlorine tolerance in antibiotic-resistant *Escherichia coli* (*E. coli*) was found when compared to antibiotic-susceptible strains (Templeton et al., 2013). Bacterial exposure to chlorine increases the expression of efflux pumps and activates *qac* genes that also export chloramphenicols, sulfonamide and β-lactams (Karumathil et al., 2014). However, chlorine tolerance and cross-resistance of *Salmonella* that are present in the poultry supply chain have not been fully investigated.

The focus of the current study was to investigate (i) chlorine tolerance and antibiotic resistance in *Salmonella* spp. isolated from poultry supply chains and (ii) to determine the level of cross-resistance in these isolates. This study examined on a broad scale the important role played by chlorine disinfectants as a non-antibiotic selective pressure for the emergence and spread of antibiotic resistance.

## MATERIALS AND METHODS

### *Salmonella* Isolates and Serovar Identification

We isolated 172 *Salmonella* spp. from poultry supply chains as follows: poultry farms ($n$ = 10), slaughter houses ($n$ = 102) and retail markets ($n$ = 60) in Guangzhou, Shandong and Zhejiang provinces during 2019–2020. *Salmonella* isolates were subcultured and serotyped by slide agglutination using commercial O and H antisera to distinguish *Salmonella* serovars (SSI-Diagnostica, Copenhagen, Denmark; Tianrun Bio-Pharmaceutical, Ningbo, China) in accordance with the White-Kauffmann-Le Minor scheme (Grimont and Weill, 2007; Slotved et al., 2016). All isolates were stored in brain heart infusion (BHI) broth (Becton Dickinson, Franklin Lakes, NJ, United States) containing 20% glycerol at −80°C until use.

### NaClO Tolerance Determinations

#### Determination of Minimum Inhibitory Concentrations and Optical Density

The MIC values for NaClO in the *Salmonella* isolates were measured using broth microdilution. The *E. coli* ATCC 29522 and *S.* Enteritidis CVCC 1806 were included as controls for susceptibility testing. Bacterial suspensions were prepared by suspending 3–5 individual overnight colonies from trypticase soy (TSA) agar (Becton Dickinson) plates into 3 mL of 0.9% saline, equivalent to the turbidity of a 0.5 McFarland standard. The 0.5 McFarland inoculum suspensions were further diluted at 1:100 in Mueller-Hinton (MH) broth (Becton Dickinson). NaClO stock solutions containing 56.8 mg/mL chlorine was purchased from Sangon Biotech (Shanghai, China) and were prepared by dilution in sterile Milli-Q water (PALL, Buckinghamshire, United Kingdom). Chlorine concentrations were determined using a Palintest ChlorSense meter (Gateshead, United Kingdom). Preliminary tests indicated the MIC of *S.* Enteritidis CVCC 1806 to NaClO was 256 mg/L and tolerance to NaClO was defined as $>$ 256 mg/L. These MIC assays for chlorine tolerance were performed in 96-well microtiter test plates containing 100 μL NaClO solution and were inoculated with 100 μL of suspended bacterial cultures to a final inoculum density of 5 Log CFU/mL per well. The plates were sealed using a perforated plate seal and incubated at 37°C for 24 h. The MIC values of NaClO were recorded as the lowest concentration of NaClO where no visible growth was observed. Control wells containing bacteria and lacking chlorine and wells with MH broth were used as positive and negative controls, respectively. Each assay was repeated three times on different days. Cell growth

in the plates was also quantified by measuring the optical density at 600 nm ($OD_{600}$) (Bernardez and de Andrade Lima, 2015; Yang et al., 2020a) using a Polarstar spectrophotometer (Omega, Ortenberg, Germany).

### Inactivation Kinetics

Chlorine susceptible and tolerant isolates were selected based on the MIC and $OD_{600}$ tests and then separately incubated in BHI at 37°C for 24 h and cultured to approximately 9 Log CFU/mL. The initial inoculum level was 5 ± 0.2 Log CFU/mL. Cell suspensions were treated with 100 mg/L of NaClO for 0, 30, 60, 90, and 120 min. Then the suspension was put into a sterile centrifuge tube containing sodium thiosulfate ($Na_2S_2O_3$) to instantaneously quench the residual disinfectant and 10-fold diluted in buffered peptone water (Becton Dickenson) and a 50 μL portion of appropriate dilutions were plated in duplicate onto TSA agar plates using a spiral plater (WASP 2, Don Whitley Scientific, Shipley, United Kingdom). The plates were incubated at 37°C for 18 h. Colonies on TSA agar plates were enumerated by a ProtoCOL 3 automated colony counter (Synbiosis, Cambridge, United Kingdom). The limit of detection was one colony per 50 μL sample (1.3 Log CFU/mL). Each treatment was repeated three times on different days and duplicate plates were used in microbial tests for each sample.

The decimal reduction time (*D* value) was used as an index to evaluate the bacterial inactivation rate vs. NaClO treatment as previously described (Luo et al., 2020). *D* values were calculated at the initial linear portion of survivor plots assuming the logarithmic numbers of bacterial growth were a linear function of the isothermal treatment time (Ahn et al., 2007). The log-linear model was used to investigate the pattern of pathogen inactivation after NaClO treatment as follows:

$$\log N_t = \log N_0 - \frac{t}{D}$$

where $N_t$ (CFU/mL) is the bacterial population at time $t$ (s), $N_0$ (CFU/mL) is the initial bacterial population, and $D$ is the decimal reduction time (min) at a specific treatment condition.

### Membrane Damage

Bacterial viability and cell properties between NaClO tolerant and susceptible bacteria were measured using flow cytometry (FCM) to quantify viable, dead and injured cells after treatment. *Salmonella* cell suspensions (8 Log CFU/mL) were treated with NaClO at 100 mg/L for 0, 10, and 30 min. A commercial live/dead staining kit (BacLight, L7012, Molecular Probes, Carlsbad, CA, United States) was used to distinguish living, dead, and damaged states of bacteria. In brief, 1 mL of bacteria solution was mixed with 1.5 μL SYTO 9 and 1.5 μL propidium iodide (PI) dyes. The mixture was kept in darkness at room temperature for 15 min. FCM tests were performed using a Accuri C6 flow cytometer (Becton Dickinson) equipped with lasers emitting at 488 and 640 nm. Some influences of impurities (such as large particles) on bacterial detection were excluded according to the difference in forward and side light scatter and 10,000 events were collected. The non-treated stained and non-stained cells served as control samples to adjust the detectors (Barros et al., 2021).

Cell structure damage under NaClO stress was observed using scanning electron (SEM, Hitachi 8010, Ibaraki, Japan) and transmission electron (TEM, Hitachi 7650, Ibaraki, Japan) microscopy. *Salmonella* cell suspensions (8 Log CFU/mL) were harvested by centrifugation following NaClO treatments (see below) and fixed with a 2.5% glutaraldehyde (Sangon Biotech, Shanghai, China) solution overnight at 4°C. The cells were centrifuged and the pellets were washed three times with 0.1 M sodium phosphate buffer solution. Each resuspension was serially dehydrated with 25, 50, 75, 90, and 100% ethanol, respectively, and examined using SEM and TEM as previously described (Tyagi and Malik, 2012).

## Antibiotic Resistance Determination

Bacterial suspensions were prepared by suspending 3–5 individual colonies grown at 37°C for 18 h on TSA into 3 mL 0.9% saline, equivalent to the turbidity of a 0.5 McFarland standard. The 0.5 McFarland inoculum suspensions were further diluted at 1:100 in MH. A panel of antibiotic agents was reconstituted by adding 200 μL/well of the inoculum and incubated at 37°C for 18 h. The *E. coli* ATCC 25922 was used as the reference strain. Antibiotic susceptibility testing was performed using the broth microdilution method with the commercial Gram-negative antibiotic panel (Biofosun, Fosun Diagnostics, Shanghai, China) consisting of ampicillin (resistant, AMP ≥ 32 μg/mL), amoxicillin/clavulanate (resistant, AMC ≥ 32 μg/mL), cefotaxime (resistant, CTX ≥ 4 μg/mL), meropenem (resistant, MEM ≥ 4 μg/mL), amikacin (resistant, AMK ≥ 64 μg/mL), gentamicin (resistant, GEN ≥ 16 μg/mL), colistin (resistant, CS ≥ 2 μg/mL), ceftiofur (resistant, CEF ≥ 8 μg/mL), ciprofloxacin (resistant, CIP ≥ 1 μg/mL), sulfamethoxazole (resistant, T/S ≥ 4/76 μg/mL), tetracycline (resistant, TET ≥ 16 μg/mL), tigecycline (resistant, TIG ≥ 8 μg/mL), and florfenicol (resistant, FFC ≥ 16 μg/mL). The breakpoints for each antibiotic agent were set by the 2019 Clinical and Laboratory Standards Institute (CLSI) and the 2017 European Committee on Antimicrobial Susceptibility Testing (EUCAST). Bacteria that were susceptible to all 13 antibiotics were classified as drug-suspectable and bacteria resistant to 1 or 2 classes of antibiotics were termed drug resistant. Multidrug resistance was defined as resistance to 3 or more different classes of antibiotics.

## Chlorine and Antibiotic Resistance Genes in *Salmonella* Isolates

### DNA Extraction

DNA was extracted from pure *Salmonella* colonies using a QIAamp1 Mini DNA Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

### PCR Detection of Chlorine and Antibiotic Resistance Genes

Antibiotic resistance and chorine resistance genes were selected based on previous studies (Hu et al., 2018, 2021; Chen et al., 2021)

and the use of the ResFinder database.[1] We screened for the presence of -eight chlorine resistance genes (*exbD, motB, qacE, qacEΔ1, qacF, sugE, ybhR,* and *yedQ*) and ten antibiotic resistance genes ($bla_{OXA}$, $bla_{CTX}$, $bla_{TEM}$, $qnr_{B6}$, $qnr_{S7}$, *tetG, tetA, floR, cat86,* and *cmx*) using PCR assays (**Table 1**) as previously described (Wu et al., 2015). Amplicons were electrophoresed in 1% gel agarose gels containing EtBr and were visualized under UV light.

## Statistical Analysis

Cross-resistance between chlorine and antibiotics was assessed by comparing antibiotic MIC data with $OD_{600}$ values growth in the presence of NaClO (128 mg/L) using the non-parametric Spearman correlation test in SPSS Statistics 20 software (IBM, Chicago, IL, United States). The Spearman coefficients ranged from −1 to + 1 indicating no association ($r = 0$) to monotonic relationships ($r = -1$ or + 1) (Schober et al., 2018). Significant differences were set at $p < 0.05$.

[1]https://cge.cbs.dtu.dk/services/ResFinder/gene_overview.php

# RESULTS

## Diversity of *Salmonella* Serotypes

We identified numerous *Salmonella* serovars from the wide range of samples we collected. These included samples from 10 farms (1 serotype), 102 slaughter houses (8 serotypes) and 60 retail markets (7 serotypes). A total of 10 serotypes were identified in 172 isolates and included *S.* Enteritidis, *S.* Kentucky, *S.* Typhimurium, *S.* Indiana, *S.* Agona, *S.* Stanley, *S.* Thompson, *S.* Derby, *S.* Mbandaka, and *S.* Montevideo. The most frequent

**TABLE 1 |** Frequency of chlorine tolerance and antibiotic resistance genes determined by PCR among *Salmonella* isolates ($n = 172$).

| | Gene | Primer sequence (5′→3′) | Amplicon size (bp) | No. (%) |
|---|---|---|---|---|
| Chlorine resistance | *exbD* | CGTTAGCGACGGTAGATGTGA | 230 | 43.6 |
| | | CAACGTCTCGTAATCGACGGTT | | |
| | *motB* | GGTGATCTCGACCAGTTGATAG | 363 | 29.7 |
| | | GCCGACGACACGTAACACTT | | |
| | *qacE* | GGGCGTAGTATGGTTACTTGTTG | 196 | 0.006 |
| | | TCCAAGGCCTGACCACATGA | | |
| | *qacEΔ1* | GTTGGCGAAGTAATCGCAACATC | 200 | 83.1 |
| | | AGCAACCAGGCAATGGCTGTA | | |
| | *qacF* | GCGATTGCTTGTGAAGTTATTGCAAC | 175 | 59.9 |
| | | TACCCGCACCCGACCAAATG | | |
| | *sugE* | AAGTACACCGACGGCTTCACT | 184 | 4.1 |
| | | TGGATTCGCCGAACAGGATGA | | |
| | *ybhR* | GCCAACTACCTGCAACAGATC | 277 | 20.3 |
| | | GTACGGCTTTGCCGATGAAGAT | | |
| | *yedQ* | CGCACAACTCCTCATCAATGG | 228 | 0 |
| | | CAGTGACCTGTGGCTCTGT | | |
| Antibiotic resistance | $bla_{OXA}$ | GAAGCACACACTACGGGTGTT | 342 | 0 |
| | | GGTACCGATATCTGCATTGCCATA | | |
| | $bla_{CTX}$ | GCACGTCAATGGGACGATGT | 326 | 65.1 |
| | | TCGCTGCACCGGTGGTAT | | |
| | $bla_{TEM}$ | CGGATGGCATGACAGTAAGAG | 324 | 76.7 |
| | | GCAGAAGTGGTCCTGCAACT | | |
| | $qnr_{B6}$ | CAGTGCGCTGGGCATTGAA | 302 | 8.7 |
| | | CCGAATTGGTCAGATCGCAATG | | |
| | $qnr_{S7}$ | CAGCGACTTTCGACGTGCTA | 305 | 37.8 |
| | | CCCTCTCCATATTGGCATAGG | | |
| | *tetG* | GCTAGAGCTGTTGAACGAGGTT | 181 | 1.7 |
| | | CTCATTCTCTTCGGGTAGCGA | | |
| | *tetA* | TGTGCTCGGTGGGCTGAT | 165 | 73.3 |
| | | AACGAAGCGAGCGGGTTGA | | |
| | *floR* | GTCCGCTCTCAGACAGAATC | 246 | 61.0 |
| | | GCACGAACGCCAGAATCGA | | |
| | *cat86* | GCTGTGTAGCCGATATTGAAAC | 222 | 0 |
| | | GTACTTGTATGGCAACGGGCAAA | | |
| | *cmx* | CATCCTGCTCGCCGTACT | 232 | 0 |
| | | CACTCTCCTGTCCATGAGGAT | | |

**TABLE 2 |** Bacterial of *Salmonella* spp. isolated from poultry supply chains.

| Sampling location | Number of isolates | Serotype distribution |
|---|---|---|
| **Farm** | | |
| Anal swabs | 10 | 10 Typhimurium |
| **Slaughter house** | | |
| Poultry carcasses | 100 | 43 Enteritidis, 33 Kentucky, 6 Typhimurium, 6 Indiana, 2 Thompson, 1 Derby, 1 Montevideo, 1 Stanley, 7 Unidentified |
| Water | 2 | 1 Enteritidis, 1 Kentucky |
| **Retail market** | | |
| Poultry carcasses | 60 | 33 Enteritidis, 10 Kentucky, 3 Indiana, 2 Agona, 2 Typhimurium, 1 Mbandaka, 1 Stanley, 8 Unidentified |
| Total | 172 | 77 Enteritidis, 44 Kentucky, 18 Typhimurium, 9 Indiana, 2 Agona, 2 Stanley, 2 Thompson, 1 Derby, 1 Mbandaka, 1 Montevideo, 15 Unidentified |

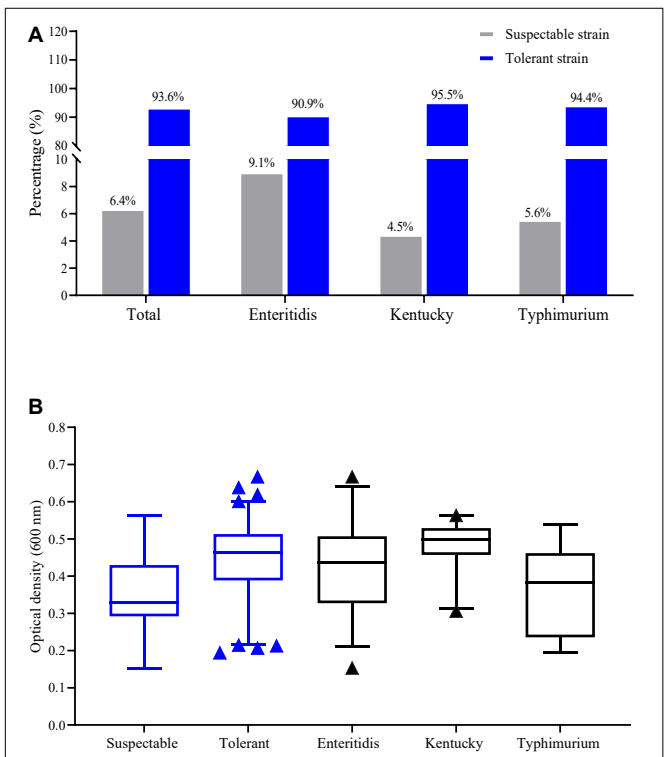


**FIGURE 1 |** Distribution of tolerance isolates by NaClO based on **(A)** MIC and **(B)** $OD_{600}$ values.

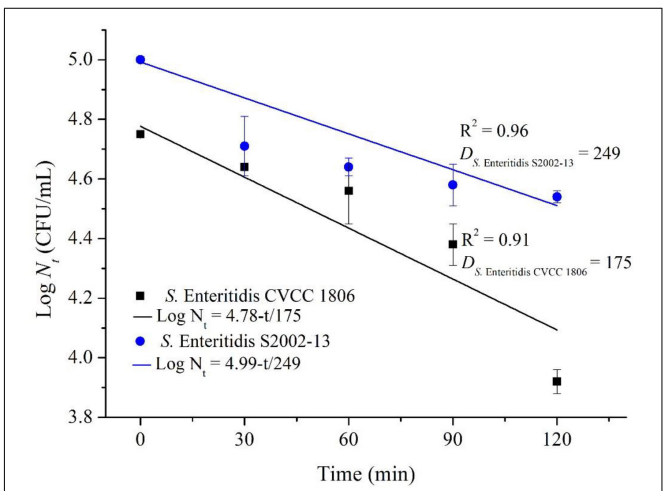


**FIGURE 2 |** Inactivation kinetics of *S.* Enteritidis CVCC 1806 and *S.* Enteritidis S2002-13 exposed to concentration of NaClO at 100 mg/L for 0, 30, 60, 90, and 120 min.

serovars in the poultry supply chain were *S.* Enteritidis (77/172, 44.8%), *S.* Kentucky (44/172, 25.6%) and *S.* Typhimurium (18/172, 10.5%) and these accounted for > 80% of the total serovars isolated (**Table 2**).

## NaClO Tolerance Evaluation

### Minimum Inhibitory Concentrations and $OD_{600}$ Determination

Tolerance to NaClO at MIC > 256 mg/L was detected in 93.6% of our *Salmonella* isolates (161/172). Tolerance based on the MIC values in the serovars *S.* Enteritidis, *S.* Kentucky and *S.* Typhimurium isolates were 90.9, 95.5, and 94.4%, respectively (**Figure 1A**). The average of $OD_{600}$ values for tolerance (MIC > 256 mg/L) and susceptibility were 0.44 ± 0.10 and 0.33 ± 0.09, respectively and this difference was significant ($p < 0.05$). The average of $OD_{600}$ values for growth in the presence of chlorine for *S.* Enteritidis, *S.* Kentucky and *S.* Typhimurium isolates were 0.42 ± 0.11, 0.48 ± 0.06, and 0.37 ± 0.11, respectively (**Figure 1B**). These data indicated that *S.* Kentucky displayed the highest chlorine tolerance compared with other two serotypes ($p < 0.05$).

### Inactivation Kinetics

We more closely examined the mechanisms for choline tolerance using two defined laboratory strains that were susceptible (*S.* Enteritidis CVCC 1806) and tolerant (*S.* Enteritidis S2002-13). We found that growth inhibition for these strains significantly ($p < 0.05$) differed when cultured for 120 min in the presence of NaClO resulting in 0.83 ± 0.04 (*S.* Enteritidis CVCC 1806) and 0.46 ± 0.02 (*S.* Enteritidis S2002-13) Log CFU/mL, respectively. The data were fitted to the log-linear model and the *D* values of NaClO required to inactivate 1 Log unit for *S.* Enteritidis CVCC 1806 and *S.* Enteritidis S2002-13 were 175, and 249 min,

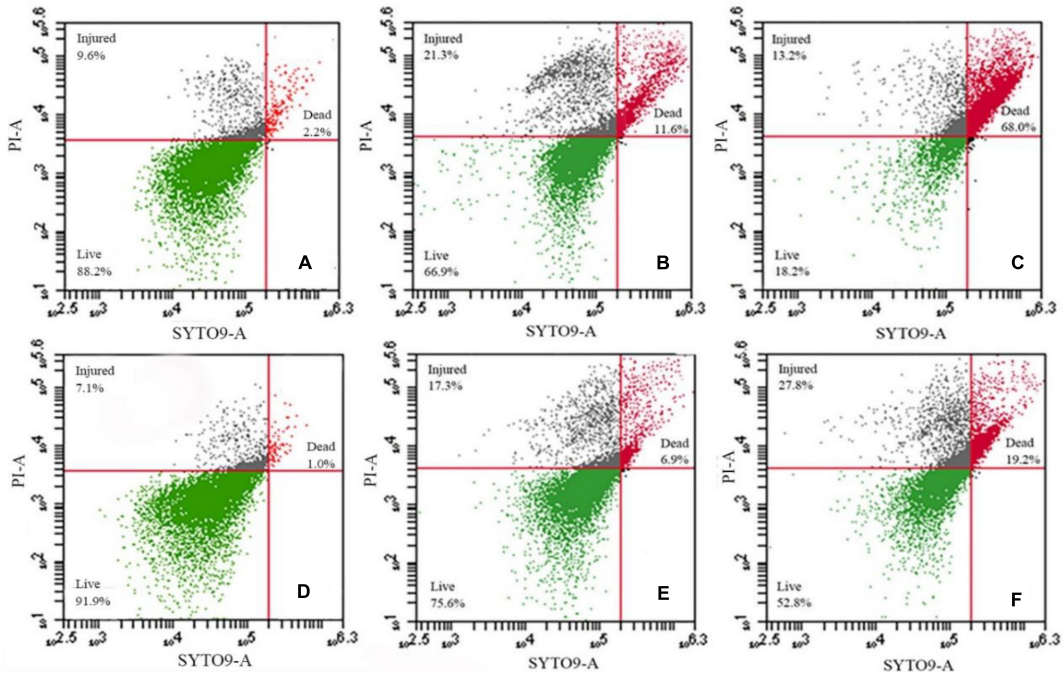


**FIGURE 3 |** *S.* Enteritidis CVCC 1806 **(A–C)** and *S.* Enteritidis S2002-13 **(D–F)** stained with SYTO9 plus PI after treatment with 100 mg/L NaClO for 0, 10 and 30 min.

respectively (**Figure 2**). These data indicated that *S.* Enteritidis S2002-13 displayed reduced susceptibility to NaClO.

### Membrane Damage

We further assessed membrane damage to the bacteria using the membrane-impermeable green fluorescent nucleic acid dye SYTO9 and chlorine-treated cells had higher levels of staining (**Figures 3A,D**). Exposure to NaClO for 10 min resulted in staining of 11.6% of the susceptible *S.* Enteritidis CVCC 1806 and 6.9% of tolerant *S.* Enteritidis S2002-13 cells (**Figures 3B,E**). Exposure for 30 min resulted in 68% of *S.* Enteritidis CVCC 1806 as positive for PI staining compared with 19.2% of *S.* Enteritidis S2002-13 (**Figures 3C,F**). These data clearly showed that *S.* Enteritidis S2002-13 was more tolerant to NaClO.

Ultrastructural analyses indicated the presence of severe cell ruptures for *S.* Enteritidis CVCC 1806 following NaClO treatment and cytoplasmic contents were displaced (**Figures 4A–D**). In contrast, structural damage was less common in *S.* Enteritidis S2002-13 and most cells showed no internal rearrangements of internal cellular content (**Figures 4E–H**). These data clearly demonstrated showed that *S.* Enteritidis S2002-13 was more tolerant to NaClO than *S.* Enteritidis CVCC 1806.

## Antibiotic Resistance Phenotypes

We also assessed our population for antibiotic resistance and 155 of our *Salmonella* isolates (90.1%) were resistant to at least one and 117 (68%) isolates were resistant to at least 3 antibiotics. These results were similar to a previous study of *Salmonella* isolates from poultry in China (Tang et al., 2020). A majority of the *Salmonella* isolates were resistant to AMC (71.5%), AMP (70.9%), and TET (71.5%) and the least prevalent resistance phenotype was found for MEM (0.58%). Additionally, we found no evidence for differences in antibiotic resistance with sampling location. However, resistance did vary by serovar and *S.* Kentucky displayed the highest prevalence of multidrug resistance (93.2%) than the other two most common serotypes (**Table 3**).

## Determination of Chlorine and Antibiotic Resistance Genes

Our *Salmonella* isolates were also screened for the presence of genes enabling chlorine resistance and *qacEΔ1* gene was the most prevalent (83.1%; $n$ = 143) and *qacF* (59.9%; $n$ = 103), *exbD* (43.6%; $n$ = 75), and *motB* (29.7%; $n$ = 51) were also highly represented. We found a positive correlation between the presence of chlorine resistance genes and higher MIC for chlorine that were associated with possession of *qacEΔ1* ($r = 0.66$, $p < 0.0001$) and to lesser extents *qacF* ($r = 0.36$, $p < 0.0001$), *exbD* ($r = 0.26$, $p < 0.0001$), and *motB* ($r = 0.19$, $p < 0.01$). We also found that the antibiotic resistance genes $bla_{TEM}$ (76.7%, $n$ = 132), *tetA* (73.3%, $n$ = 126), $bla_{CTX}$ (65.1%, $n$ = 112), and *floR* (61%, $n$ = 105) were highly represented in our isolates from the different nodes of the poultry supply chain (**Table 1**).

## Association Between Chlorine and Antibiotic Resistance

We compared our *Salmonella* isolates based on the antibiotic MIC values against $OD_{600}$ values for NaClO (128 mg/L) using non-parametric Spearman correlation tests to determine whether

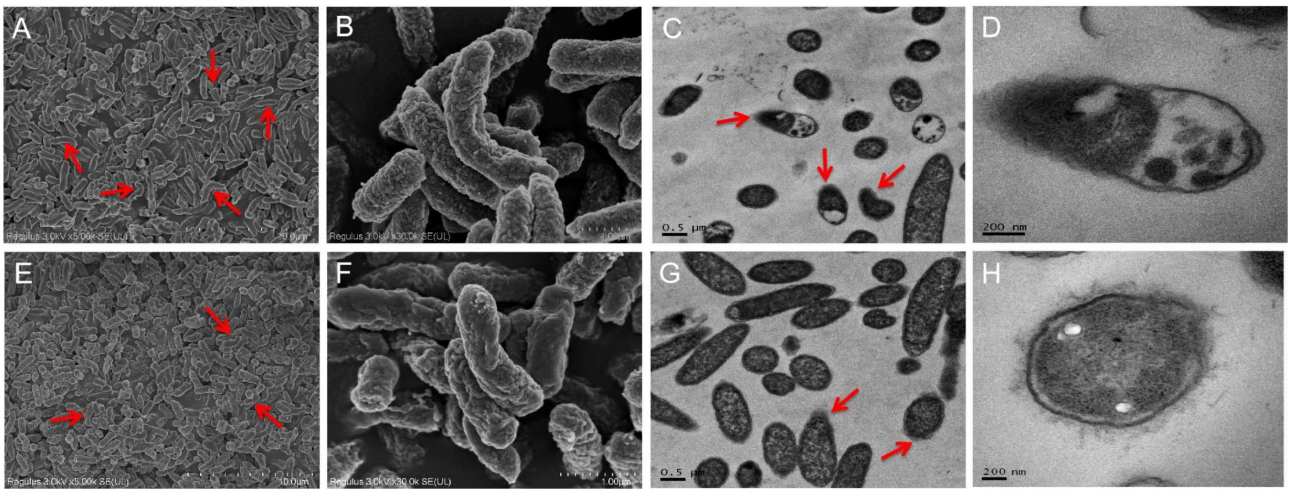


**FIGURE 4 |** SEM and TEM photomicrographs of *S.* Enteritidis CVCC 1806 **(A–D)** and *S.* Enteritidis S2002-13 **(E–H)** after treatment with 100 mg/L NaClO for 30 min. The red arrows indicate regions of bacterial cell damage.

chlorine and antibiotic resistance were correlated. We found significant positive relationships between chlorine and ceftiofur, tetracycline, ciprofloxacin and florfenicol resistance and was most likely related to the over-expression of drug efflux pumps (**Table 4**). *Salmonella* isolates with resistance to chlorine and antibiotics were analyzed for their content of resistance genes. In our group of 172 isolates, chlorine tolerant strains were more likely to also be antibiotic resistant ($p < 0.05$) (**Figure 5A**). This was especially for the *qac* genes that displayed a relatedness with correlation coefficients in the range of 0.10–0.44 (**Figure 5B**).

# DISCUSSION

## Chlorine Tolerance

There is no widely accepted scientific definition of chlorine tolerance in bacteria or for quantification of the chlorine tolerance phenotype. Previous studies have utilized MIC, inhibition zone, logarithmic removal rate and membrane damage methods for determining bacterial chlorine tolerance (Jia et al., 2020; Luo et al., 2020). The MIC method is suitable for comparison of chlorine tolerance among isolates, but it cannot reflect the inactivation effect of typical MIC value (Garcia and Pelaz, 2008). Growth inhibition can be measured using 96 well plates and chlorine potency can be judged from growth inhibition quantified using absorbance at 600 nm (Kolarević et al., 2016). In our study, we combined MIC and $OD_{600}$ methods to evaluate chlorine tolerance. Therefore, the disadvantage that for the MIC method that it does not reflect inactivation effects at the same MIC values was overcome.

The inhibition zone method is suitable for comparison of chlorine tolerance of a large number of isolates but becomes problematic with bacteria with differing growth rates and test results are often inaccurate (Khan et al., 2016; Luo et al., 2020). A previous study had isolated 87 bacterial strains in 22 genera from drinking water and chlorine tolerance could be differentiated at the species and genus levels (Khan et al., 2016). Logarithmic inactivation rates are suitable for comparison of chlorine tolerance between studies but disinfection concentration and time have not been standardized between studies (Zeng et al., 2020). Additionally, the antimicrobial activity of disinfectants is routinely tested by determination of survival curves and this procedure is labor-intensive and time consuming (Puangseree et al., 2021).

Culture-independent flow cytometry combined with fluorescent dyes has been used to investigate the effects of chlorine disinfection on bacterial physiological properties including membrane integrity and potential and respiratory activity (Song et al., 2019). Chlorine inactivates microorganisms by reacting and damaging cellular components including cell walls, membranes and nucleic acids. Chlorine-susceptible and chlorine-tolerant bacteria possess differences in the relative percentages of fatty acids in cell walls and membranes. Chlorine tolerance has been explained by replacement of 95.7% of membranes lipids as saturated long-chain fatty acids that act to limit chlorine diffusion (Chen et al., 2012).

## Mechanisms of Chlorine Tolerance in Bacteria

Chlorine disinfection induces the expression of many functional gene families including responses to oxidative stress, DNA repair, energy metabolism, membrane damage and efflux pumps (Hou et al., 2019; Luo et al., 2020). The SOS response (a conserved response to DNA damage) triggered by oxidative stress renders bacteria chlorine resistant (Tong et al., 2021). Nutrient limitations result in quiescence of growth and metabolism that may subsequently lead to bacteria in the viable but non-culturable (VBNC) state for chlorine stress adaptations (Zhu et al., 2022). *E. coli, Enterococcus and Salmonella* can enter into a VBNC state after disinfection, and *Salmonella* was more

**TABLE 3 |** Frequency (% of total isolates) of resistance to antibiotic agents among *Salmonella* isolates from poultry supply chains.

| Antibiotic | % (No. of resistant isolates) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Sampling location | | | Predominant serotype | | | Total (%) (*n* = 172) |
| | Farm (*n* = 10) | Slaughter house (*n* = 102) | Retail (*n* = 60) | Enteritidis (*n* = 77) | Kentucky (*n* = 44) | Typhimurium (*n* = 18) | |
| **β -lactams** | | | | | | | |
| AMP | 6 (60.0) | 78 (76.5) | 38 (63.3) | 53 (68.8) | 38 (86.4) | 9 (50.0) | 122 (70.9) |
| AMC | 7 (70.0) | 79 (77.5) | 37 (61.7) | 54 (70.1) | 37 (84.1) | 11 (61.1) | 123 (71.5) |
| CTX | 3 (30.0) | 38 (37.3) | 17 (28.3) | 5 (6.5) | 34 (77.3) | 3 (16.7) | 58 (33.7) |
| MEM | 0 (0) | 0 (0) | 1 (1.7) | 0 (0) | 0 (0) | 0 (0) | 1 (0.58) |
| CEF | 2 (20.0) | 45 (44.1) | 19 (31.7) | 11 (14.3) | 35 (79.5) | 3 (16.7) | 66 (38.4) |
| **Aminoglycosides** | | | | | | | |
| AMK | 0 (0) | 29 (28.4) | 8 (13.3) | 3 (3.9) | 28 (63.6) | 0 (0) | 37 (21.5) |
| GEN | 3 (30.0) | 38 (37.3) | 18 (30.0) | 5 (6.5) | 36 (81.8) | 3 (16.7) | 59 (34.3) |
| **Sulfonamides** | | | | | | | |
| T/S | 6 (60.0) | 48 (47.1) | 19 (31.7) | 12 (15.6) | 36 (81.8) | 6 (33.3) | 73 (42.4) |
| **Quinolones** | | | | | | | |
| CIP | 5 (50.0) | 46 (45.1) | 20 (33.3) | 5 (6.5) | 40 (90.9) | 6 (33.3) | 71 (41.3) |
| **Tetracyclines** | | | | | | | |
| TET | 7 (70.0) | 58 (56.9) | 29 (48.3) | 16 (20.8) | 44 (100) | 12 (66.7) | 94 (54.7) |
| TIG | 1 (10.0) | 13 (12.7) | 6 (10.0) | 1 (1.3) | 13 (29.6) | 2 (11.1) | 20 (11.6) |
| **Polymyxins** | | | | | | | |
| CS | 4 (40.0) | 12 (11.8) | 18 (30.0) | 25 (32.5) | 2 (4.5) | 4 (22.2) | 34 (19.8) |
| **Chloramphenicol** | | | | | | | |
| FFC | 6 (60.0) | 47 (46.1) | 22 (36.7) | 6 (7.8) | 38 (86.4) | 8 (44.4) | 75 (43.6) |
| DS* | 1 (10.0) | 10 (9.8) | 6 (10.0) | 11 (14.3) | 0 (0) | 2 (11.1) | 17 (9.9) |
| DR** | 1 (10.0) | 20 (19.6) | 17 (28.3) | 29 (37.7) | 3 (6.8) | 4 (22.2) | 38 (22.1) |
| MDR*** | 8 (80.0) | 72 (70.6) | 37 (61.7) | 37 (48.1) | 41 (93.2) | 12 (66.7) | 117 (68.0) |

**DS, bacterial susceptible to all 13 antibiotics. **DR, bacteria resistant to 1 or 2 classes of antibiotic**s**. ***MDR, multidrug resistance was defined as resistance to three or more different classes of antibiotics. AMP, Ampicillin; AMC, Amoxicillin/clavulanate; CTX, Cefotaxime; MEM, Meropenem; AMK, Amikacin; GEN, Gentamicin; CS, Colistin; CEF, Ceftiofur; CIP, Ciprofloxacin; T/S, Sulfamethoxazole; TET, Tetracycline; TIG, Tigecycline; and FFC, Florfenicol.*

**TABLE 4 |** Spearman correlation analysis for $OD_{600}$ (NaClO) and MIC (antibiotic) measurements.

| Antibiotic | β -lactams (CEF) | Quinolones (CIP) | Tetracyclines (TET) | Chloramphenicol (FFC) |
|---|---|---|---|---|
| Spearman correlation | 0.18 | 0.15 | 0.21 | 0.25 |
| *p*-value | 0.01 | 0.03 | 0.03 | 0.001 |

*CEF, Ceftiofur; CIP, Ciprofloxacin; TET, Tetracycline; and FFC, Florfenicol. Significant differences, $p < 0.05$.*

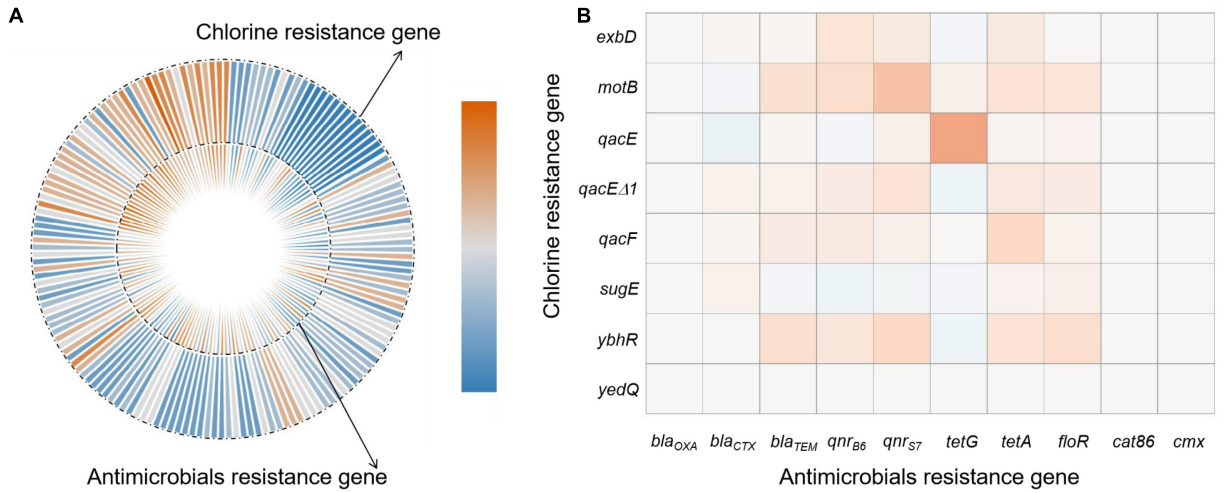


**FIGURE 5 |** Correlations of bacterial NaClO and antibiotic I resistance genes based on **(A)** correlation coefficient and **(B)** frequency of resistance gene detection.

resistant to chlorine in reclaimed water (Li et al., 2013). In addition, membrane permeability is a primary barrier to uptake of foreign or extracellular DNA and NaClO exposure alters the expression of cell membrane-related genes and especially those regulating cell membrane permeability as has been demonstrated in *Pseudomonas* spp. (Tong et al., 2021). The upregulation of efflux genes also contributes to the persistence of chlorine-treated cells (Hou et al., 2019). In our current study, *qacEΔ1* was tightly linked to elevated MIC for NaClO. Previous studies had reported that the *qacEΔ1* gene was the most frequently present in *E. coli* (69.70%), followed by *K. pneumoniae* (50.00%) and *Salmonella* (39.62%), respectively. The *qacEΔ1* gene is common in enteric bacteria and encodes an efflux pump conferring resistance to chlorine disinfectants via an electrochemical proton gradient (Wu et al., 2015). The gene is also frequently associated with mobile genetic elements such as class I integrons resulting in co-selection of antibiotic resistance genes (Hu et al., 2018).

## Cross-Resistance Between Chlorine Disinfectants and Antibiotics

Cross-resistance between chlorine and antibiotics has become a particular concern due to the possible contribution to the persistence of antibiotic resistance despite antibiotic withdrawn (Liao et al., 2020). In this study, exposure to chlorine resulted in cross-resistance to ceftiofur, tetracycline, ciprofloxacin, and florfenicol. Expression of multidrug efflux pumps is a major mechanism mediating such cross-resistance (Hou et al., 2019; Jin et al., 2020; Puangseree et al., 2021). The over-expression of the drug efflux pump MexEF-OprN in *Pseudomonas aeruginosa* following chlorine exposure promoted resistance to diverse antibiotics including tetracyclines, fluoroquinolones, β-lactams, chloramphenicol, macrolides, novobiocin, trimethoprim, and sulfonamides (Lister et al., 2009). This directly linked increased antibiotic resistance to chlorine exposure. Similarly, pretreatment of *S.* Enteritidis with chlorine, sodium nitrite, sodium benzoate or acetic acid induced the over-expression of the marRAB operon, a global antibiotic resistance regulator involved in the production of AcrAB efflux pumps that extrude antibiotics (Potenski et al., 2003). The development of VBNC bacteria via chlorination increases their resistance to antibiotics (Lin et al., 2017). Chlorine-tolerant injured bacteria that are physiologically competent cells present higher plasmid transformation frequencies than the corresponding untreated bacteria. Since the transferable plasmids released from killed sensitive antibiotic resistant bacteria have a consistent resistance to degradation through disinfection, the chlorination process can promote the horizontal transfer of the released plasmid into chlorine-injured bacteria through natural transformation. This leads to the enrichment of antibiotic resistance genes in viable bacteria (Jin et al., 2020). Therefore, using chlorine disinfectant could provide selection pressure for strains that acquire antibiotic resistance.

## CONCLUSION

*Salmonella* spp. from poultry supply chains and possessed tolerance to NaClO at MIC > 256 mg/L in > 90% of the

172 isolates. These tolerant strains displayed higher *D* values and a more integrated cellular shape. The presence of NaClO tolerance genes and the MIC for NaClO were positively correlated especially for *qacEΔ1* ($r = 0.66$, $p < 0.0001$). Significant positive relationships existed between NaClO and the antibiotics ceftiofur, tetracycline, ciprofloxacin, and florfenicol indicating that chlorine-tolerant bacteria were more likely to also be antibiotic resistant. The generated data could provide parts of the input data for microbial risk assessment of *Salmonella* with different chlorine tolerance profile to capture the variability in the strains of interest while decreasing the uncertainty in some model input parameters.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## AUTHOR CONTRIBUTIONS

WW and HY: writing—review and editing. XX and LB: investigation and writing—original draft preparation. SW and LL: data curation. XQ and JZ: software. YX, BT, and YL: resources. All authors contributed to manuscript revision, read, and approved the submitted version.

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## REFERENCES

Ahn, J., Balasubramaniam, V., and Yousef, A. (2007). Inactivation kinetics of selected aerobic and anaerobic bacterial spores by pressure-assisted thermal processing. *Int. J. Food Microbiol.* 113, 321–329. doi: 10.1016/j.ijfoodmicro.2006.08.012

Andoh, L., Dalsgaard, A., Obiri-Danso, K., Newman, M., Barco, L., and Olsen, J. (2016). Prevalence and antimicrobial resistance of *Salmonella* serovars isolated from poultry in Ghana. *Epidemiol. Infect.* 144, 3288–3299. doi: 10.1017/S0950268816001126

Barros, C. P., Pires, R. P. S., Guimarães, J. T., Abud, Y. K. D., Almada, C. N., Pimentel, T. C., et al. (2021). Ohmic heating as a method of obtaining paraprobiotics: impacts on cell structure and viability by flow cytometry. *Food Res. Int.* 140:110061. doi: 10.1016/j.foodres.2020.110061

Bernardez, L., and de Andrade Lima, L. (2015). Improved method for enumerating sulfate-reducing bacteria using optical density. *MethodsX* 2, 249–255. doi: 10.1016/j.mex.2015.04.006

Bhojani, G., Binod Kumar, S., Kumar Saha, N., and Haldar, S. (2018). Membrane biofouling by chlorine resistant *Bacillus* spp.: effect of feedwater chlorination on bacteria and membrane biofouling. *Biofouling* 34, 426–439. doi: 10.1080/08927014.2018.1461213

Carey, D. E., and McNamara, P. J. (2015). The impact of triclosan on the spread of antibiotic resistance in the environment. *Front. Microbiol.* 5:780. doi: 10.3389/fmicb.2014.00780

Chen, H., Wilson, J., Ercanbrack, C., Smith, H., Gan, Q., and Fan, C. (2021). Genome-wide screening of oxidizing agent resistance genes in *Escherichia coli*. *Antioxidants* 10:861. doi: 10.3390/antiox10060861

Chen, Y. Q., Chao, C., Zhang, X. J., Zheng, Q., and Liu, Y. Y. (2012). Inactivation of resistant *Mycobacteria mucogenicum* in water: chlorine resistance and mechanism analysis. *Biomed. Environ. Sci.* 25, 230–237. doi: 10.3967/0895-3988.2012.02.016

Elnekave, E., Hong, S., Mather, A. E., Boxrud, D., Taylor, A. J., Lappi, V., et al. (2018). *Salmonella enterica* serotype 4,[5], 12: i:-in swine in the United States Midwest: an emerging multidrug-resistant clade. *Clin. Infect. Dis.* 66, 877–885. doi: 10.1093/cid/cix909

Garcia, M., and Pelaz, C. (2008). Effectiveness of disinfectants used in cooling towers against *Legionella pneumophila*. *Chemotherapy* 54, 107–116. doi: 10.1159/000118662

Grimont, P. A., and Weill, F. X. (2007). *Antigenic formulae of the Salmonella serovars*, 9th Edn. (Paris: Institut Pasteur), 1–166.

Hou, A., Yang, D., Miao, J., Shi, D., Yin, J., Yang, Z., et al. (2019). Chlorine injury enhances antibiotic resistance in *Pseudomonas aeruginosa* through over expression of drug efflux pumps. *Water Res.* 156, 366–371. doi: 10.1016/j.watres.2019.03.035

Hu, Y., Jiang, L., Zhang, T., Jin, L., Han, Q., Zhang, D., et al. (2018). Occurrence and removal of sulfonamide antibiotics and antibiotic resistance genes in conventional and advanced drinking water treatment processes. *J. Hazard. Mater.* 360, 364–372. doi: 10.1016/j.jhazmat.2018.08.012

Hu, Z., Peng, Z., Zhang, X., Li, Z., Jia, C., Li, X., et al. (2021). Prevalence and molecular characterization of antimicrobial-resistant *Escherichia coli* in pig farms, slaughterhouses, and terminal markets in henan province of China. *Foodborne Pathog. Dis.* 18, 733–743. doi: 10.1089/fpd.2021.0011

Jia, S., Jia, R., Zhang, K., Sun, S., Lu, N., Wang, M., et al. (2020). Disinfection characteristics of *Pseudomonas* peli, a chlorine-resistant bacterium isolated from a water supply network. *Environ. Res.* 185:109417. doi: 10.1016/j.envres.2020.109417

Jin, M., Liu, L., Wang, D., Yang, D., Liu, W., Yin, J., et al. (2020). Chlorine disinfection promotes the exchange of antibiotic resistance genes across bacterial genera by natural transformation. *ISME J.* 14, 1847–1856. doi: 10.1038/s41396-020-0656-9

Jun, W., Guo, Y. C., and Ning, L. (2013). Prevalence and risk assessment of Campylobacter jejuni in chicken in China. *Biomed. Environ. Sci.* 26, 243–248. doi: 10.3967/0895-3988.2013.04.002

Karumathil, D. P., Yin, H. B., Kollanoor-Johny, A., and Venkitanarayanan, K. (2014). Effect of chlorine exposure on the survival and antibiotic gene expression of multidrug resistant *Acinetobacter baumannii* in water. *Int. J. Environ. Res. Public Health* 11, 1844–1854. doi: 10.3390/ijerph110201844

Khan, S., Beattie, T. K., and Knapp, C. W. (2016). Relationship between antibiotic- and disinfectant-resistance profiles in bacteria harvested from tap water. *Chemosphere* 152, 132–141. doi: 10.1016/j.chemosphere.2016.02.086

Kolarević, S., Milovanović, D., Avdović, M., Oalde, M., Kostić, J., Sunjog, K., et al. (2016). Optimisation of the microdilution method for detection of minimum inhibitory concentration values in selected bacteria. *Bot. Serb.* 40, 29–36. doi: 10.5281/zenodo.48751

Langsrud, S., Sidhu, M. S., Heir, E., and Holck, A. L. (2003). Bacterial disinfectant resistance-a challenge for the food industry. *Int. Biodeter. Biodegr.* 51, 283–290. doi: 10.1016/S0964-8305(03)00039-8

Lee, N., Park, S., Kang, I., and Ha, S. (2014). The evaluation of combined chemical and physical treatments on the reduction of resident microorganisms and *Salmonella* Typhimurium attached to chicken skin. *Poultry Sci.* 93, 208–215. doi: 10.3382/ps.2013-03536

Li, D., Zeng, S., Gu, A. Z., He, M., and Shi, H. (2013). Inactivation, reactivation and regrowth of indigenous bacteria in reclaimed water after chlorine disinfection of a municipal wastewater treatment plant. *J. Environ. Sci.* 25, 1319–1325. doi: 10.1016/S1001-0742(12)60176-4

Liao, X., Ma, Y., Daliri, E. B., Koseki, S., Wei, S., Liu, D., et al. (2020). Interplay of antibiotic resistance and food-associated stress tolerance in foodborne pathogens. *Trends Food Sci. Technol.* 95, 97–106. doi: 10.1016/j.tifs.2019.11.006

Lin, H., Ye, C., Chen, S., Zhang, S., and Yu, X. (2017). Viable but non-culturable *E. coli* induced by low level chlorination have higher persistence to antibiotics than their culturable counterparts. *Environ. Pollut.* 230, 242–249. doi: 10.1016/j.envpol.2017.06.047

Lister, P. D., Wolter, D. J., and Hanson, N. D. (2009). Antibacterial-resistant *Pseudomonas aeruginosa*: clinical impact and complex regulation of chromosomally encoded resistance mechanisms. *Clin. Microbiol. Rev.* 22, 582–610. doi: 10.1128/CMR.00040-09

Luo, L., Wu, Y., Yu, T., Wang, Y., Chen, G. Q., Tong, X., et al. (2020). Evaluating method and potential risks of chlorine-resistant bacteria (CRB): a review. *Water Res.* 188:116474. doi: 10.1016/j.watres.2020.116474

Mechesso, A. F., Moon, D. C., Kim, S. J., Song, H. J., Kang, H. Y., Na, S. H., et al. (2020). Nationwide surveillance on serotype distribution and antimicrobial resistance profiles of non-typhoidal *Salmonella* serovars isolated from food-producing animals in South Korea. *Int. J. Food Microbiol.* 335:108893. doi: 10.1016/j.ijfoodmicro.2020.108893

Potenski, C. J., Gandhi, M., and Matthews, K. R. (2003). Exposure of *Salmonella* enteritidis to chlorine or food preservatives increases susceptibility to antibiotics. *FEMS Microbiol. Lett.* 220, 181–186. doi: 10.1016/S0378-1097(03)00099-5

Puangseree, J., Jeamsripong, S., Prathan, R., Pungpian, C., and Chuanchuen, R. (2021). Resistance to widely-used disinfectants and heavy metals and cross resistance to antibiotics in *Escherichia coli* isolated from pigs, pork and pig carcass. *Food Control* 124:107892. doi: 10.1016/j.foodcont.2021.107892

Roy, P. K., and Ghosh, M. (2017). Chlorine resistant bacteria isolated from drinking water treatment plants in West Bengal. *Desal. Water Treat.* 79, 103–107. doi: 10.5004/dwt.2017.20697

Schober, P., Boer, C., and Schwarte, L. A. (2018). Correlation coefficients: appropriate use and interpretation. *Anesth. Analg.* 126, 1763–1768. doi: 10.1213/ANE.0000000000002864

Slotved, H. C., Dalby, T., Harboe, Z. B., Valentiner-Branth, P., Casadevante, V. F. D., Espenhain, L., et al. (2016). The incidence of invasive pneumococcal serotype 3 disease in the Danish population is not reduced by PCV-13 vaccination. *Heliyon* 2:00198. doi: 10.1016/j.heliyon.2016.e00198

Song, Y., Mao, G., Gao, G., Bartlam, M., and Wang, Y. (2019). Resistance and resilience of representative low nucleic acid-content bacteria to free chlorine exposure. *J. Hazard. Mater.* 365, 270–279. doi: 10.1016/j.jhazmat.2018.10.080

Tang, B., Chang, J., Zhang, L., Liu, L., Xia, X., Hassan, B. H., et al. (2020). Carriage of distinct mcr-1-harboring plasmids by unusual serotypes of *Salmonella*. *Adv. Biosyst.* 4:1900219. doi: 10.1002/adbi.201900219

Templeton, M. R., Oddy, F., Leung, W. K., and Rogers, M. (2013). Chlorine and UV disinfection of ampicillin-resistant and trimethoprim-resistant *Escherichia coli*. *J. Environ. Eng.* 8, 231–237. doi: 10.1139/L09-040

Tong, C., Hu, H., Chen, G., Li, Z., Li, A., and Zhang, J. (2021). Chlorine disinfectants promote microbial resistance in *Pseudomonas* sp. *Environ. Res.* 199, 111296–111296. doi: 10.1016/j.envres.2021.111296

Tyagi, A. K., and Malik, A. (2012). Morphostructural damage in food-spoiling bacteria due to the lemon grass oil and its vapour: SEM, TEM, and AFM investigations. *Evid Based Complement. Alternat. Med.* 2012:692625. doi: 10.1155/2012/692625

Wang, J., Sui, M., Yuan, B., Li, H., and Lu, H. (2019). Inactivation of two mycobacteria by free chlorine: effectiveness, influencing factors, and mechanisms. *Sci. Total Environ.* 648, 271–284. doi: 10.1016/j.scitotenv.2018.07.451

Wu, G., Yang, Q., Long, M., Guo, L., Li, B., Meng, Y., et al. (2015). Evaluation of agar dilution and broth microdilution methods to determine the disinfectant susceptibility. *J. Antibiot.* 68, 661–665. doi: 10.1038/ja.2015.51

Xiao, X., Wang, W., Zhang, J., Liao, M., Yang, H., Fang, W., et al. (2019). Modeling the reduction and cross-contamination of *Salmonella* in poultry chilling process in China. *Microorganisms* 7:448. doi: 10.3390/microorganisms7100448

Yang, X., Guo, P., Li, M., Li, H., Hu, Z., Liu, X., et al. (2020a). Optimization of culture conditions for amoxicillin degrading bacteria screened from pig manure. *Int. J. Environ. Res. Public Health* 17:1973. doi: 10.3390/ijerph17061973

Yang, X., Huang, J., Zhang, Y., Liu, S., Chen, L., Xiao, C., et al. (2020b). Prevalence, abundance, serovars and antimicrobial resistance of *Salmonella* isolated from retail raw poultry meat in China. *Sci. Total Environ.* 713:136385. doi: 10.1016/j.scitotenv.2019.136385

Zeng, F., Cao, S., Jin, W., Zhou, X., Ding, W., Tu, R., et al. (2020). Inactivation of chlorine-resistant bacterial spores in drinking water using UV irradiation, UV/Hydrogen peroxide and UV/Peroxymonosulfate: efficiency and mechanism. *J. Clean. Prod.* 243:118666. doi: 10.1016/j.jclepro.2019.118666

Zeng, X., Lv, S., Qu, C., Lan, L., Tan, D., Li, X., et al. (2021). Serotypes, antibiotic resistance, and molecular characterization of non-typhoidal *Salmonella* isolated from diarrheic patients in Guangxi Zhuang Autonomous Region, China, 2014–2017. *Food Control* 120:107478. doi: 10.1016/j.foodcont.2020.107478

Zhu, L., Shuai, X., Xu, L., Sun, Y., Lin, Z., Zhou, Z., et al. (2022). Mechanisms underlying the effect of chlorination and UV disinfection on VBNC state *Escherichia coli* isolated from hospital wastewater. *J. Hazard. Mater.* 423:127228. doi: 10.1016/j.jhazmat.2021.127228

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# Single Molecule Real-Time Sequencing and Traditional Cultivation Techniques Reveal Complex Community Structures and Regional Variations of Psychrotrophic Bacteria in Raw Milk

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In this study, we investigated the species composition and diversity of psychrotrophic bacteria in raw milk from Heilongjiang, Inner Mongolia, Gansu, Henan, Anhui, Jiangsu, Chongqing, and Hunan provinces in China using traditional cultivation and PacBio Single Molecule Real-Time sequencing methods. The isolated psychrotrophic bacteria were highly diverse, which composed of 21 genera and 59 species. *Pseudomonas* accounted for 58.9% of the total genera while *Stenotrophomonas* and *Enterococcus* were also highly represented (above 5.0%). In particular, *P. azotoformans* occurred at a level of 16.9% and *P. paralactis, P. lactis, E. faecalis, and P. marginalis* were present in relatively high proportions (above 4.0%). Regional differences were found significantly among the test regions except samples from Heilongjiang and Inner Mongolia were similar. Additionally, differences were observed between days in Henan, Anhui, and Jiangsu samples. Therefore, control strategies must be implemented on regional and season basis.

**Keywords: milk, PacBio single molecule real-time sequencing, psychrophilic bacteria, community structures, regional**

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## INTRODUCTION

Milk is an indispensable food for humans and its high nutrient content plays a key role in maintaining health especially for the elderly and children. The quality and safety of dairy products is deeply trusted by consumers (America Department of Health and Human Services, 2019) and the high level of safety procedures in the dairy industry have all but eliminated any physical and chemical dangers of milk consumption (Wang et al., 2018; Du et al., 2019). In contrast, milk contamination by microorganisms is still a constant threat and raw milk may be exposed to

microorganisms during milking, storage, transportation, and processing. This risk is offset by the use of rapid cooling equipment and cold chain systems in the dairy industry. The temperature of the raw milk after extrusion is rapidly reduced to <6°C and kept at this level during storage and transportation. Interestingly, the presence of psychrotrophic bacteria can result in contamination of the milk even at these storage temperatures (Ahmed et al., 2014; Fusco and Quero, 2014; Hahne et al., 2019; Du et al., 2020).

Psychrophilic bacteria are widely distributed in the environment and can contaminate cow udders, milking equipment and storage tanks. Cold storage conditions provide ideal conditions for their growth (Yuan et al., 2019). These organisms also secrete heat-resistant proteases and lipases that can significantly alter the quality of dairy products. For example, lipid hydrolysis by contaminating lipases can lead to rancidity while released proteases hydrolyze casein and produces peculiar smells and cause aging and gelation thereby shortening the shelf life (Xin et al., 2017; Meng et al., 2018; Yang et al., 2020; Bu et al., 2022).

The primary species of psychrotrophic bacteria in raw milk have been identified in the genera *Pseudomonas, Chromobacterium, Clostridium, Lactobacillus, Alcaligenes, Flarobacterium, Micrococcus, Corynebacterium, Streptococcus,* and *Enterobacterium* (Wang et al., 2018). This diversity was further cataloged in raw milk samples from Heilongjiang Province in Northern China and psychrophilic milk bacteria were primarily found for the genera *Chryseobacterium, Pseudomonas, Staphylococcus, Acinetobacter, Clostridium, Flavobacterium, Lactococcus, Kocuria, Bacillus, Serratia, Sphingobacterium,* and *Aerococcus* (Yang et al., 2020). However, the diversity of psychrotrophic bacteria in raw milk for different regions of China was not clear. Therefore, this study was focused on the identification of psychrophilic bacterial community structures in raw milk from different regions of China through a combination of traditional culture and PacBio single-molecule real-time sequencing. We also examined whether regional differences in the community structure of psychrophiles were present. These results provide suggestions for the prevention and control of psychrotrophic bacteria in raw milk.

# MATERIALS AND METHODS

## Sampling

From December 2018 to 2020, eight farms were selected from eight regions in China for raw milk collection. The regions included Hunan, Henan, Heilongjiang, Chongqing, Inner Mongolia, Gansu, Anhui, and Jiangsu. At each ranch, one batch of samples was collected every day for 3 consecutive days except for Henan when sample collection was 4 consecutive days. The milk was immediately placed in 200 mL sterile bottles and refrigeration at 4°C for pending analysis.

## Psychrophilic Bacterial Culture

Psychrophiles were cultured using 25 mL raw milk that was diluted 10 times and 200 μL of three suitable dilutions were spread on Psychrophilic bacterial selective agar plates (Qingdao Hope Bio-Technology Co., Ltd., Qingdao, China). The plates were incubated at 6.5°C incubator for 10 d according to IDF method (IDF 101:2005) (International Dairy Federation, 2005). Single colonies were then streak-purified until pure cultures were obtained. Randomly selected colonies were used for identification in triplicate.

**TABLE 1** | Primer sequences used in the study.

| Gene | Primers (5′ to 3′) | Reference |
|---|---|---|
| 16S rRNA | AGAGTTTGATCCTGGCTCAG<br>CTACGGCTACCTTGTTACGA | Scarpellini et al., 2004 |

## Sequence Analysis of Bacterial Colonies

DNA was extracted from individual bacterial colonies on agar plates and directly lysed in PCR tubes containing 50 μL lysis buffer (Takara Biomedical Technology, Beijing, China) that was heated at 80°C for 15 min. Bacterial species were identified by PCR using 16S rRNA universal primers (**Table 1**). The PCR reactions were carried out using Emerald Amp Max PCR Master Mix (Takara Biomedical Technology, Beijing, China) using 2 μL DNA template and other conditions as specified by the manufacturer. The PCR amplification program for 16S rRNA was as follows: 94°C 4 min, 30 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 90 s and a final step of 72°C for 10 min.

Amplicons were electrophoresed through 1.5% agarose gels to determine whether a target band was present. The PCR products of 16S rRNA amplification were purified and sequenced by a commercial company [BGI Tech Solutions (Beijing Liuhe), Beijing, China].

## Direct Sequence Analysis of Milk Samples

Milk samples (10 mL each) were centrifuged at 14,000 × *g* for 5 min and the precipitate was used for DNA extraction using the PowerSoil DNA Isolation kit (Mobio, Carlsbad, CA, United States) according to the manufacturer's instructions. Extracted DNA was amplified using the following PCR cycling program: 95°C 5 min, followed by 30 cycles of 95°C 30 s, 50°C 30 s, 72°C 90 s and a final extension at 72°C for 7 min. The PCR primers (5′- 3′) specific for the 16S rDNA were 27F (GAGAGTTTGATCCTGGCTCAG) and 1492R (AAGGAGGTGATCCAGCCGCA) (Wang et al., 2018). The amplified products were purified using MagicPure Size Selection DNA Beads (TransGen Biotech, Beijing, China). Electrophoresis results were quantified using Image J software[1] and a Sequel II kit (PacBio, Menlo Park, CA, United States) was used for sequence analysis following the instructions of the manufacturer.

## Statistical Analyses

The raw data reads were corrected to obtain the CCS (Circular Consensus Sequencing) sequence using SMRT Link (version 8.0) and lima (v1.7.0) software to identify the CCS sequence through

[1]https://imagej.nih.gov/ij/download.html

**TABLE 2 |** Identification and distribution of psychrotrophic bacteria isolated from raw milk samples collected from different regions in China.

| Genus (Total No.) | Species (No.) | Genus (Total No.) | Species (No.) |
|---|---|---|---|
| *Pseudomonas*(146) | *Azotoformans* (42) | *Chryseobacterium* (12) | *hominis*(8) |
| | *Paralactis* (19) | | *lactis*(2) |
| | *lactis*(13) | | *cucumeris*(1) |
| | *marginalis*(10) | | *haifense*(1) |
| | *helmanticensis*(9) | *Serratia*(9) | *marcescens*(7) |
| | *hibiscicola*(8) | | *surfactantfaciens*(2) |
| | *gessardii*(7) | *Bacillus*(8) | *galactosidilyticus*(6) |
| | *oryzihabitans*(5) | | *licheniformis*(1) |
| | *mucidolens*(4) | | *kochii*(1) |
| | *lundensis*(3) | *Moraxella*(8) | *osloensis*(8) |
| | *fluorescens*(3) | *Lactococcus*(5) | *raffinolactis*(2) |
| | *reinekei*(3) | | *lactis*(2) |
| | *koreensis*(3) | | *garvieae*(1) |
| | *canadensis*(3) | *Rothia*(5) | *endophytica*(5) |
| | *lurida*(3) | *Comamonas*(3) | *testosteroni*(3) |
| | *weihenstephanensis*(2) | *Citrobacter*(3) | *freundii*(3) |
| | *rhodesiae*(1) | *Staphylococcus*(3) | *xylosus*(2) |
| | *synxantha*(1) | | *gallinarum*(1) |
| | *kilonensis*(1) | *Macrococcus*(3) | *goetzii*(2) |
| | *coleopterorum*(1) | | *caseolyticus*(1) |
| | *poae*(1) | *Acinetobacter*(2) | *baumannii*(2) |
| | *fildesensis*(1) | *Enterobacter*(1) | *bugandensis*(1) |
| | *tolaasii*(1) | *Psychrobacter*(1) | *faecalis*(1) |
| | *baetica*(1) | *Sporosarcina*(1) | *luteola*(1) |
| | *orientalis*(1) | *Streptococcus*(1) | *suis*(1) |
| *Stenotrophomonas*(18) | *maltophilia*(9) | *Kocuria* (1) | *arsenatis*(1) |
| | *pavanii*(6) | *Galactobacter*(1) | *valiniphilus*(1) |
| | *rhizophila*(3) | *Pantoea*(1) | *agglomerans*(1) |
| *Enterococcus*(16) | *faecalis*(12) | | |
| | *pseudoavium*(3) | | |
| | *hirae*(1) | | |

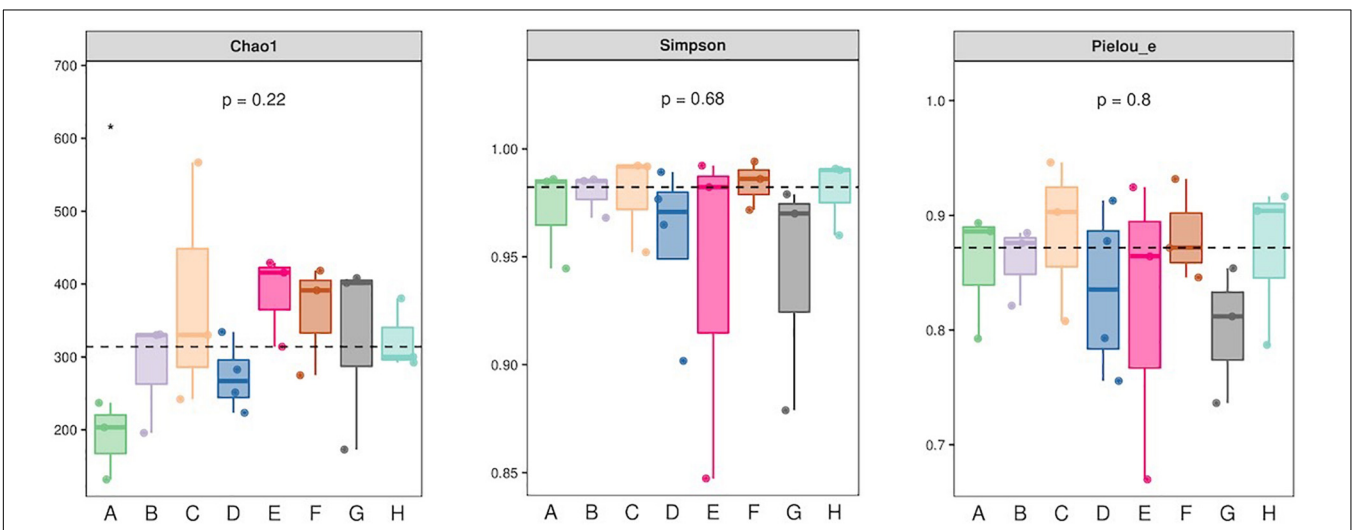


**FIGURE 1 |** Alpha analysis metrics for microbiota present in milk samples collected in eight locations for this study. A. Heilongjiang, B. Inner Mongolia, C. Gansu, D. Henan, E. Anhui, F. Jiangsu, G. Chongqing, and H. Hunan. *Significant difference.

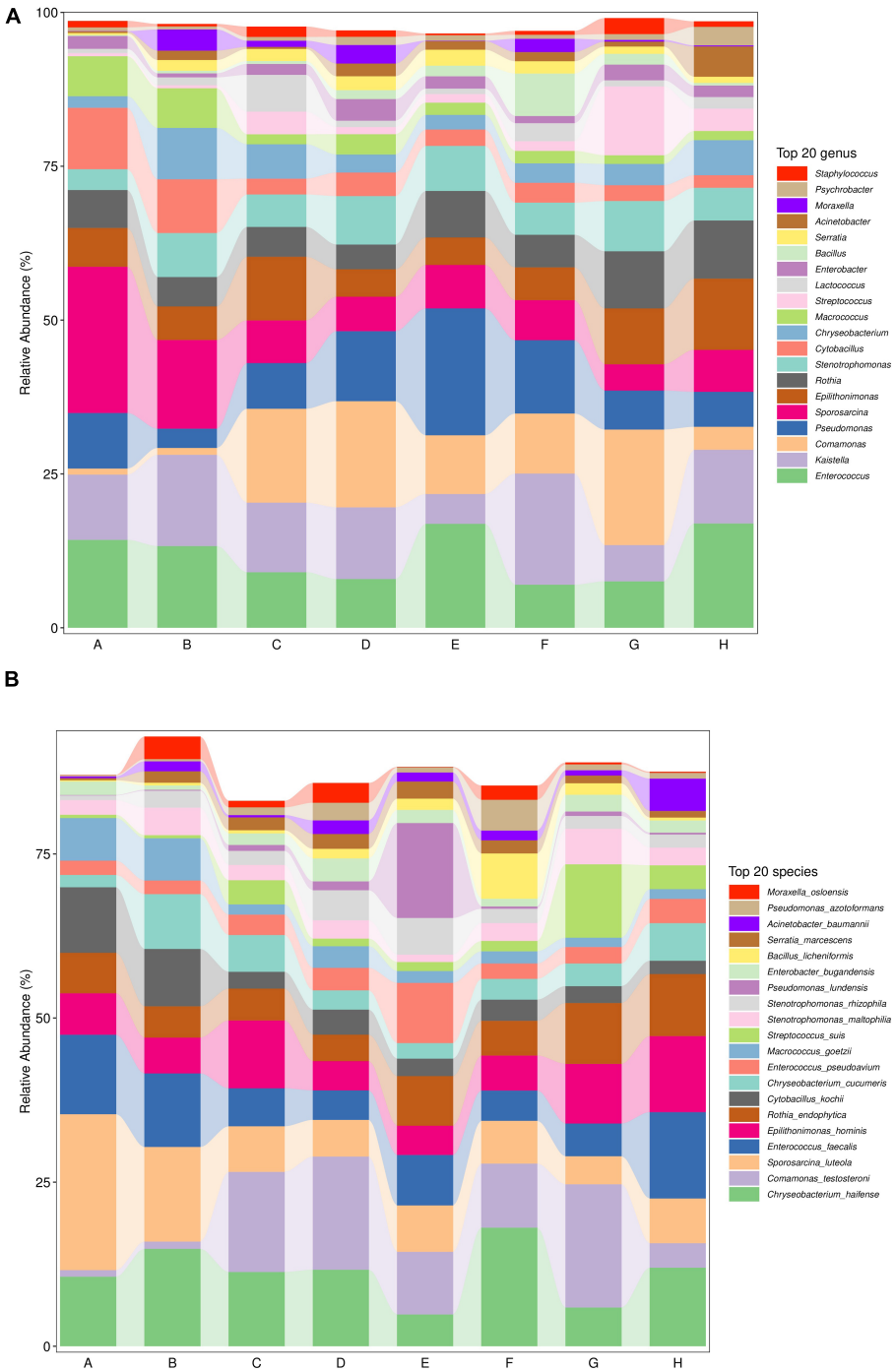


**FIGURE 2 |** Relative OTU abundance at the **(A)** genus and **(B)** species levels from raw milk samples used for this study. A. Heilongjiang, B. Inner Mongolia, C. Gansu, D. Henan, E. Anhui, F. Jiangsu, G. Chongqing, and H. Hunan.

the barcode sequence. Chimeras were removed using UCHIME version 8.1 to obtain the final high-quality CCS sequences (Edgar et al., 2011). The composition of psychrotrophic bacterial microbiota was investigated by submitting the psychrotrophic bacteria database to the raw milk microbiota database. Using the sequence similarity relationship between valid data, different data clusters were grouped in operational taxonomic units (OTU) at a 97% level and annotated based on OTU abundance and sequence information (Dixon, 2003). The relative abundance map of microbial community composition was carried out in R ggplot2 (version 2.2.1) (Wickham, 2016), the diversity index was calculated using https://view.qiime2.org, and the principal

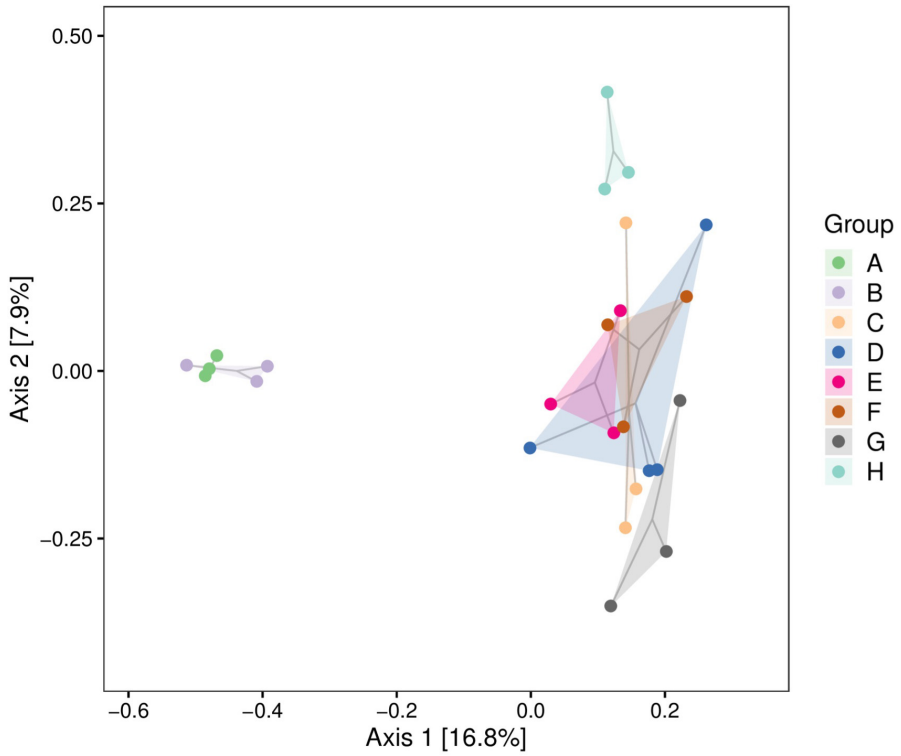


**FIGURE 3 |** Principal coordinates analysis (PCoA) plot of raw milk samples used in this study. A. Heilongjiang, B. Inner Mongolia, C. Gansu, D. Henan, E. Anhui, F. Jiangsu, G. Chongqing, and H. Hunan.

coordinate analysis (PCoA) was generated in the R project Vegan package version 2.5.3 (Dixon, 2003), Heat map, Venny, and linear discriminant analysis were generated using the online software at https://www.genescloud.cn.

# RESULTS

## Identification of Cultivable Psychrophilic Bacteria in the Sample

In the current study we examined 248 different psychrophilic bacterial colonies that were isolated from 25 raw milk samples, which identified as 59 species in 21 genera. The dominant genus was *Pseudomonas* (58.9%) with *Stenotrophomonas* (7.3%), *Enterococcus* (6.5%), *Chryseobacterium* (4.8%), and *Serratia* (3.6%) as secondary dominants. *P. azotoformans* accounted for 16.9% of the total species followed by *P. paralactis* (7.7%), *P. lactis* (5.2%), *E. faecalis* (4.8%), *and P. marginalis* (4.0%) (**Table 2**).

## Community Structure Analysis

Single-molecule real-time sequencing was used to determine the identities of microorganisms in the raw milk samples. The richness, uniformity and coverage of the sample sequencing were sufficient to predict the diversity within each sample. Interestingly, the samples from Heilongjiang possessed significantly lower Chao 1 species richness scores than samples from the other regions. However, the Simpson and Pielou evenness indices were not significantly different (**Figure 1**). These data indicated that the microbial diversity between the sampling locations was similar.

Milk samples were also used to culture psychrotrophic bacteria that were then identified using 16S rDNA sequencing from pure cultures. We then combined this data with the single-molecule sequencing data to analyze the diversity of psychrotrophic bacteria in raw milk. The most abundant genera in our samples were *Enterococcus*, *Kaistella*, *Commonas*, *Pseudomonas*, *Sporosarcina*, *Epilithonimonas*, *Rothia*, *Stenotrophomonas*, *Cytobacillus*, *Chryseobacterium*, *Macrococcus*, *Streptococcus*, *Lactococcus*, *Enterobacter*, *Bacillus*, *Serratia*, *Acinetobacter*, *Moraxella*, *Psychrobacter,* and *Staphylococcus*. In particular, *Sporosarcina* was the most abundant psychrophilic genus (23.8%) in raw milk in Heilongjiang. *Comamonas* was the most abundant (18.8%) in samples from Hunan and *Pseudomonas* (20.6%) from Anhui. Overall, *Pseudomonas* accounted for 9.0, 3.1, 7.4, 11.4, 20.6, 11.9, 6.3, and 5.7% from Heilongjiang, Inner Mongolia, Gansu, Henan, Jiangsu, Hunan, and Chongqing, respectively (**Figure 2A**).

The most abundant species were *Chryseobacterium haifense, Comamonas testosteroni, Sporosarcina luteola, Enterococcus faecalis, Epilithonimonas hominis, Rothia endophytica, Cytobacillus kochii, Chryseobacterium cucumeris, Enterococcus pseudoavium, Macrococcus goetzii, Streptococcus suis, Stenotrophomonas maltophilia, Stenotrophomonas rhizophila, Pseudomonas lundensis, Enterobacter bugandensis, Bacillus*

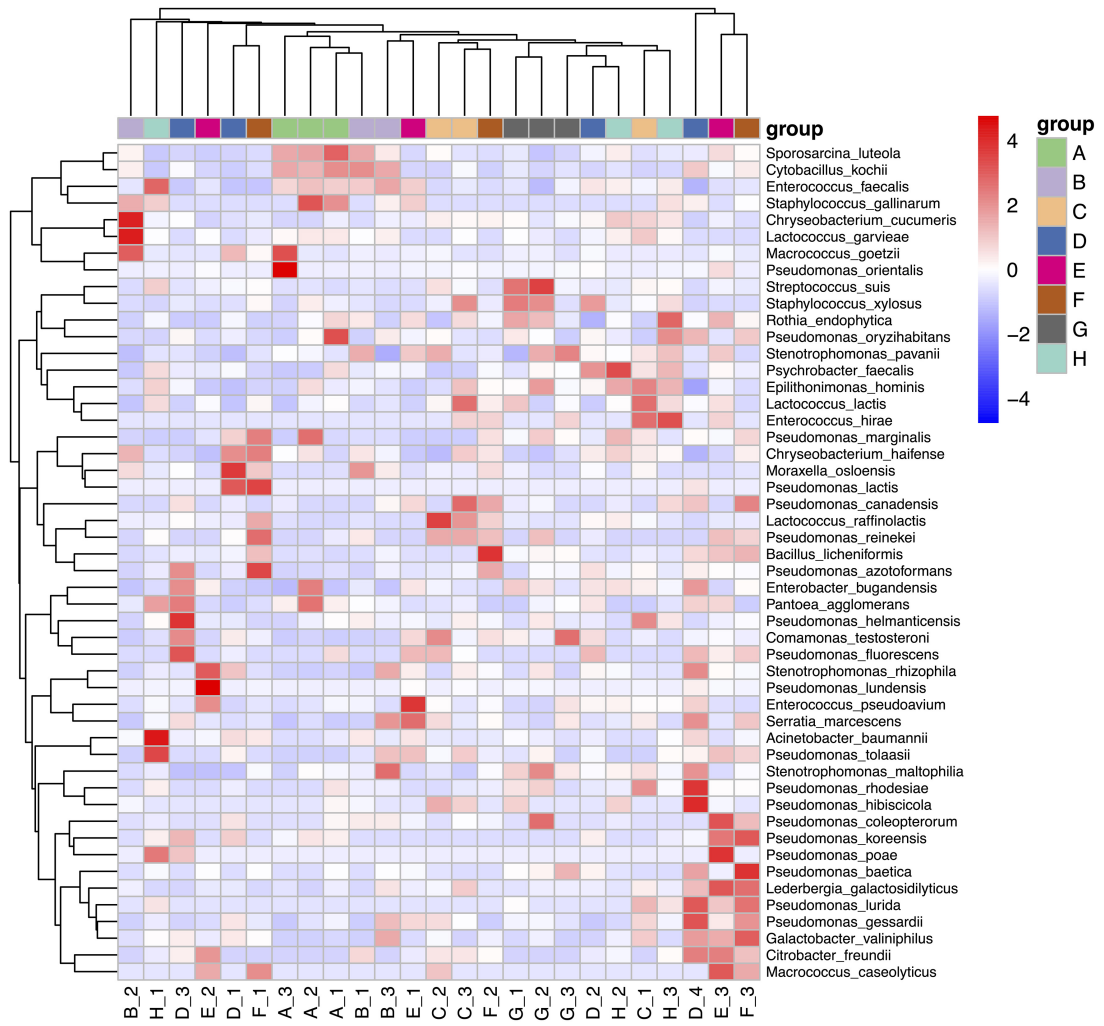


**FIGURE 4 |** Heatmap analysis of raw milk samples used in this study. A. Heilongjiang, B. Inner Mongolia, C. Gansu, D. Henan, E. Anhui, F. Jiangsu, G. Chongqing, and H. Hunan.

*licheniformis, Serratia marcescens, Acinetobacter baumannii, Pseudomonas azotoformans,* and *Moraxella osloensis* (**Figure 2B**). *S. luteola* was the most abundant in Heilongjiang (23.8%) and *C. testosteroni* in Henan (17.2%) and *P. lundii* in Anhui (14.5%) raw milk samples (**Figure 2B**).

## Principal Coordinates Analysis

We analyzed the correlations between community structures of the psychrophilic bacteria in raw milk samples from different regions using PCoA. The Heilongjiang and Inner Mongolia raw milk samples clustered indicating similar communities. Interestingly, these two groups were clearly separated from the remaining sample regions indicating significant differences in community structures (**Figure 3**).

## Clustering Heat Map Analysis

We used clustering heat map analysis to further explore the differences in the community structure of psychrotrophic bacteria using the top 50 relative abundance scores. This analysis utilized the Euclidean distance of the microbial composition data and species were clustered according to Pearson correlation coefficient data. We found that Heilongjiang and Inner Mongolia samples were clustered together and separated from the other groups due to the presence of *S. luteola*, *C. kochii,* and *E. faecalis*. The clustering of Henan, Anhui, and Jiangsu raw milk samples were due to the presence of *P. lurida*, *P. gessardii*, *L. galactosidilyticus*, *G. valiniphilus*, and *C. freundii* (**Figure 4**).

## Species Difference Analysis of Psychrotrophic Bacteria in Samples

We used Venny and linear discriminant analyses to categorize the different raw milk bacterial species that we identified from our eight study areas. We found that *E. faecalis* and *R. endophytica* were common species identified in samples from all regions. In addition, the relative abundance of *Bacillus* in Jiangsu samples was significantly greater than for the other regions

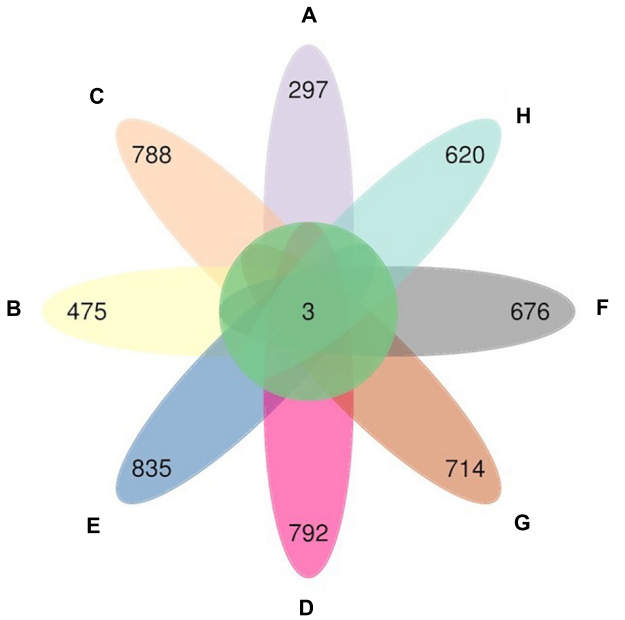


**FIGURE 5 |** Venny analysis of raw milk samples used in this study. A. Heilongjiang, B. Inner Mongolia, C. Gansu, D. Henan, E. Anhui, F. Jiangsu, G. Chongqing, and H. Hunan.

while *Streptococcus* and *Comamonas* in Hunan samples were significantly greater than other regions (**Figures 5**, **6**).

## DISCUSSION

We isolated 248 psychrophilic bacterial colonies using traditional culture methods and found 21 genera and 59 species that were identified by 16 rDNA gene sequencing. At the genus level, *Pseudomonas*, *Stenotrophomonas*, *Enterococcus*, *Chryseobacterium*, and *Serratia* were the present in the highest proportions and *Pseudomonas* accounted for 58.9% of the total. At the species level, *P. azotoformans*, *P. paralactis*, *P. lactis*, *E. faecalis,* and *P. marginalis* were the present in the highest proportions and *P. azotoformans* accounted for to 16.9% of the total species numbers in our samples. These results were consistent with a previous study where *Pseudomonas*, *Lactococcus,* and *Acinetobacter* accounted for 62% of the total (Von Neubeck et al., 2015). Another study found that the *Pseudomonas* was the most prevalent genus (74.79%) in raw milk and was represented by 25 species, the most common of these species were *P. fragi (*10.92%), *P. lundensis* (6.72%), and *P. fluorescens* (6.72%) (Zhang et al., 2020). Additionally, a study utilizing 595 strains of psychrotrophic bacteria from raw milk samples indicated 90% of the samples contained *Pseudomonas* at proportions as high as 25.7% (Yang et al., 2020).

The traditional culture of microorganisms has numerous advantageous over other methods and is an indispensable technical means for the in-depth exploration of microbial resources in raw milk. The composition of psychrotrophic bacterial microbiota was investigated by submitting the psychrotrophic bacteria database to the raw milk microbiota database. We used single-molecule real-time sequencing to also identify un-culturable microorganisms to understand the total diversity of microorganisms in raw milk. These combined data indicated that *Enterococcus, Kaistella, Comamonas, Pseudomonas, Sporosarcina, Epilithonimonas, Rothia, Stenotrophomonas, Cytobacillus*, *Chryseobacterium*, *Macrococcus*, *Streptococcus*, *Lactococcus*, *Enterobacter*, *Bacillus*, *Serratia*, *Acinetobacter, Moraxella*, *Psychrobacter,* and *Staphylococcus* were the 20 most abundant genus in all samples. This was consistent with the results of previous studies that found *Pseudomonas*, *Acinetobacter*, *Lactococcus*, *Corynebacterium,* and *Streptococcus* as the most abundant genus in raw milk (McHugh et al., 2020). An additional study indicated that *Lactococcus*, *Bacillus*, *Streptococcus*, *Sporosarcina*, *Pseudomonas,* and *Acinetobacter* were the primary psychrophilic bacteria at the genus level in raw milk (Hou et al., 2015). Raw milk samples analyzed in another study indicated and *Pseudomonas*, *Lactococcus,* and *Acinetobacter* were the most common genera (Li et al., 2018). In our study we found that *Enterococcus* posed a high risk for contamination and a previous study of 1,584 batches of raw milk samples from four dairy factories found that *Enterococcus* was presence at levels ranging from 40.6 to 79.7% (Yoon and Lee, 2021). This agreed with another study where the relative abundance of *Enterococcus* in raw yak milk reached levels as high as 36% (Qazalbash et al., 2021).

At the species level we found that the most abundant organisms were *C. haifense, C. testosterone, S. luteola, E. faecalis, E. hominis, R. endophytica, C. kochii, C. cucumeris, E. pseudoavium, M. goetzii, S. suis, S. maltophilia, S. rhizophila, P. lundensis, E, bugandensis, B. licheniformis, S. marcescens, A. baumannii, P. azotoformans,* and *M. osloensis*. A previous study indicated that *E. faecalis*, *S. rhizophila*, *P. lundensis,* and *M. osloensi* were the primary psychrophilic bacteria at the species level in raw milk (Yang et al., 2020). The most common source for *A. baumannii* is environmental pollution and this bacterium can cause pneumonia, meningitis, and wound infections (Engür et al., 2014). We found that the relative abundance of *A. baumannii* in Chongqing was relatively high and this was consistent with previous studies implicating raw milk and commercial infant formula milk powder as sources (Tamang et al., 2014). *Streptococcus* originates primarily in the farm environment and is a common cause of dairy cow mastitis and most animals have no obvious clinical symptoms at the time of infection (Moroni et al., 2006). *S. suis* has been also identified in raw milk in cows with mastitis (Ma et al., 2021). We found *S. suis* in Hunan samples and this may be related to farm management practices.

China is a vast country with many farms, we also analyzed the influence of geographic regions factors on the community structure of psychrotrophic bacteria in raw milk, and the results showed that geographic regions factors significantly influenced the community structure of psychrotrophic bacteria in raw milk, with Heilongjiang and Inner Mongolia clustered together in both principal coordinates analysis and cluster heat map analysis due to their location in the north of China, while Henan, Anhui, and Jiangsu clustered together due to their proximity.

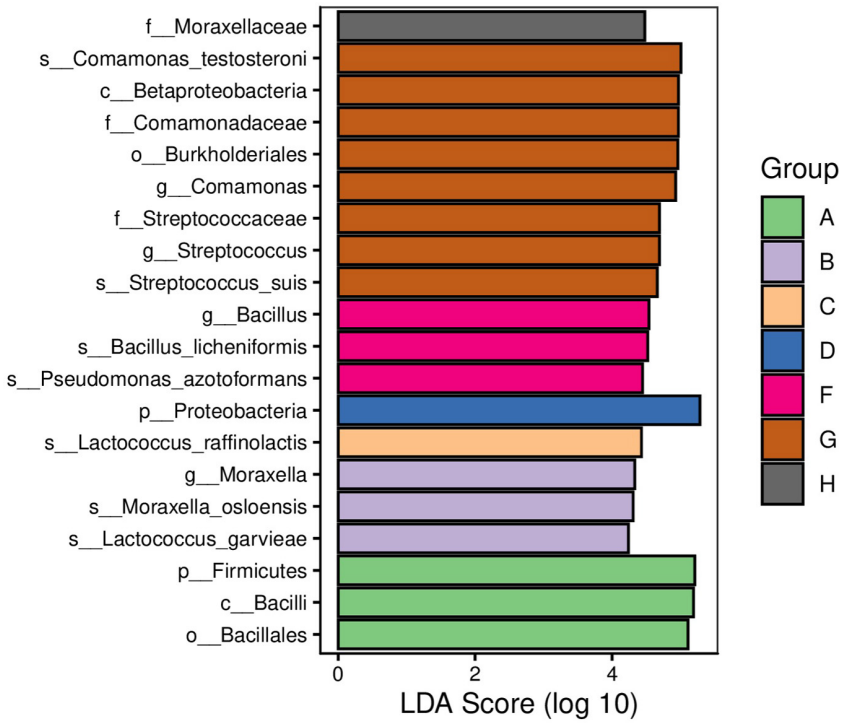


**FIGURE 6 |** Linear discriminant analysis (LDA) of raw milk samples used in this study. A. Heilongjiang, B. Inner Mongolia, C. Gansu, D. Henan, E. Anhui, F. Jiangsu, G. Chongqing, and H. Hunan.

This is consistent with previous findings that geographic factors were significant factors influencing the milk microbiota (Guo et al., 2021), while studies on psychrotrophic bacteria had also shown that geographic factors were determining factors attributing to significant influence in the raw milk psychrotrophic bacteria (Yap et al., 2021). In addition, differences in the community structure of psychrotrophic bacteria in raw milk between different geographic regions may be influenced by storage temperature (Vithanage et al., 2016; Hahne et al., 2019; Quinto et al., 2020). The low-temperature storage resulted in an increasing relative abundance of psychrotrophic bacteria (Yang et al., 2020), the psychotropic bacterial community structure diversity in the summer was higher than that in the winter (Vithanage et al., 2016). Cold storage tests using raw water buffalo milk indicated that the microbial community structure changed with storage time. The first 24 h were dominated by *Lactococcus* and *Streptococcus* while at 72 h *Pseudomonas* and *Acinetobacter* dominated and latter achieved levels more than 60% (Li et al., 2016). A comparison of the effects of different cold storage conditions on the microorganism content of raw milk indicated that *Pseudomonas* isolates also dominated all temperatures and were more than 80% at 2 and 4°C (Vithanage et al., 2017), however, only 8–10% at 10 and 30°C (Hahne et al., 2019), however, another study found that all *Pseudomonas* can grow at 2–25°C (Meng et al., 2019), 67% *Pseudomonas* can grow at 40°C (Caldera et al., 2016). Differences were observed between days in Henan, Anhui, and Jiangsu samples that may be related to farm management or disinfection procedures. Disinfection of farm milking equipment and acid or alkaline disinfection are usually performed every other day.

## CONCLUSION

This is the first study to analyze the diversity of psychrophilic bacteria in raw milk in different regions of China by a combination of traditional culture and PacBio single-molecule real-time sequencing. The community structure of psychrotrophic bacteria in raw milk was very different across regions. In this study, we found that the community structure and diversity of psychrotrophic bacteria in raw milk in Heilongjiang and Inner Mongolia were similar although they differed significantly from the other study regions. Additionally, differences were observed between days in Henan, Anhui, and Jiangsu samples. These procedures assisted in a more accurate understanding of the community structure of psychrophilic bacteria in raw milk from different regions and can be used to develop timely individual control strategies on a case or a reginal basis in order to ensure milk safety.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://bigd.big.ac.cn, CRA005869.

## AUTHOR CONTRIBUTIONS

BD: conceptualization, methodology, investigation, and writing – original draft. LM: methodology. HL: data curation. NZ: conceptualization and supervision. YZ and SZ: writing – review, supervision, and funding acquisition. JW: conceptualization and funding acquisition. All authors contributed to the article and approved the submitted version.

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## REFERENCES

Ahmed, M. M. M., Hafez, E. E., Mona, A. M., Abdelrrassoul, H. A., and Mabrouk, Y. M. (2014). Detection of baby milk powder contamination by microorganisms. *World Appl. Sci. J.* 30, 93–98. doi: 10.5829/idosi.wasj.2014.30.01.81255

America Department of Health and Human Services (2019). *Grade "A" Pasteurized Milk Ordinance*. Washington, DC: America Department of Health and Human Services.

Bu, Y., Qiao, W., Zhai, Z., Liu, T., Gong, P., Zhang, L., et al. (2022). Establishment and evaluation of a loop-mediated isothermal amplification assay for rapid detection of *Pseudomonas* fluorescens in raw milk. *Front. Microbiol.* 12:810511. doi: 10.3389/fmicb.2021.810511

Caldera, L., Franzetti, L., Van Coillie, E., De Vos, P., Stragier, P., De Block, J., et al. (2016). Identification, enzymatic spoilage characterization and proteolytic activity quantification of *Pseudomonas* spp. isolated from different foods. *Food Microbiol.* 54, 142–153. doi: 10.1016/j.fm.2015.10.004

Dixon, P. (2003). VEGAN, a package of R functions for community ecology. *J. Veg. Sci.* 14, 927–930. doi: 10.1111/j.1654-1103.2003.tb02228.x

Du, B., Meng, L., Liu, H., Zheng, N., Zhang, Y., Guo, X., et al. (2020). Impacts of milking and housing environment on milk microbiota. *Animals* 10, 1–12. doi: 10.3390/ani10122339

Du, B., Wen, F., Zhang, Y., Zheng, N., Li, S., Li, F., et al. (2019). Presence of tetracyclines, quinolones, lincomycin and streptomycin in milk. *Food Control* 100, 171–175. doi: 10.1016/j.foodcont.2019.01.005

Edgar, R. C., Haas, B. J., Clemente, J. C., Quince, C., and Knight, R. (2011). UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27, 2194–2200. doi: 10.1093/bioinformatics/btr381

Engür, D., Çakmak, B. Ç, Kaynak Türkmen, M., Telli, M., Eyigör, M., and Güzünler, M. (2014). A milk pump as a source for spreading *Acinetobacter baumannii* in a neonatal intensive care unit. *Breastfeed. Med.* 9, 551–554. doi: 10.1089/bfm.2014.0054

Fusco, V., and Quero, G. M. (2014). Culture-dependent and culture-independent nucleic-acid-based methods used in the microbial safety assessment of milk and dairy products. *Compr. Rev. Food Sci. Food Safety* 13, 493–537. doi: 10.1111/1541-4337.12074

Guo, X., Yu, Z., Zhao, F., Sun, Z., Kwok, L.-Y., and Li, S. (2021). Both sampling seasonality and geographic origin contribute significantly to variations in raw milk microbiota, but sampling seasonality is the more determining factor. *J. Dairy Sci.* 104, 10609–10627. doi: 10.3168/jds.2021-20480

Hahne, J., Isele, D., Berning, J., and Lipski, A. (2019). The contribution of fast growing, psychrotrophic microorganisms on biodiversity of refrigerated raw cow's milk with high bacterial counts and their food spoilage potential. *Food Microbiol.* 79, 11–19. doi: 10.1016/j.fm.2018.10.019

Hou, Q., Xu, H., Zheng, Y., Xi, X., Kwok, L., Sun, Z., et al. (2015). Evaluation of bacterial contamination in raw milk, ultra-high temperature milk and infant formula using single molecule, real-time sequencing technology. *J. Dairy Sci.* 98, 8464–8472. doi: 10.3168/jds.2015-9886

International Dairy Federation (2005). *IDF 101:2005, Milk-Enumeration of Colony-Forming Units of Psychrotrophic Microorganisms-Colony-Count Technique at 6.5°C*. Brussels: International Dairy Federation.

Li, L. Jr., Renye, J. A., Feng, L., Zeng, Q., Tang, Y., Huang, L., et al. (2016). Characterization of the indigenous microflora in raw and pasteurized buffalo milk during storage at refrigeration temperature by high throughput sequencing. *J. Dairy Sci.* 99, 7016–7024. doi: 10.3168/jds.2016-11041

Li, N., Wang, Y., You, C., Ren, J., Chen, W., Zheng, H., et al. (2018). Variation in raw milk microbiota throughout 12 months and the impact of weather conditions. *Sci. Rep.* 8, 1–10. doi: 10.1038/s41598-018-20862-8

Ma, T., Shen, L., Wen, Q., Lv, R., Hou, Q., Kwok, L. Y., et al. (2021). PacBio sequencing revealed variation in the microbiota diversity, species richness and composition between milk collected from healthy and mastitis cows. *Microbiology* 167, 1–10. doi: 10.1099/mic.0.000968

McHugh, A. J., Feehily, C., Fenelon, M. A., Gleeson, D., Hill, C., and Cotter, P. D. (2020). Tracking the dairy microbiota from farm bulk tank to skimmed milk powder. *MSystems* 5, 1–16. doi: 10.1128/mSystems.00226-20

Meng, L., Liu, H., Dong, L., Zheng, N., Xing, M., Zhang, Y., et al. (2018). Identification and proteolytic activity quantification of *Pseudomonas* spp. isolated from different raw milks at storage temperatures. *J. Dairy Sci.* 101, 2897–2905. doi: 10.3168/jds.2017-13617

Meng, L., Zhang, Y., Liu, H., Zhao, S., Wang, J., and Zheng, N. (2019). Characterization of *Pseudomonas* spp. and associated proteolytic properties in raw milk stored at low temperatures. *Front. Microbiol.* 8:2158. doi: 10.3389/fmicb.2017.02158

Moroni, P., Sgoifo Rossi, C., Pisoni, G., Bronzo, V., Castiglioni, B., and Boettcher, P. J. (2006). Relationships between somatic cell count and intramammary infection in buffaloes. *J. Dairy Sci.* 89, 998–1003. doi: 10.3168/jds.S0022-0302(06)72165-8

Qazalbash, M., Masud, T., Ahmad, A., Hayat, R., Ibrahim, M., Mujtaba, A., et al. (2021). Diversity of lactic acid bacteria associated with raw yak (*Bos grunniens*) milk produced in Pakistan. *J. Anim. Feed Sci.* 30, 42–51. doi: 10.22358/jafs/133201/2021

Quinto, E. J., Marín, J. M., Caro, I., Mateo, J., and Schaffner, D. W. (2020). Modelling growth and decline in a two-species model system: pathogenic *Escherichia coli* O157:H7 and psychrotrophic spoilage bacteria in milk. *Foods* 9, 1–15. doi: 10.3390/foods9030331

Scarpellini, M., Franzetti, L., and Galli, A. (2004). Development of PCR assay to identify *Pseudomonas fluorescens* and its biotype. *FEMS Microbiol. Lett.* 236, 257–260. doi: 10.1016/j.femsle.2004.05.043

Tamang, M. D., Gurung, M., Nam, H. M., Kim, S. R., Jang, G. C., Jung, S. C., et al. (2014). Short communication: genetic characterization of antimicrobial resistance in Acinetobacter isolates recovered from bulk tank milk. *J. Dairy Sci.* 97, 704–709. doi: 10.3168/jds.2013-7403

Vithanage, N. R., Dissanayake, M., Bolge, G., Palombo, E. A., Yeager, T. R., and Datta, N. (2016). Biodiversity of culturable psychrotrophic microbiota in raw milk attributable to refrigeration conditions, seasonality and their spoilage potential. *Int. Dairy J.* 57, 80–90. doi: 10.1016/j.idairyj.2016.02.042

Vithanage, N. R., Dissanayake, M., Bolge, G., Palombo, E. A., Yeager, T. R., and Datta, N. (2017). Microbiological quality of raw milk attributable to prolonged refrigeration conditions. *J. Dairy Res.* 84, 92–101. doi: 10.1017/s0022029916000728

Von Neubeck, M., Baur, C., Krewinkel, M., Stoeckel, M., Kranz, B., Stressler, T., et al. (2015). Biodiversity of refrigerated raw milk microbiota and their enzymatic spoilage potential. *Int. J. Food Microbiol.* 211, 57–65. doi: 10.1016/j.ijfoodmicro.2015.07.001

Wang, J., Zheng, Y., Xi, X., Hou, Q., Xu, H., Zhao, J., et al. (2018). Application of PacBio Single Molecule Real-Time (SMRT) sequencing in bacterial source tracking analysis during milk powder production. *Food Control* 93, 226–234. doi: 10.1016/j.foodcont.2018.05.044

Wickham, H. (2016). *ggplot2: Elegant Graphics for Data Analysis*. New York, NY: Springer.

Xin, L., Meng, Z., Zhang, L., Cui, Y., Han, X., and Yi, H. (2017). The diversity and proteolytic properties of psychrotrophic bacteria in raw cows' milk from North China. *Int. Dairy J.* 66, 34–41. doi: 10.1016/j.idairyj.2016.10.014

Yang, X., Guo, X., Liu, W., Tian, Y., Gao, P., Ren, Y., et al. (2020). The complex community structures and seasonal variations of psychrotrophic bacteria in raw milk in Heilongjiang Province, China. *LWT Food Sci. Technol.* 134, 1–12. doi: 10.1016/j.lwt.2020.110218

Yap, M., Gleeson, D., O'Toole, P. W., O'Sullivan, O., Cotter, P. D., and Björkroth, J. (2021). Seasonality and geography have a greater influence than the use of chlorine-based cleaning agents on the microbiota of bulk tank raw milk. *Appl. Environ. Microbiol.* 87, 1–11. doi: 10.1128/AEM.01081-21

Yoon, S., and Lee, Y. J. (2021). Molecular characteristics of enterococcus faecalis and enterococcus faecium from bulk tank milk in Korea. *Animals* 11, 1–9. doi: 10.3390/ani11030661

Yuan, L., Sadiq, F., Burmolle, M., Wang, N., and He, G. (2019). Insights into psychrotrophic bacteria in raw milk: a review. *J. Food Prot.* 82, 1148–1159. doi: 10.4315/0362-028x.Jfp-19-032

Zhang, D., Palmer, J., Teh, K. H., and Flint, S. (2020). Identification and selection of heat-stable protease and lipase-producing psychrotrophic bacteria from fresh and chilled raw milk during up to five days storage. *LWT Food Sci. Technol.* 134:110165. doi: 10.1016/j.lwt.2020.110165

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# Genotyping Based on CRISPR Loci Diversity and Pathogenic Potential of Diarrheagenic *Escherichia coli*

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Diarrheagenic *Escherichia coli* (DEC) can cause epidemic diarrhea worldwide. The pathogenic potential of different strains is diverse and the continuous emergence of pathogenic strains has brought serious harm to public health. Accurately distinguishing and identifying DEC with different virulence is necessary for epidemiological surveillance and investigation. Clustered regularly interspaced short palindromic repeats (CRISPR) typing is a new molecular method that can distinguish pathogenic bacteria excellently and has shown great promise in DEC typing. The purpose of this study was to investigate the discrimination of CRISPR typing method for DEC and explore the pathogenicity potential of DEC based on CRISPR types (CT). The whole genome sequences of 789 DEC strains downloaded from the database were applied CRISPR typing and serotyping. The D value (Simpson's index) with 0.9709 determined that CRISPR typing had a higher discrimination. Moreover, the same H antigen strains with different O seemed to share more identical spacers. Further analyzing the strains CRISPR types and the number of virulence genes, it was found that there was a significant correlation between the CRISPR types and the number of virulence genes ($p < 0.01$). The strains with the largest number of virulence genes concentrated in CT25 and CT56 and the number of virulence genes in CT264 was the least, indicating that the pathway potential of different CRISPR types was variable. Combined with the Caco-2 cell assay of the laboratory strains, the invasion capacity of STEC strains of different CRISPR types was different and there was no significant difference in the invasion rate between different CRISPR type strains ($p > 0.05$). In the future, with the increase of the number of strains that can be studied experimentally, the relationship between CRISPR types and adhesion and invasion capacities will be further clarified.

**Keywords: diarrheagenic *Escherichia coli*, CRISPR typing, serotyping, virulence genes, Caco-2 cell**

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## INTRODUCTION

Diarrhea illness is one of the global healthcare problems. Diarrheagenic *Escherichia coli* (DEC) is an important agent of epidemic diarrhea worldwide especially in some underdeveloped areas with poor sanitation among bacterial pathogens (Gomes et al., 2016). DEC can be divided into five categories according to its virulence characteristics: enterotoxigenic *E. coli* (ETEC),

enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), and Shiga toxin–producing *E. coli* (STEC) (Shahbazi et al., 2021). Strains can be identified according to the characteristic virulence genes of different DEC categories: *stx1*, *stx2*, and *eae* in STEC; *aggR* and *astA* in EAEC; *invE* and *ipaH* in EIEC; *est* and *elt* in ETEC. Furthermore, typical ($eae^+$, $bfp^+$) EPEC and atypical ($eae^+$, $bfp^-$, $stx1^-$, $stx2^-$) EPEC can be distinguished (Fujioka et al., 2013; Rodrigues et al., 2016; Spano et al., 2021). Some studies have shown the association between EPEC and diarrhea. Moreover, EPEC was the main cause of death in children with diarrhea in developing countries (Scaletsky et al., 2002; Afset et al., 2004; Hernandes et al., 2009). ETEC was the most common cause of travelers' diarrhea followed by EAEC in developed and developing countries (Daniels, 2005; Croxen and Finlay, 2010). Large outbreaks of diarrheal diseases were caused by EIEC and proved to be indeed associated with diarrhea (Manuel et al., 1992). STEC was first associated with hemolytic uremic syndrome (HUS) and hemorrhagic colitis (HC) in the 1980s, and subsequently with uncomplicated diarrhea (Karmali et al., 1983; Riley et al., 1983; Pai et al., 1988). Both *stx1* and *stx2* carried by STEC are the most common in HUS patients. The *stx* were proved to reach endothelial cells and induce cascade thrombosis and inflammatory changes, leading to HUS in kidney and other organs (Keir et al., 2012). Both *eae* and *ehxA* carried by STEC also can cause serious diseases in humans (Shen et al., 2015). STEC can lead to typical colonic attachment and elimination of lesions by adhesion to intestinal epithelial cells, which can cause diarrhea or bloody diarrhea (Bruyand et al., 2018). This feature is primarily related to the intima of a protein encoded by the *eae* carried by the chromosomal locus of enterocyte effacement (LEE). Ruminants such as cattle and sheep are the main hosts of STEC. STEC infections are mainly foodborne, and from bovine feces and water contaminated by animal feces (Boerlin et al., 1999; Muniesa et al., 2006).

Different *E. coli* pathogenic categories have different characteristic virulence genes, and virulence genes can be transferred horizontally in pathogenic *E. coli*, allowing pathogenic strains to acquire new biological characteristics and even lead to the generation of new strains (Kyle et al., 2012). Horizontal gene transfer (HGT) between bacteria by Mobile Genetic Elements (MGE) carrying virulence genes has resulted in the continuous emergence of highly virulent strains, which has brought serious harm to public health (Xiong et al., 2012; Yan et al., 2015). Therefore, it is an important task for epidemiological surveillance and investigation to accurately distinguish and identify diarrheagenic *E. coli* with different virulence. Serotypes can be used as a characteristic indicator of bacteria and long term for DEC typing in view of most of the pathogenic strains contained in the known O:H serotypes. For example, O157:H7 caused severe disease harming to patients. In addition, some studies have shown that O26:H11, O45:H2, O103:H2, O111:H8(NM), O121:H19, and O145:NM are also the main serotypes of major foodborne diseases (Karmali, 2018; Spano et al., 2021). Consequently, O:H serotyping plays an important role in bacterial classification, epidemiological monitoring, epidemic detection, and other aspects, and it can also provide information directly related to antigen response (Joensen et al., 2015). However, serotyping still has limitations, so it is necessary to use new typing methods to compensate for the shortcomings of isolates serotyping.

Clustered regularly interspaced short palindromic repeats (CRISPR) is one of the most rapidly evolving components of the genome, mainly composed of almost identical repeats and highly specific spacers. The insertion and deletion of spacers in CRISPR arrays reflect the polymorphism of bacterial evolution (Deveau et al., 2010; Makarova et al., 2011). CRISPR and CRISPR-associated sequence (CAS) proteins together form the CRISPR/CAS system (Díez-Villaseñor et al., 2010; Toro et al., 2014), which can acquire immunity for prokaryotes through invading phages and plasmids, thereby resisting phage infection and limiting the HGT (Marraffini and Sontheimer, 2008, 2010). Some researchers found that spacers are derived from foreign genetic elements such as exogenous plasmids or phages (Bolotin et al., 2005; Mojica et al., 2005; Pourcel et al., 2005), which is related to the mechanism of action of CRISPR/CAS system. The CRISPRs of different strains are divergence and the high polymorphism of spacers can be used as high discrimination biomarkers for genotyping. Four CRISPR loci have been identified in *E. coli*, namely CRISPR1, CRISPR2, CRISPR3, and CRISPR4 (Díez-Villaseñor et al., 2010; Yin et al., 2013; Toro et al., 2014). A recent study (Long et al., 2019) classified 413 *E. coli* strains into 23 types according to CRISPR3 and CRISPR4 loci. In addition, CRISPR typing method has been applied to *Salmonella*, *Campylobacter jejuni*, *Cronobacter*, and other foodborne pathogens (Li et al., 2018; Zeng et al., 2019; Yeh and Awad, 2020), proving that CRISPR typing method has become popular. However, pathogenicity potential of DEC based on CRISPR types seldom focus on and needs further studies.

The purpose of this study was to investigate the distinguishing ability of CRISPR typing for DEC and explore the pathogenicity potential of DEC strains. We performed CRISPR typing of DEC strains and analyzed virulence genes of different CRISPR types. In addition, we combined the results of Caco-2 cell assay of laboratory *E. coli* to further explore the adhesion and invasion capacities of STEC strains based on CRISPR types.

# MATERIALS AND METHODS

## Strains

### Sequence Collection

The whole genome sequences of DEC were downloaded from the National Center for Biotechnology Information (NCBI) database (updated before May 19, 2021). The strains were classified according to the characteristic genes of DEC.

### Laboratory Strains

Twelve STEC strains were screened in the laboratory. A total of 8 strains were isolated from ground beef and 4 strains were isolated from cattle feces. The whole genomes of the strains have been sequenced.

## Serotyping

SerotypeFinder 2.0 Web-based tool[1] was applied to analyze the serotype of the whole genome sequence of laboratory strains and DEC downloaded by NCBI with the following parameters: 85% threshold for %ID and 60% minimum length.

## Clustered Regularly Interspaced Short Palindromic Repeats Typing

The identification of CRISPR loci and the spacers were extracted using CRISPRfinder (Grissa et al., 2007) and BLAST. The comparison of spacer sequences and obtaining unique spacers were identified by ClustalX (Larkin et al., 2007). Each unique spacer was recorded associated a single number beginning with 1. Then, every CRISPR array with multiple spacers was assigned a number as a spacer code. CRISPR typing was performed by combining CRISPR1, CRISPR2, CRISPR3, and CRISPR4 into one allele and displayed this as an arrangement of CRISPR spacers. The CRISPR type (CT) of each strain was defined using a specific number beginning with 1. The discrimination index (D) was calculated based on the Simpson's index of diversity with the equation as previously defined (Hunter and Gaston, 1988; Yeh and Awad, 2020).

## Identification of Virulence Genes

VirulenceFinder 2.0 Web-based tool[2] was used to determine virulence genes of DEC genomes with the following parameters: 90% threshold for %ID and 60% minimum length.

## Adherence and Invasion Assay of Caco-2 Cells

### Cell Culture

Caco-2 cells (FH0029) were purchased from the FuHeng BioLogy (Shanghai, China). The cells were removed from the liquid nitrogen and placed in 37°C water bath, thawed by shaking. The Caco-2 cells were placed in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10–20% (v/v) fetal bovine serum (FBS), 1% (v/v) non-essential amino acids (Coolaber, Beijing, China), and 1% (v/v) penicillin–streptomycin solution. Cells were grown at 37°C in a humidified atmosphere of 5% $CO_2$ and 95% air conditions (Klingberg et al., 2008). For the experimental assays, Caco-2 cells were grown in 12-well tissue culture plates (Greiner Bio-One, Frickenhausen, Germany) until monolayers were developed.

### Adhesion Assay

*In vitro*, the adhesion capacity of DEC to Caco-2 cells was studied, and DEC in this study belonged to STEC. The STEC isolates grow overnight at 37°C in Luria–Bertani (LB) culture. Bacteria suspension (1 ml) was added to each well of the tissue culture plates containing monolayers of Caco-2 cells and the precise number of bacteria in the inoculum ($10^7$–$10^8$ CFU/ml) added to monolayers was determined retrospectively by serial dilutions and plate counting. After 2 h of incubation at 37°C, the Caco-2 cells were washed three times with PBS to remove non-attached bacterial cells (Olesen and Jespersen, 2010). One milliliter 1% Triton X-100 (Applichem, Darmstadt, Germany) was added to each well to loosen and lyse the Caco-2 monolayers. Appropriate serial dilutions of the Caco-2/DEC mixtures were plated on LB agar plates to determine the number of adhered Caco-2 cells. For each assay, the mean value of adherent bacteria was determined and the SEM from triplicate experiments was calculated. The adhesion index was calculated as the ratio of the number of adhered cells to the amount of inoculation × 100%. Results were expressed as% bacteria adhered relative to inoculum.

### Invasion Assay

To determine bacterial invasion, the bacteria solution was added to each well of a tissue culture plate containing Caco-2 monolayer cells, incubated at 37°C for 2 h and then washed with PBS 3 times. Then 1 ml 100 μg/L penicillin–streptomycin solution was added and incubated for 1 h to kill remaining viable extracellular bacteria. The cells were lysed with 1% Triton X-100 for 5 min after washing the penicillin–streptomycin solution and a serial dilution method was used to quantify viable intracellular bacteria. LB agar plates were used to determine the number of invasive bacteria. The mean value of invasive bacteria was determined and the SEM from triplicate experiments was calculated. Results were expressed as% of invasive bacteria relative to inoculum.

## Statistics

Results were analyzed using GraphPad Prism 8 and SPSS 25. The ANOVA analysis followed by a Duncan test at 95% confidence limits were applied to determine the differences in the STEC adhesive and invasion capacities, and the difference in the number of virulence genes among strains of different CRISPR types. *P*-values of $<0.05$ were considered statistically significant. Pearson test was used to analyze the correlation between CRISPR typing and virulence gene number of strains.

# RESULTS AND DISCUSSION

## Sequence Collection

Complete genome sequences of 789 DEC strains were downloaded from NCBI database, including 521 strains of STEC, 116 strains of EAEC, 105 strains of EPEC, 30 strains of EIEC, and 17 strains of ETEC. The NCBI accession numbers of all genomes analyzed in the current work were listed in **Supplementary Table 1**.

## Identification of Clustered Regularly Interspaced Short Palindromic Repeats Loci

The CRISPR1, CRISPR2, CRISPR3, and CRISPR4 loci of DEC were identified and extracted. A total of 733 DEC strains had CRISPR loci. The upstream and downstream sequences of CRISPR loci were relatively conserved. The CRISPR1 locus was located between *iap* and *cysH*, the CRISPR2 locus was located between *ygcE* and *ygcF*, and CRISPR3 and CRISPR4 [named

[1]https://cge.cbs.dtu.dk/services/SerotypeFinder/

[2]https://cge.cbs.dtu.dk/services/VirulenceFinder/

CRISPR4.1 and CRISPR4.2, respectively, by Díez-Villaseñor et al. (2010)] were located between *clpA* and *infA*. The results are consistent with a previous study (Díez-Villaseñor et al., 2010). The number of CRISPR loci varies with DEC types, as shown in **Figure 1**.

The number of CRISPR1 and CRISPR2 loci was the largest with 713 respectively, the number of CRISPR3 loci was 94, and the number of CRISPR4 loci was 16. STEC, EAEC, EPEC, EIEC, and ETEC had more CRISPR1 and CRISPR2 loci than CRISPR3 and CRISPR4, and the number of CRISPR4 loci was the least.

## Serotyping

Among 789 DEC strains, the serotypes of 747 strains were identified. The strains were divided into 212 serotypes, of which O157:H7 accounted for a large proportion with 182 strains. The isolates of O157:H7 were mainly isolated from the United States, Netherlands, Sweden, and other places, which were associated with some food-borne disease outbreaks caused by STEC. O157:H7, O111:H8, O26:H11, O145:H28, and O121:H19 were the dominant serotypes of STEC. These serotypes most frequently implicated in outbreaks and sporadic cases of HC and HUS. The dominant serotypes of EAEC were O6:H1, O153:H30, and the dominant serotypes of EPEC were O55:H51 and O157:H16. The serotypes of EIEC and ETEC were dispersed. In addition, 119 strains had 119 serotypes, individually. The D value (Simpson's index) for DEC strains serotyping was 0.9316.

All 12 STEC strains from our laboratory were divided into different serotypes. Among them, 4 strains isolated from cattle feces belong to O136:H12, and 3 strains isolated from ground beef were from O157:H7, O26:H11, O181:H31, O178:H19, O105:H8, and ONT:H10 distributed in single strain respectively.

## Clustered Regularly Interspaced Short Palindromic Repeats Typing

The downloaded genome sequences of DEC strains were classified by CRISPR typing method, and a total of 1,878 unique CRISPR spacers were obtained through alignment. The distributions of spacer numbers from each location seemed to be random. According to the different arrangement of these spacers to determine the CRISPR classification, the bacteria were divided into 397 CRISPR types (CT). CRISPR typing results of DEC are shown in **Supplementary Table 2**. CT7 ($n$ = 86) was the most prevalent followed by CT48 ($n$ = 85) and two kinds of CT were STEC serotype O157:H7. The strain number including CT6, CT32, and CT287 were 17, 16, and 10, respectively. The strains of other CTs were less than 10. 325 types of CRISPR typing were the least ($n$ = 1). The D value (Simpson's index) with 0.9709 determining the discriminatory power of CRISPR typing method indicated that CRISPR typing method had a higher discriminatory power than serotyping for strains, which was related to the polymorphism of CRISPR. In addition, the same serotype can be divided into different CRISPR types (**Figure 2**).

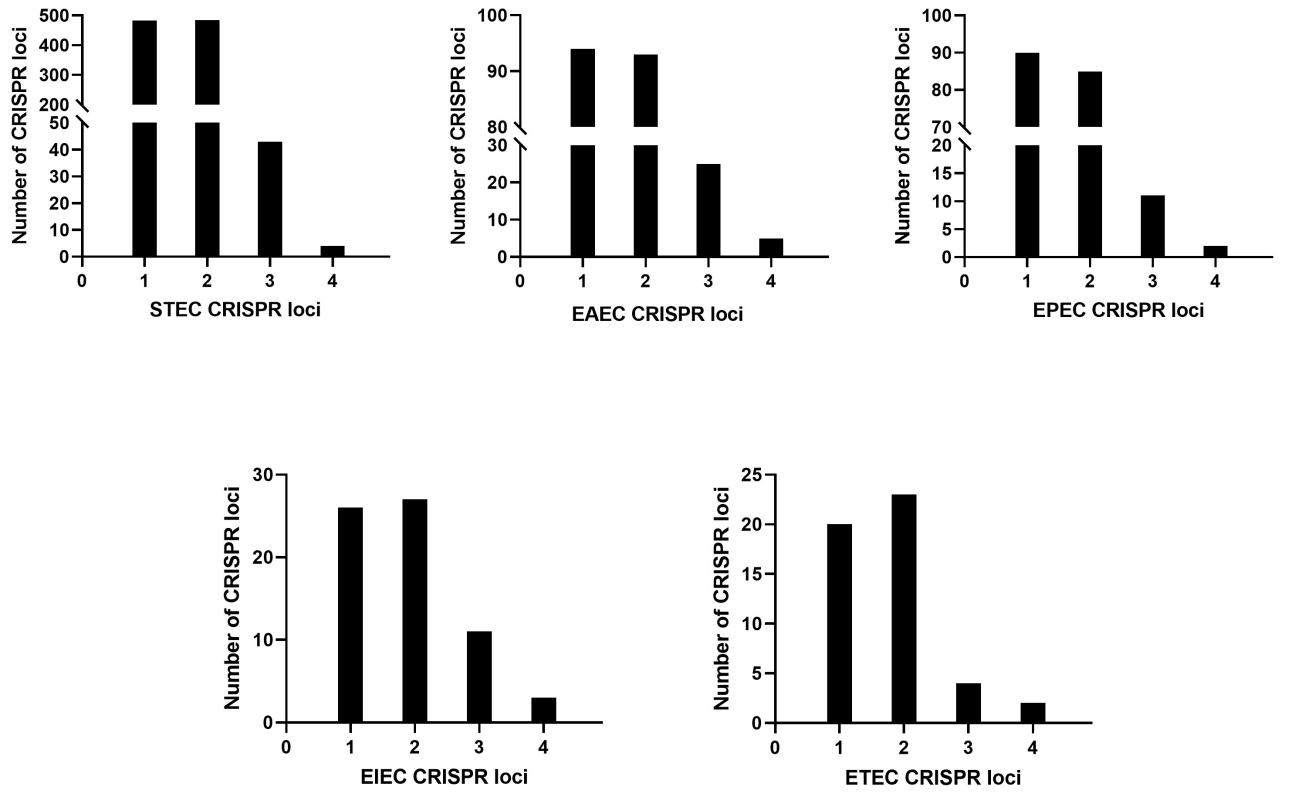


**FIGURE 1 |** Number of DEC CRISPR loci.

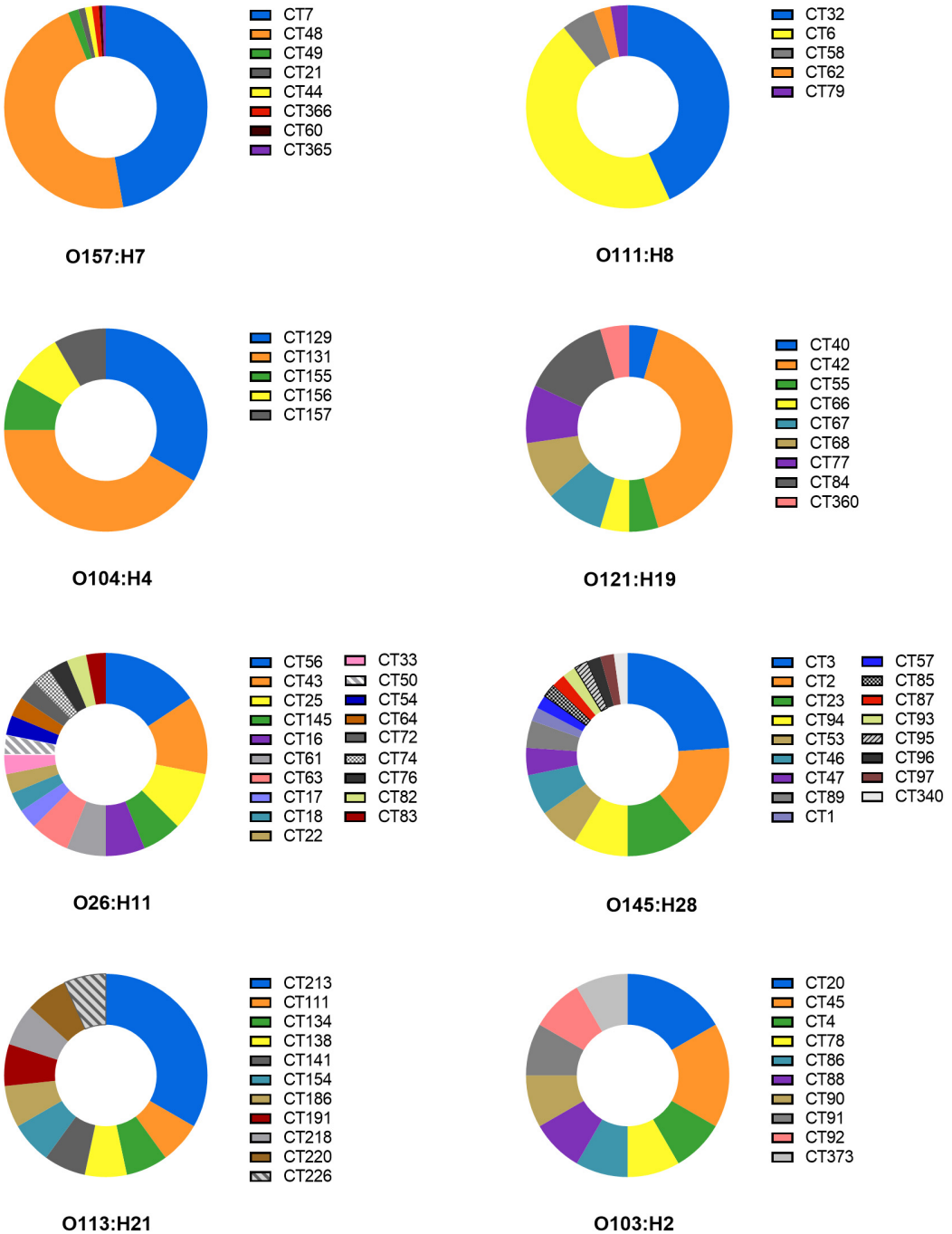


**FIGURE 2 |** CRISPR typing distribution of the same serotype strains.

O157:H7 serotype was divided into 8 CRISPR types including CT7, CT21, CT44, CT48, CT49, CT60, CT365, CT366; O111:H8 contained CT6, CT32, CT58, CT62, and CT79; O104:H4 was separated by CT129, CT131, CT155, CT156, and CT157; O121:H19 included CT40, CT42, CT55, CT66, CT67, CT68, CT77, CT84, and CT360. Otherwise, O26:H11, O145:H28, O113:H21, and O103:H2 contained more than 10 CRISPR types. CRISPR typing method had a higher ability to distinguish strains than serotypes. The study suggests the possibility of serotype prompting through CRISPR arrays. It is worth mentioning that the same H antigen strains with different O seemed to share more identical spacers, while the same O group with different H could not share spacers or had fewer spacers in CRISPR. For example, O26:H11 and O116:H11 had the same CRISPR typing (CT145), which means that spacers were arranged exactly the same. O111:H11 and O69:H11 had 6 identical spacers in CRRSPR1

**TABLE 1 |** CRISPR types and serotypes of laboratory STEC.

| Strains | Serotype | CRISPR type | Number of spacers | CRISPR loci |
|---|---|---|---|---|
| MRL380001 | O157:H7 | CT48 | 4 | CRISPR1/CRISPR2 |
| MRL380002 | O26:H11 | CT398 | 15 | CRISPR1/CRISPR2 |
| MRL380003 | O157:H7 | CT48 | 4 | CRISPR1/CRISPR2 |
| MRL380004 | O157:H7 | CT7 | 4 | CRISPR1/CRISPR2 |
| MRL380005 | ONT:H10 | CT399 | 26 | CRISPR1/CRISPR2 |
| MRL380006 | O81:H31 | CT400 | 20 | CRISPR1/CRISPR2 |
| MRL380007 | O178:H19 | CT401 | 13 | CRISPR1/CRISPR2 |
| MRL380008 | O105:H8 | CT402 | 22 | CRISPR1/CRISPR2 |
| MRL380009 | O136:H12 | CT403 | 12 | CRISPR1/CRISPR2/CRISPR3 |
| MRL380010 | O136:H12 | CT404 | 12 | CRISPR1/CRISPR2/CRISPR3 |
| MRL380011 | O136:H12 | CT405 | 12 | CRISPR1/CRISPR2/CRISPR3 |
| MRL380012 | O136:H12 | CT406 | 12 | CRISPR1/CRISPR2/CRISPR3 |

and completely consistent spacers in CRISPR2, while O26:H11 and O26:H36 had only one common spacer (**Supplementary Table 2**). Similar results in the study of STEC were found by another study (Toro et al., 2014), indicating that H antigen loci are more evolutionarily stable than O antigen because of the diversity of O antigen due to the O antigen transformation caused by gene level transfer (Rump et al., 2010; Wang et al., 2012).

CRISPR1, CRISPR2, CRISPR3, and CRISPR4 loci and spacers of STEC screened by our laboratory were identified and extracted, as shown in **Table 1**. A total of 156 spacers in 12 STEC strains were checked, including 107 unique spacers. Also, 18 unique spacers from 12 STEC strains were not found in the 789 DEC genomes downloaded from the database. Therefore, the CRISPR typing of strains was acquired after spacers of STEC were sorted based on assigning new unique spacers. The spacer arrangement is shown in **Figure 3**. All 3 strains (MRL380004, MRL380001, and MRL380003) were the same CRISPR typing as the downloaded strains, and two belong to CT48 and one belongs to CT7. In addition, all the strains of CT7 and CT48 were O157:H7 serotype.

The results indicated that the spacers of STEC are relatively diverse. The spacers of CRISPR1 loci of MRL380009, MRL380010, MRL380011, and MRL380012 strains were completely consistent, and the spacer sequences of MRL380009 and MRL380012 strains were consistent at CRISPR2 loci. However, the distribution of spacers at CRISPR3 loci was inconsistent. Similarly, MRL380010 and MRL380011 strains had the same spacer sequences at CRISPR2 loci, while the spacer sequences at CRISPR3 loci were different. MRL380009, MRL380010, MRL380011, and MRL380012 belonged to O136:H12 serotype, but belonged to four different CTs.

## Pathogenic Potential of the Strains

### Relationship Between Diarrheagenic *Escherichia coli* Virulence Gene Quantity and Clustered Regularly Interspaced Short Palindromic Repeats Typing

The type and number of virulence genes of foodborne pathogenic bacteria represent the pathogenic potential of the strain, and the strain with strong pathogenic factors is often more likely to bring pathogenic risk to human. For example, STEC has caused many foodborne disease outbreaks and public health challenges (Karmali, 2017) mostly from *stx*, the main virulence factor of STEC. In this study, 123 strains were positive for *stx1* only and 237 were positive for *stx2* only. There was difference in CRISPR types between strains carrying only *stx1* or *stx2*. The virulence genes were analyzed and it was found that *stx2* was more prevalent than *stx1* in STEC. In STEC strains isolated from animals, food, and clinical samples by Kruger et al. (2015), *stx2* was more popular, while other studies have shown that the prevalence of *stx1* was higher than that of *stx2* (Wani et al., 2007; Michel and Kase, 2009). These results may be caused by the differences between the isolation place and source.

Typical virulence genes of EAEC strains are *astA*, *pic*, and *aggR*, and these virulence factors are commonly used as important indicators for EAEC isolation and identification (Hebbelstrup Jensen et al., 2017). Researchers have investigated

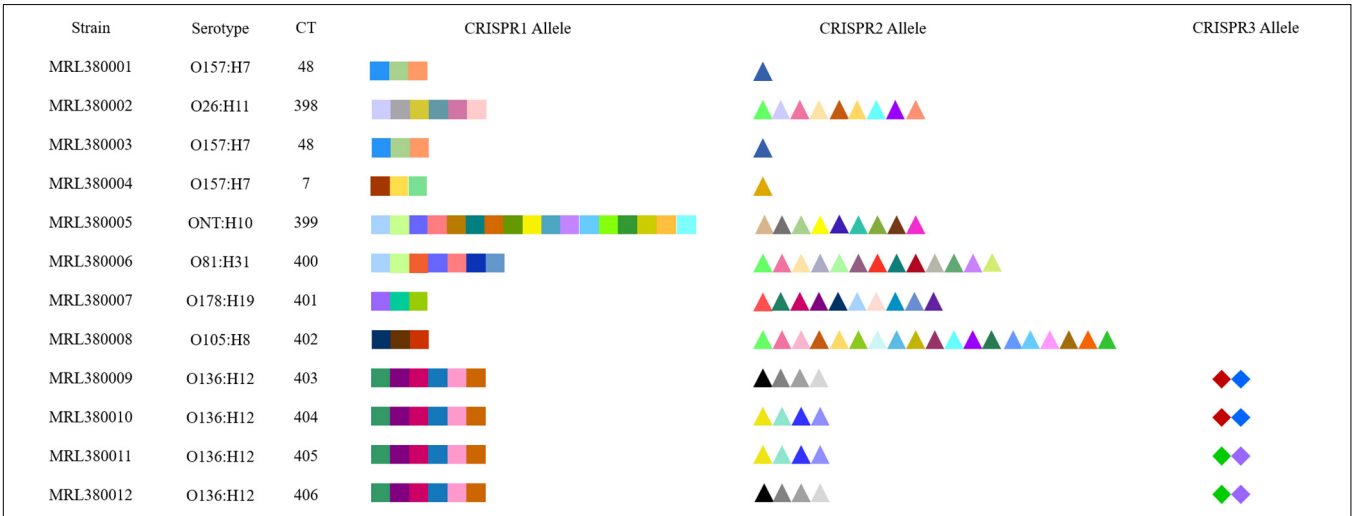


**FIGURE 3 |** CRISPR spacer arrangements of STEC strains. Elements with different colors and shapes represent different spacers.

the prevalence of diarrheagenic *Escherichia coli* pathotypes among diarrhea and healthy children up to 5 years of age in Brazil, and found that EAEC was the most common pathological type, indicating that the pathogenicity of EAEC should not be underestimated (Dias et al., 2016). In a Brazilian study that investigated childhood diarrhea, the toxin gene *astA* was associated with acute diarrhea (Pereira et al., 2007). In our study, *astA* was more popular than *pic* and *aggR* in EAEC ($n$ = 116); the clinical strains containing *astA* that were downloaded in this study can cause diarrhea (not mentioned whether children are involved), indicating that EAEC with *astA* possibly possessed high potential pathogenicity.

The strains with $eae^+$ and $bfp^+$ were marked as typical EPEC (tEPEC), and the strains with $eae^+$, $bfp^-$, $stx1^-$, and $stx2^-$ were recorded as atypical EPEC (aEPEC) (Spano et al., 2021). Among the EPEC ($n$ = 105) downloaded in this study, atypical EPEC covered a relatively high proportion (75.24%), which was similar to the results of other studies (Afset et al., 2004).

A potential contributor to the less reports than other DEC strains, is that EIEC is often observed as a rare cause of diarrhea compared with other DECs (Vieira et al., 2007). However, EIEC is a leading cause of bacterial dysentery with fever, abdominal cramps, diarrhea, and other symptoms in places with poor sanitation (Farajzadeh-Sheikh et al., 2020). Therefore, its pathogenic risk should not be ignored, and it is necessary that the research of the distribution of virulence genes is more conducive to understand the pathogenic potential of bacteria.

Enterotoxigenic *E. coli* is a global diarrhea pathogen that causes disease in mammalian hosts infected by adhesins and secreted enterotoxins (Crofts et al., 2018). ETEC was first recognized as a cause of human illness in the 1960s (Bradley et al., 1971). ETEC is the most common cause of traveler's diarrhea and rarely found in meat products, and contaminated food and water have been implicated as vehicles for transmission of ETEC infection. Moreover, ETEC has been studied in India, the United States, and other regions (Daniels, 2005; Crofts et al., 2018; Mondal et al., 2022). While the amount of ETEC collected in this study was limited and originated from Bangladesh, the United States, China, Chile, and other countries, it was impossible to evaluate the geographical specificity of strains in each region.

Combined with the results of CRISPR typing of DEC strains, there was a significant correlation between CRISPR typing and the number of virulence genes ($p < 0.01$). The number of virulence genes between strains of different CTs was significantly different ($p < 0.01$), indicating that the pathogenicity potential of different CT strains was different. The distribution of virulence gene quantity of DEC strains with different CTs is shown in **Figure 4**. Virulence genes can not only be used for characteristic identification of strains, but also can make prospective judgments on the potential pathogenic properties of strains. To investigate the virulence potential of different CT strains, we analyzed the distribution of virulence gene quantity in different CTs of DEC strains downloaded (**Figure 4**). **Figure 4** shows the CT of more than ten strains. The strains with the largest number of virulence genes were concentrated in CT25 (H11, H16) and CT56 (H11). CT264 (H14) was the least prevalent followed by CT213 (H21). CT25 and CT56 only had CRISPR1 and CRISPR2 locus, while CT264 had CRISPR3 locus, and the number of spacers in CRISPR1 locus of CT264 is more than that of CT25 and CT56. CT48 (O157:H7) had significant difference with CT25 (H11, H16), CT56 (H11), CT131 (H4), CT213 (H21), and CT264 (H4) ($p < 0.01$), but had no significant difference from other CTs. It can also be found that the number of virulence genes varied with different CRISPR types, and the CRISPR types that were significantly different from other types were H4, H11, H14, and H21 serotypes, indicating that CRISPR typing can distinguish the number of virulence genes of these serotype strains excellently.

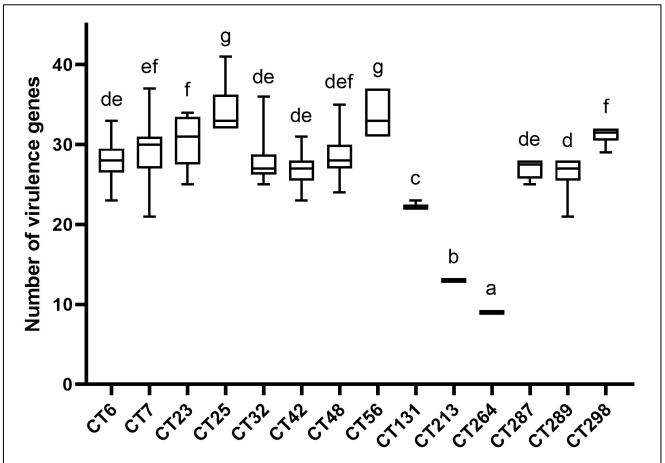


**FIGURE 4 |** Number of virulence genes among different CRISPR types of DEC strains. The same letter in the figure indicates that there is no significant difference between each other, and the absence of the same letter indicates that there is a significant difference between each other ($p < 0.01$).

The distribution of virulence genes in different CRISPR types of laboratory STEC strains is shown in **Figure 5**. CT398 (O26:H11) was the most prevalent followed by CT7 (O157:H7), and CT402 (O105:H8) had the least number of virulence genes. As the number of strains increases in the future, it is expected to obtain a more accurate relationship between CT and the number of virulence genes.

## Caco-2 Cell Assay

The virulence potential of the strain is not only reflected in the distribution of virulence genes. To investigate the pathogenic potential of the STEC more comprehensively, the adhesion and invasion capacities of STEC to Caco-2 cells were studied (**Figures 6**, **7**).

MRL380002 and MRL380007 behaved similarly and presented a significantly higher adhesive capacity ($p < 0.05$). MRL380010 had the least adhesive capacity and was not significantly different from that of MRL380001, MRL380006, MRL380008, MRL380009, MRL380010, MRL380011, and MRL380012, while it was significantly lower than MRL380002, MRL380003, MRL380004, MRL380005, and MRL380007 adhesion rate ($p < 0.05$).

The serotype of MRL380002 was O26:H11, which also carried *eae*, *stx1*, and *stx2* virulence genes. The serotype of MRL380007 was O178:H19, which carried *stx2* but did not carry *eae* and

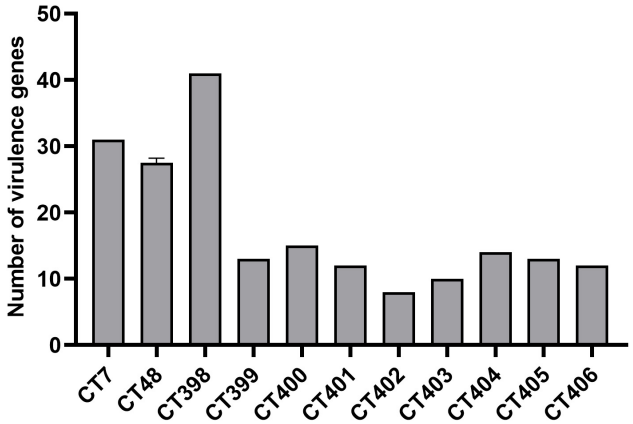


**FIGURE 5 |** Number of virulence genes among different CRISPR types of laboratory STEC strains.

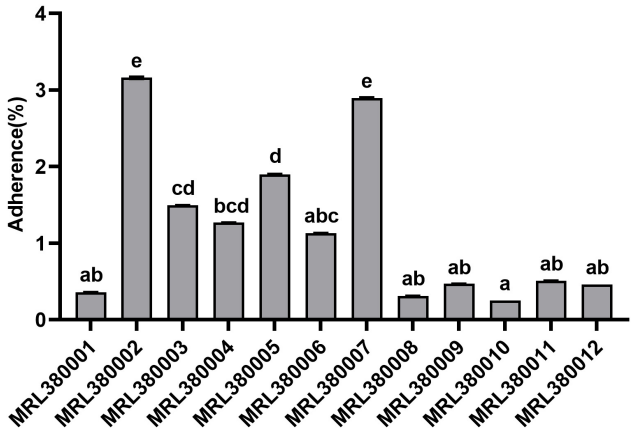


**FIGURE 6 |** Adherence to of Caco-2 cells by different strains of STEC. The same letter indicates that there was no significant difference between each other, and the absence of the same letter indicates that there was a significant difference between each other ($p < 0.05$). Results were expressed as percent of adherent bacteria with respect to inoculum.

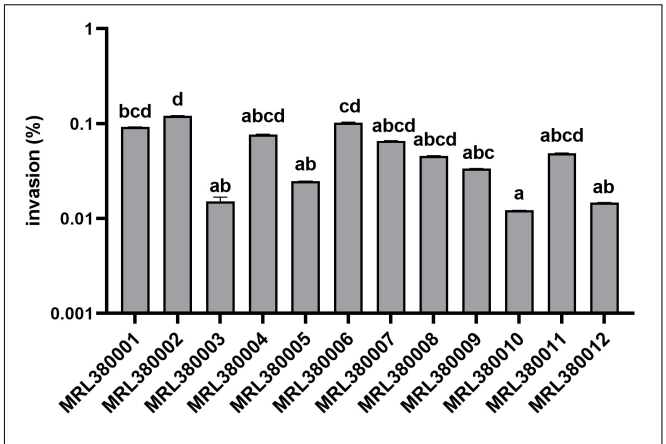


**FIGURE 7 |** Invasion of Caco-2 cells by different strains of STEC. The same letter indicates that there was no significant difference between each other, and the absence of the same letter indicates that there was a significant difference between each other ($p < 0.05$). Results were expressed as percent of invasive bacteria respect to inoculum.

*stx1*. MRL380010 had the lowest adhesion rate, and its serotype was O136:H12, which did not carry *eae* and *stx2* genes, but carried *stx1* genes. We found that there were also differences in adhesion ability between strains of the same serotype. The serotypes of MRL380001, MRL380003, and MRL380004 were O157:H7, among which MRL380001 and MRL380003 showed significant differences in adhesion ability to cells. Kobayashi et al. (2016) measured the cell adhesion capacity of 11 STEC strains with serotype O103:H2, and the results showed that there were significant differences in adhesion capacity among strains of the same serotype, which was similar to our results.

MRL380002 had the greatest invasive capacity and it was significantly higher than that of MRL380003, MRL380005, MRL380009, MRL380010, and MRL380012 ($p < 0.05$). The invasion rates of MRL380004, MRL380007, MRL380008, and MRL380011 were not significantly different from other strains. MRL380001, MRL380003, and MRL380004 belonging to O157:H7 showed no significant difference in invasive capacity. Serotypes of MRL380009, MRL380010, MRL380011, and MRL380012 were O136:H12. There was no significant difference in the invasion rate of Caco-2 cells. The adhesion and invasion abilities of MRL380002 were the highest among strains, but the order of adhesion and invasion abilities of other strains was not consistent, indicating that there was not necessarily a correlation between the adhesion ability and invasion ability. In addition, the outstanding adherence to and invasion of Caco-2 epithelial cells by O26:H11 confirmed that the virulence of non-O157 strain might not be underestimated. The serotypes O26:H11 and O103:H2 were used for comparison together with O157:H7 because these serotypes are considered the most important non-O157 STEC serotypes associated with increasing frequency in patients with bloody diarrhea and HUS (Jelacic et al., 2003; Alexander et al., 2005).

Combined with the CRISPR typing results of STEC, this study also analyzed the differences of adhesion and invasion capacities among strains with different CRISPR type. The distribution of cell adhesion capacity of different CT type STEC strains is shown in **Figure 8**.

The mean adhesion rate of CT398 was the highest followed by CT401, and adhesion rates of CT398 and CT401 strains to Caco-2 cells were significantly higher than that of other CRISPR types ($p < 0.05$). CT399 adhesion ability was significantly higher than CT400, CT402, CT403, CT404, CT405, and CT406 ($p < 0.05$), but with CT7 and CT48, there was no significant difference between strains. Whether the results of CRISPR typing can predict the adhesive capacity of the strains needs to be further studied by increasing the number of strains.

CT398 had the largest quantity of virulence genes, including *eae* and *stx1*, and CT402 and CT404 with the lowest adhesion rate did not have *eae* because *eae* was a factor related to adhesive capacity (Kobayashi et al., 2016), the lack of *eae* had a certain impact on the adhesive capacity of the strain. Including intimin

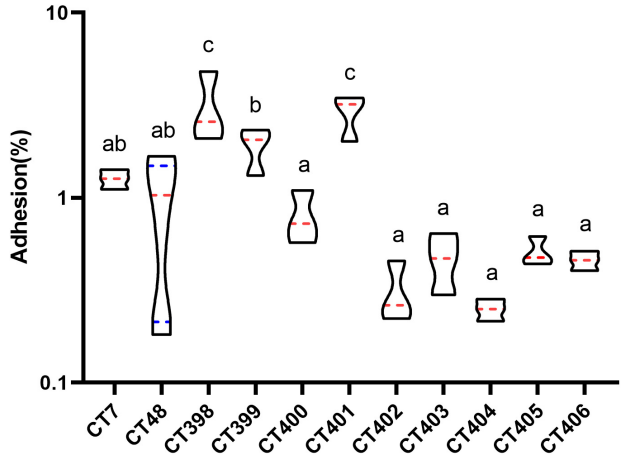


**FIGURE 8 |** Distribution of adhesive capacity of different CRISPR typing STEC strains. The same letter indicates that there was no significant difference between each other, and the absence of the same letter indicates that there was a significant difference between each other ($p < 0.05$). The red lines represent the median, and the blue lines represent the quartile.

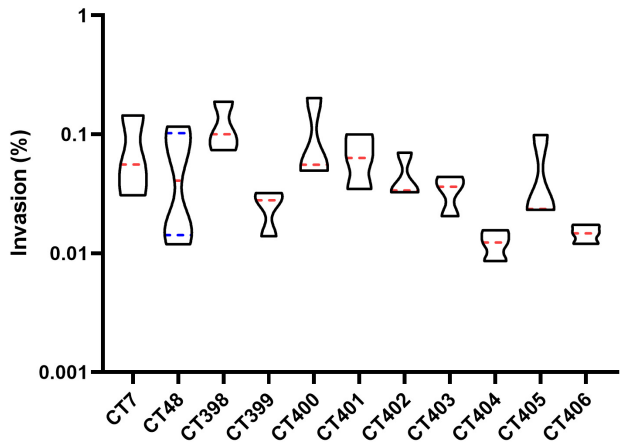


**FIGURE 9 |** Distribution of invasion capacity of different CRISPR typing STEC strains. The red lines represent the median, and the blue lines represent the quartile.

(*eae*), translocated intimin receptor (*tir*), the type III secretion apparatus (*espB* and *espD*), and homolog adhesion (*iha*) are also associated with adherence to the intestinal epithelium (Tarr et al., 2000; Kobayashi et al., 2016). CT398 with the highest adhesion rate carried *tir* and *espB*. CT7 and CT48 carried *tir* and *espB*, and *iha* had a higher adhesion rate. CT402, CT403, CT404, CT405, CT406 *tir*, *espB*, and *iha* were negative, and their adhesive capacities were inferior. The largest number of spacer sequences were identified in CT402, but CT402 had the lowest number of virulence genes and a low adhesive capacity. How the spacer affects the adhesive capacity of strains is indistinct, and further investigation is needed by increasing the number of strains.

The invasion capacity of STEC strains of different CRISPR types is shown in **Figure 9**. CT398 showed the highest invasion value followed by CT400, and CT404 was generally lower than other CT types. CT398 carried *eae* and *stx1*; moreover, the number of virulence genes and adhesion rate are both the highest. CT404 had the least virulence genes quantity and adhesion rate carried *stx1* without *eae*. There was no significant difference in the invasion rate between different CT type strains ($p > 0.05$), which was different from the adhesion capacity.

## CONCLUSION

In conclusion, the existence rate of CRISPR1 and CRISPR2 loci in DEC strains was generally higher than that of CRISPR3 and CRISPR4 loci. The strains with the same H antigen shared more spacers, while the same O group did not share spacer sequences or shared spacers less. The CRISPR spacers polymorphism showed the potential for DEC typing, providing clues for inferring virulence of strains. The number of virulence genes was different among DEC strains with different CRISPR types, indicating that the pathogenicity potential of CT strains was different. For STEC, the adhesion capacity of different CT strains was significantly different, which provided a basis for CRISPR typing to distinguish the pathogenicity of strains. As a whole, with the increase in the number of DEC strains and the popularization of CRISPR typing method, it is expected to judge the pathogenic potential of the strain by CRISPR type. Moreover, paying attention to CT strains with strong virulence potential in advance is beneficial to reduce the occurrence of food safety problems.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

## AUTHOR CONTRIBUTIONS

QD and HL conceived of the study and modified the first draft of the article. ZB was responsible for the experimental work, article writing, and data analysis. SZ isolated laboratory strains. XW and WW provided help with research ideas. MA modified the article. All authors reviewed and approved the final article.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.852662/full#supplementary-material

## REFERENCES


Afset, J. E., Bevanger, L., Romundstad, P., and Bergh, K. (2004). Association of atypical enteropathogenic *Escherichia coli* (EPEC) with prolonged diarrhoea. *J. Med. Microbiol.* 53(Pt 11), 1137–1144. doi: 10.1099/jmm.0.45719-0

Alexander, M., Martina, B., Zimmerhackl, L. B., Rita, P., Dag, H., Helmut, T., et al. (2005). Enterohemorrhagic *Escherichia coli* in human infection: *in vivo* evolution of a bacterial pathogen. *Clin. Infect. Dis.* 6, 785–792. doi: 10.1086/432722

Boerlin, P., Mcewen, S. A., Boerlin-Petzold, F., Wilson, J. B., and Gyles, C. (1999). Associations between virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans. *J. Clin. Microbiol.* 37, 497–503. doi: 10.1128/JCM.37.3.497-503.1999

Bolotin, A., Quinquis, B., Sorokin, A., and Ehrlich, S. D. (2005). Clustered regularly interspaced short palindrome repeats (CRISPRs) have spacers of extrachromosomal origin. *Microbiology* 151(Pt 8), 2551–2561. doi: 10.1099/mic.0.28048-0

Bradley, S. R., Gorbach, S. L., Banwell, J. G., Benedicta, J., Chatterjee, B. D., and Mitra, R. (1971). Enterotoxigenic *Escherichia coli* isolated from patients with severe cholera-like disease. *J. Infect. Dis.* 123, 378–385. doi: 10.1093/infdis/123.4.378

Bruyand, M., Mariani-Kurkdjian, P., Gouali, M., de Valk, H., King, L. A., Le Hello, S., et al. (2018). Hemolytic uremic syndrome due to Shiga toxin-producing *Escherichia coli* infection. *Med. Mal. Infect.* 48, 167–174. doi: 10.1016/j.medmal.2017.09.012

Crofts, A. A., Giovanetti, S. M., Rubin, E. J., Poly, F. M., Gutierrez, R. L., Talaat, K. R., et al. (2018). Enterotoxigenic *E. coli* virulence gene regulation in human infections. *Proc. Natl. Acad. Sci. U.S.A.* 115, E8968–E8976. doi: 10.1073/pnas.1808982115

Croxen, M. A., and Finlay, B. B. (2010). Molecular mechanisms of *Escherichia coli* pathogenicity. *Nat. Rev. Microbiol.* 8, 26–38. doi: 10.1038/nrmicro2265

Daniels, N. A. (2005). Enterotoxigenic *Escherichia coli*: traveler's diarrhea comes home. *Clin. Infect. Dis.* 42, 335–336. doi: 10.1086/499249

Deveau, H., Garneau, J. E., and Moineau, S. (2010). CRISPR/Cas system and its role in phage-bacteria interactions. *Annu. Rev. Microbiol.* 64, 475–493. doi: 10.1146/annurev.micro.112408.134123

Dias, R. C., Dos Santos, B. C., Dos Santos, L. F., Vieira, M. A., Yamatogi, R. S., Mondelli, A. L., et al. (2016). Diarrheagenic *Escherichia coli* pathotypes investigation revealed atypical enteropathogenic *E. coli* as putative emerging diarrheal agents in children living in Botucatu. Sao Paulo State, Brazil. *APMIS* 124, 299–308. doi: 10.1111/apm.12501

Díez-Villaseñor, C., Almendros, C., García-Martínez, J., and Mojica, F. J. M. (2010). Diversity of CRISPR loci in *Escherichia coli*. *Microbiology* 156, 1351–1361. doi: 10.1099/mic.0.036046-0

Farajzadeh-Sheikh, A., Savari, M., Ahmadi, K., Hosseini Nave, H., Shahin, M., and Afzali, M. (2020). Distribution of genes encoding virulence factors and the genetic diversity of enteroinvasive *Escherichia coli* (EIEC) Isolates from Patients with Diarrhea in Ahvaz, Iran. *Infect. Drug Resist.* 13, 119–127. doi: 10.2147/IDR.S235009

Fujioka, M., Otomo, Y., and Ahsan, C. R. (2013). A novel single-step multiplex polymerase chain reaction assay for the detection of diarrheagenic *Escherichia coli*. *J. Microbiol. Methods* 92, 289–292. doi: 10.1016/j.mimet.2012.12.010

Gomes, T. A., Elias, W. P., Scaletsky, I. C., Guth, B. E., Rodrigues, J. F., Piazza, R. M., et al. (2016). Diarrheagenic *Escherichia coli*. *Braz. J. Microbiol.* 47(Suppl. 1), 3–30. doi: 10.1016/j.bjm.2016.10.015

Grissa, I., Vergnaud, G., and Pourcel, C. (2007). CRISPRFinder: a web tool to identify clustered regularly interspaced short palindromic repeats. *Nucleic Acids Res.* 35, W52–W57. doi: 10.1093/nar/gkm360

Hebbelstrup Jensen, B., Poulsen, A., Hebbelstrup Rye Rasmussen, S., Struve, C., Engberg, J. H., Friis-Moller, A., et al. (2017). Genetic virulence profile of enteroaggregative *Escherichia coli* strains isolated from danish children with either acute or persistent diarrhea. *Front. Cell Infect. Microbiol.* 7:230. doi: 10.3389/fcimb.2017.00230

Hernandes, R. T., Elias, W. P., Vieira, M. A., and Gomes, T. A. (2009). An overview of atypical enteropathogenic *Escherichia coli*. *FEMS Microbiol. Lett.* 297, 137–149. doi: 10.1111/j.1574-6968.2009.01664.x

Hunter, P. R., and Gaston, M. A. (1988). Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. *J. Clin. Microbiol.* 26, 2465–2466. doi: 10.1128/jcm.26.11.2465-2466.1988

Jelacic, J. K., Damrow, T., Chen, G. S., Jelacic, S., Bielaszewska, M., Ciol, M., et al. (2003). Shiga toxin–producing *Escherichia coli* in montana: bacterial genotypes and clinical profiles. *J. Infect. Dis.* 188, 719–729. doi: 10.1086/376999

Joensen, K. G., Tetzschner, A. M., Iguchi, A., Aarestrup, F. M., and Scheutz, F. (2015). Rapid and easy *in silico* serotyping of *Escherichia coli* isolates by use of whole-genome sequencing data. *J. Clin. Microbiol.* 53, 2410–2426. doi: 10.1128/JCM.00008-15

Karmali, M. A. (2017). Emerging public health challenges of shiga toxin-producing *escherichia coli* related to changes in the pathogen, the population, and the environment. *Clin. Infect. Dis.* 64, 371–376. doi: 10.1093/cid/ciw708

Karmali, M. A. (2018). Factors in the emergence of serious human infections associated with highly pathogenic strains of shiga toxin-producing *Escherichia coli*. *Int. J. Med. Microbiol.* 308, 1067–1072. doi: 10.1016/j.ijmm.2018.08.005

Karmali, M., Petric, M., Steele, B., and Lim, C. J. L. (1983). Sporadic cases of haemolytic-uraemic syndrome associated with faecal cytotoxin and cytotoxin-producing *Escherichia coli* in stools. *Lancet* 321, 619–620. doi: 10.1016/s0140-6736(83)91795-6

Keir, L. S., Marks, S. D., and Kim, J. J. (2012). Shigatoxin-associated hemolytic uremic syndrome: current molecular mechanisms and future therapies. *Drug Des. Dev. Ther.* 6, 195–208. doi: 10.2147/DDDT.S25757

Klingberg, T. D., Lesnik, U., Arneborg, N., Raspor, P., and Jespersen, L. (2008). Comparison of Saccharomyces cerevisiae strains of clinical and nonclinical origin by molecular typing and determination of putative virulence traits. *FEMS Yeast Res.* 8, 631–640. doi: 10.1111/j.1567-1364.2008.00365.x

Kobayashi, N., Maeda, E., Saito, S., Furukawa, I., and Hara-Kudo, Y. J. (2016). Association of cell-adhesion activities with virulence in shiga toxin-producing *Escherichia coli* O103:H2. *Biocontrol Sci.* 21, 57–61. doi: 10.4265/bio.21.57

Kruger, A., Lucchesi, P. M., Sanso, A. M., Etcheverria, A. I., Bustamante, A. V., Burgan, J., et al. (2015). Genetic characterization of Shiga toxin-producing *Escherichia coli* O26:H11 strains isolated from animal, food, and clinical samples. *Front. Cell Infect. Microbiol.* 5:74. doi: 10.3389/fcimb.2015.00074

Kyle, J. L., Cummings, C. A., Parker, C. T., Quinones, B., Vatta, P., Newton, E., et al. (2012). *Escherichia coli* serotype O55:H7 diversity supports parallel acquisition of bacteriophage at Shiga toxin phage insertion sites during evolution of the O157:H7 lineage. *J. Bacteriol.* 194, 1885–1896. doi: 10.1128/JB.00120-12

Larkin, M. A., Blackshields, G., Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., et al. (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* 23, 2947–2948. doi: 10.1093/bioinformatics/btm404

Li, Q., Wang, X., Yin, K., Hu, Y., Xu, H., Xie, X., et al. (2018). Genetic analysis and CRISPR typing of *Salmonella enterica* serovar Enteritidis from different sources revealed potential transmission from poultry and pig to human. *Int. J. Food Microbiol.* 266, 119–125. doi: 10.1016/j.ijfoodmicro.2017.11.025

Long, J., Xu, Y., Ou, L., Yang, H., Xi, Y., Chen, S., et al. (2019). Polymorphism of Type I-F CRISPR/Cas system in *Escherichia coli* of phylogenetic group B2 and its application in genotyping. *Infect. Genet. Evol.* 74:103916. doi: 10.1016/j.meegid.2019.103916

Makarova, K. S., Haft, D. H., Barrangou, R., Brouns, S. J., Charpentier, E., Horvath, P., et al. (2011). Evolution and classification of the CRISPR-Cas systems. *Nat. Rev. Microbiol.* 9, 467–477. doi: 10.1038/nrmicro2577

Manuel, E., Gordillo Gordon, R., Reeve Janeene, P., Mathewson, J. J., Dupont, H. L., and Murray, B. E. (1992). Molecular characterization of strains of enteroinvasive *Escherichia coli* O143, including isolates from a large outbreak in Houston, Texas. *J. Clin. Microbiol.* 30, 889–893. doi: 10.1128/jcm.30.4.889-893.1992

Marraffini, L. A., and Sontheimer, E. J. (2008). CRISPR interference limits horizontal gene transfer in staphylococci by targeting DNA. *Science* 322, 1843–1845. doi: 10.1126/science.1165771

Marraffini, L. A., and Sontheimer, E. J. (2010). CRISPR interference: RNA-directed adaptive immunity in bacteria and archaea. *Nat. Rev. Genet.* 11, 181–190. doi: 10.1038/nrg2749

Michel, P. A., and Kase, J. A. (2009). Genetic profiles of Shiga toxin and intimin genes found in stool broth cultures: a 2-year reference laboratory study. *Diagn. Microbiol. Infect. Dis.* 65, 85–92. doi: 10.1016/j.diagmicrobio.2009.06.006

Mojica, F. J., Diez-Villasenor, C., Garcia-Martinez, J., and Soria, E. (2005). Intervening sequences of regularly spaced prokaryotic repeats derive from

foreign genetic elements. *J. Mol. Evol.* 60, 174–182. doi: 10.1007/s00239-004-0046-3

Mondal, I., Bhakat, D., Chowdhury, G., Manna, A., Samanta, S., Deb, A. K., et al. (2022). Distribution of virulence factors and its relatedness towards the antimicrobial response of enterotoxigenic *Escherichia coli* strains isolated from patients in Kolkata, India. *J. Appl. Microbiol.* 132, 675–686. doi: 10.1111/jam.15206

Muniesa, M., Jofre, J., García-Aljaro, C., and Blanch, A. R. (2006). Occurrence of *Escherichia coli* O157:H7 and other enterohemorrhagic *Escherichia coli* in the environment. *Environ. Sci. Technol.* 40, 7141–7149. doi: 10.1021/es060927k

Olesen, I., and Jespersen, L. (2010). Relative gene transcription and pathogenicity of enterohemorrhagic *Escherichia coli* after long-term adaptation to acid and salt stress. *Int. J. Food Microbiol.* 141, 248–253. doi: 10.1016/j.ijfoodmicro.2010.05.019

Pai, C. H., Ahmed, N., Lior, H., Johnson, W. M., Sims, H. V., and Woods, D. E. (1988). Epidemiology of sporadic diarrhea due to verocytotoxin-producing *Escherichia coli*: a two-year prospective study. *J. Infect. Dis.* 157, 1054–1057. doi: 10.1093/infdis/157.5.1054

Pereira, A. L., Ferraz, L. R., Silva, R. S., and Giugliano, L. G. (2007). Enteroaggregative *Escherichia coli* virulence markers: positive association with distinct clinical characteristics and segregation into 3 enteropathogenic *E. coli* Serogroups. *J. Infect. Dis.* 195, 366–374. doi: 10.1086/510538

Pourcel, C., Salvignol, G., and Vergnaud, G. (2005). CRISPR elements in *Yersinia pestis* acquire new repeats by preferential uptake of bacteriophage DNA, and provide additional tools for evolutionary studies. *Microbiology* 151(Pt 3), 653–663. doi: 10.1099/mic.0.27437-0

Riley, L. W., Remis, R. S., Helgerson, S. D., Mcgee, H. B., and Wells, J. G. (1983). Hemorrhagic colitis associated with a rare *Escherichia coli* Serotype. *NEJM* 308, 681–685. doi: 10.1056/NEJM198303243081203

Rodrigues, V. F. V., Rivera, I. N. G., Lim, K. Y., and Jiang, S. C. (2016). Detection and risk assessment of diarrheagenic *E. coli* in recreational beaches of Brazil. *Mar. Pollut. Bull.* 109, 163–170. doi: 10.1016/j.marpolbul.2016.06.007

Rump, L. V., Feng, P. C., Fischer, M., and Monday, S. R. (2010). Genetic analysis for the lack of expression of the O157 antigen in an O Rough:H7 *Escherichia coli* strain. *Appl. Environ. Microbiol.* 76, 945–947. doi: 10.1128/AEM.02046-09

Scaletsky, I. C. A., Fabbricotti, S. H., Silva, S. O. C., Morais, M. B., and Fagundes-Neto, U. (2002). HEp-2–Adherent *Escherichia coli* strains associated with acute infantile diarrhea, São Paulo, Brazil. *Emerg. Infect. Dis.* 8, 855–858. doi: 10.3201/eid0808.010492

Shahbazi, G., Rezaee, M. A., Nikkhahi, F., Ebrahimzadeh, S., Hemmati, F., Namarvar, B. B., et al. (2021). Characteristics of diarrheagenic *Escherichia coli* pathotypes among children under the age of 10 years with acute diarrhea. *Gene Rep.* 25:101318. doi: 10.1016/j.genrep.2021.101318

Shen, J., Rump, L., Ju, W., Shao, J., Zhao, S., Brown, E., et al. (2015). Virulence characterization of non-O157 Shiga toxin-producing *Escherichia coli* isolates from food, humans and animals. *Food Microbiol.* 50, 20–27. doi: 10.1016/j.fm.2015.02.007

Spano, L. C., Guerrieri, C. G., Volpini, L. P. B., Schuenck, R. P., Goulart, J. P., Boina, E., et al. (2021). EHEC O111:H8 strain and norovirus GII.4 Sydney [P16] causing an outbreak in a daycare center. Brazil, 2019. *BMC Microbiol.* 21:95. doi: 10.1186/s12866-021-02161-x

Tarr, P. I., Bilge, S. S., Vary, J. C., Jelacic, S., Habeeb, R. L., Ward, T. R., et al. (2000). Iha: a novel *Escherichia coli* O157:H7 adherence-conferring molecule encoded on a recently acquired chromosomal island of conserved structure. *Infect. Immun.* 68, 1400–1407. doi: 10.1128/IAI.68.3.1400-1407.2000

Toro, M., Cao, G., Ju, W., Allard, M., Barrangou, R., Zhao, S., et al. (2014). Association of clustered regularly interspaced short palindromic repeat (CRISPR) elements with specific serotypes and virulence potential of shiga toxin-producing *Escherichia coli*. *Appl. Environ. Microbiol.* 80, 1411–1420. doi: 10.1128/AEM.03018-13

Vieira, N., Bates, S. J., Solberg, O. D., Ponce, K., Howsmon, R., Cevallos, W., et al. (2007). High prevalence of enteroinvasive *Escherichia coli* isolated in a remote region of northern coastal Ecuador. *Am. J. Trop. Med. Hyg.* 76, 528–533. doi: 10.4269/ajtmh.2007.76.528

Wang, Q., Perepelov, A. V., Wen, L., Shashkov, A. S., Wang, X., Guo, X., et al. (2012). Identification of the two glycosyltransferase genes responsible for the difference between *Escherichia coli* O107 and O117 O-antigens. *Glycobiology* 22, 281–287. doi: 10.1093/glycob/cwr137

Wani, S. A., Hussain, I., Nabi, A., Fayaz, I., and Nishikawa, Y. (2007). Variants of eae and stx genes of atypical enteropathogenic *Escherichia coli* and non-O157 Shiga toxin-producing *Escherichia coli* from calves. *Lett. Appl. Microbiol.* 45, 610–615. doi: 10.1111/j.1472-765X.2007.02235.x

Xiong, Y., Wang, P., Lan, R., Ye, C., and Wang, H. (2012). A Novel *Escherichia coli* O157:H7 clone causing a major hemolytic uremic syndrome outbreak inChina. *PLoS One* 7:e36144. doi: 10.1371/journal.pone.0036144

Yan, X., Fratamico, P., Bono, J., Baranzoni, G., and Chen, C. J. (2015). Genome sequencing and comparative genomics provides insights on the evolutionary dynamics and pathogenic potential of different H-serotypes of Shiga toxin-producing *Escherichia coli* O104. *BMC Microbiol.* 15:83. doi: 10.1186/s12866-015-0413-9

Yeh, H. Y., and Awad, A. (2020). Genotyping of *Campylobacter jejuni* isolates from poultry by clustered regularly interspaced short palindromic repeats (CRISPR). *Curr. Microbiol.* 77, 1647–1652. doi: 10.1007/s00284-020-01965-w

Yin, S., Jensen, M. A., Bai, J., Debroy, C., Barrangou, R., and Dudley, E. G. (2013). The evolutionary divergence of Shiga toxin-producing *Escherichia coli* is reflected in clustered regularly interspaced short palindromic repeat (CRISPR) spacer composition. *Appl. Environ. Microbiol.* 79, 5710–5720. doi: 10.1128/AEM.00950-13

Zeng, H., Li, C., He, W., Zhang, J., Chen, M., Lei, T., et al. (2019). *Cronobacter sakazakii, Cronobacter malonaticus, and Cronobacter dublinensis* genotyping based on CRISPR locus diversity. *Front. Microbiol.* 10:1989. doi: 10.3389/fmicb.2019.01989

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# Analysis of Key Control Points of Microbial Contamination Risk in Pork Production Processes Using a Quantitative Exposure Assessment Model

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Pork is one of the most common foods causing microbial foodborne diseases. Since pork directly enters the market after slaughtering, the control of microbial contamination in the slaughtering processes is the key to ensuring the quality and safety of pork. The contamination level of *Escherichia coli*, a health-indicator bacterium, can reflect the risk level of potential pathogens. In order to assess the *E. coli* exposure risk of pork during slaughtering and to identify the key control points, we established an *E. coli* quantitative exposure assessment model for swine-slaughtering processes in slaughterhouses of different sizes. The model simulation data indicated the *E. coli* contamination pattern on the surfaces of swine carcasses during slaughtering. The changes in *E. coli* contamination were analyzed according to the simulation data of each slaughtering process. It was found that the number of *E. coli* after trimming in big and small slaughterhouses increased to the maximum values for the whole processes, which were 3.63 and 3.52 $\log_{10}$ CFU/100 $cm^2$, respectively. The risk contribution of each slaughtering process to the *E. coli* contamination on the surface of terminal swine carcasses can be determined by correlation analysis. Because the absolute value of correlation coefficient during the trimming process was maximum (0.49), it was regarded as the most important key control point. This result can be further proved via the multilocus sequence typing of *E. coli*. The dominant sequence type before trimming processes was ST10. ST1434 began to appear in the trimming process and then became the dominant sequence type in the trimming and pre-cooling processes. The model can provide a theoretical basis for microbial hygiene supervision and risk control in swine-slaughtering processes.

**Keywords: swine-slaughtering processes, microbial contamination risk, quantitative exposure assessment model, key control points, multilocus sequence typing**

# INTRODUCTION

Pork has played a dominant role in China's meat consumption for a long time, accounting for more than 60% of the total meat consumption since 2000 (Min et al., 2015). Pork directly enters the market after slaughtering, and it is directly exposed to the surrounding environment from the beginning of the slaughtering process. Since the microbial contamination caused by the devices and appliances used for slaughtering is inevitable (Van Ba et al., 2019), the hygienic control of the slaughtering processes is indispensable. Some scholars pointed out that the number of microorganisms on the carcass surface after slaughtering has a significant impact on the shelf life and safety of products (Gill et al., 1998). Therefore, controlling microbial contamination during slaughtering processes is the key to ensure the quality and safety of meat.

*Escherichia coli* is an intestinal symbiotic bacterium in humans and animals, and most of its strains are harmless to people. In the case of poor environmental sanitation, it is often scattered in the surrounding environment with feces. Because there are some normal flora in addition to intestinal pathogenic bacteria in the feces, *E. coli* can be used as a health-indicator bacterium to reflect food hygiene and the risk to human health. The detection of *E. coli* indicates that there may be fecal contamination. Repeated detection of *E. coli* in multiple processes of food production indicates that the risk of food safety is increasing, and the risk of possible cross-contamination of various pathogenic microorganisms is high (Kusturov et al., 2017).

Key control points are important steps or processes determined by analyzing raw materials, production processes, and human factors affecting product quality and safety (Setiabuhdi et al., 1997). Analysis of key control points can control or eliminate food safety hazards to acceptable levels. Quantitative microbiological risk assessment (QMRA) has been increasingly investigated and paid more attention in food safety management and control worldwide, including China (Dong et al., 2015; Wu et al., 2018). As an important part of risk assessment, exposure assessments can provide useful information for identifying key control points. An exposure assessment model is a more scientific assessment method based on monitoring data, which is formed by data fitting, logical operation, and random sampling simulation. In order to explore the key control points affecting the risk of microbial contamination in pork during slaughtering more scientifically, it can be realized by establishing an *E. coli* quantitative exposure assessment model in different swine-slaughtering processes. Some studies have aimed at establishing microbial risk assessment models for the "farm-to-fork" food chain or retail and subsequent links (Dang-Xuan et al., 2018; Zhang et al., 2018). In addition, because pork has been identified as one of the main sources of *Salmonella* infection in humans, the risk assessment of microorganisms in pork also focused on *Salmonella* (Swart et al., 2016; Gurman et al., 2018). However, there are other unknown risks posed by pathogenic microorganisms in pork. Insufficient understanding of the application of health-indicator bacteria in exposure assessment and the difficulties of sample collection in swine-slaughtering lines hinder the establishment of such an exposure assessment model. Therefore, exposure assessment of health-indicator bacteria such as *E. coli* used to analyze the overall contamination risk of pathogenic microorganisms during swine-slaughtering is urgently needed.

In this study, an *E. coli* exposure assessment model suitable for China's general swine-slaughtering processes was established, and the key control points of microbial contamination were analyzed. Our study provides a scientific basis for effectively controlling the risk of pathogenic microorganisms and improving the quality of pork products.

# MATERIALS AND METHODS

## Data Information During Swine Slaughtering

### Sample Collection

All contamination data of *E. coli* from various sources in the slaughterhouses were from the monitoring data of our laboratory. From August to September 2020, we chose five swine slaughterhouses of different sizes in Shandong Province, including three large slaughterhouses and two small slaughterhouses. Large slaughterhouses slaughter more than 300 swine per hour, and small slaughterhouses slaughter between 30 and 70 swine per hour (GB 50317-2009, 2009). We collected 359 surface samples of swine carcasses and 139 environmental samples from six slaughtering processes [skinning, washing (1), eviscerating, washing (2), trimming, and pre-cooling] and from the environment (workers' hands, slaughterhouses' ground, and appliances) in five slaughterhouses, using PBS sampling swabs (SWAB-10 PBS, ELAB Scientific, Escondido, United States), and transported them to the laboratory at 4°C on the same day.

### Isolation and Identification of Microorganisms

#### *Escherichia coli Counts*

The swabs collected from the five swine slaughterhouses were fully vortexed and diluted according to a gradient. Sample solutions (1 mL) of three suitable dilutions were vertically transferred into the center of the *E. coli*/Coliform Count Plate (3M Petrifilm 6414, 3M Health Care, Saint Paul, United States) using a pipette and cultured for about 24 h at 37°C. From to the instructions of 3M Petrifilm 6414, the blue and dark blue colonies with bubbles were identified as *E. coli*. The colonies with the above morphological characteristics were selected for *E. coli* count.

#### *Salmonella Detection*

Two slaughterhouses were randomly selected for isolation of *Salmonella* on swine carcass surfaces during slaughtering. The specific isolation method referred to ISO 6579-1:2017 (2017), and we used *inv*A primer for PCR identification (Rahn et al., 1992).

#### *Statistical Analysis*

Monitoring data of *E. coli* contamination during swine-slaughtering processes were statistically analyzed using SPSS 22 (IBM, Qingdao, China). *T*-test was used to investigate the statistical differences between adjacent processes.

### Multilocus Sequence Typing of *Escherichia coli*

We picked out *E. coli* on the count plates and inoculated them on MacConkey culture medium (CM908, Lu Qiao Technology, Beijing, China). All strains on MacConkey medium were cultured for 18 to 24 h at 37°C and purified two times. Strains were randomly selected from all of the *E. coli* isolates for multilocus sequence typing. Seven housekeeping genes of *E. coli* (*adk, fumC, gyrB, icd, mdh, purA,* and *recA*) were amplified according to the recommended PCR amplification procedure and annealing temperature (Wirth et al., 2006). PCR-amplified products were submitted to Beijing Qingke Biotechnology Co., Ltd. for sequencing. The cut sequences were inputted into the multilocus sequence typing database,[1] and the numerical sequence numbers of each allele was searched. The final seven sequence numbers determined the sequence type (ST) of a strain. The profile including seven gene loci of all strains was entered into BioNumerics software (v.7.6, Applied Maths, Kortrijk, Belgium) to construct the minimum spanning tree of different slaughtering processes. In the minimum spanning tree, circles correspond to STs, and the size of each circle is proportional to the number of isolates in each ST. The maximum risk process was determined according to the typing results.

## Exposure Assessment Model of *Escherichia coli*

We selected a single swine as the object for *E. coli* exposure assessment. The carcasses of swine after skinning were directly exposed to air and the environment for the subsequent processing. Therefore, we regarded skinning as the starting point of the evaluation procedure, and the downstream processes included washing (1), eviscerating, washing (2), trimming, and pre-cooling.

### Exposure Assessment Tool

The distribution fitting function of the risk assessment software @Risk (v.7.0, Palisade, NY, United States) was applied to process the data. The parameters and variables involved in the exposure assessment were expressed by formulas, specific values, or distributions. The model was established in Excel 2007 worksheet (Microsoft, Redmond, United States), and the Latin hypercube sampling method in @Risk was used for Monte Carlo simulation during model simulation.

### Data Fitting

This model for *E. coli* exposure assessment described the changes in *E. coli* concentration and the positive rate in a swine-slaughtering line [processes of skinning, washing (1), eviscerating, washing (2), trimming, and pre-cooling were included]. We used the monitoring data on *E. coli* prevalence rate in a swine-slaughtering line for data fitting by Fit Distribution, which formed the basis distributions in this exposure assessment model.

The data of *E. coli* concentration for quantitative fitting are shown in **Supplementary Table 1**. Results of *E. coli* quantitative data fitting in differently sized slaughterhouses are shown in the "Distribution" part of **Table 1** and **Supplementary Figures 1–5**. In each slaughtering process, the minimum, maximum, mean, standard test dose (std), and deviation (dev) of *E. coli* concentration of our data were input for data fitting, and optimum distributions which could describe the variation of *E. coli* concentration in each slaughtering process were output. The quantitative data-fitting distribution of *E. coli* adopted the data-fitting function in @Risk software to obtain the best-fitting function expression. Results of *E. coli* qualitative data fitting in differently sized slaughterhouses are also shown in the "Distribution" part of **Table 1**. We adopted RiskDiscrete distribution to describe the changed *E. coli* prevalence at each slaughtering process: RiskDiscrete ({0, 1},{a, b}), where "0" represents *E. coli* negative, "1" represents *E. coli* positive, "a" is the value of the *E. coli* negative rate, and "b" is the value of the *E. coli* positive rate.

### Monte Carlo Simulation and Model Establishment

On the basis of the fitting functions generated above, an exposure assessment model was established by @Risk and Monte Carlo simulation. In the logic relationship of this *E. coli* exposure assessment model, the output of the previous process was set as the input of the next process. The data of samples after skinning were set as the initial contamination load, and then the *E. coli* concentration or prevalence of other slaughtering processes was outputted successively through the Monte Carlo simulation. The number of iterations per simulation calculation was 10, 000 (Huang et al., 2017b). The system extracted a value from the distribution of each input variable to complete each iterative computation randomly.

### Sensitivity Analysis

Through the correlation coefficient of model fitting, the correlation between *E. coli* contamination in pork after pre-cooling and after each slaughtering process was analyzed to determine the risk contribution of each process to *E. coli* contamination in terminal pork products. Sensitivity analyses were performed in @Risk software. The Spearman level correlation coefficient was calculated to be between −1 and +1, where + and - denote positive correlation and negative correlation, respectively. The positive correlation coefficient indicated that the process had a risk elimination effect, while the negative correlation coefficient indicated that the process had a risk introduction effect. The higher the absolute value of the correlation coefficient, the greater the impact of this slaughtering process on the risk posed by terminal pork products.

# RESULTS

## Monitoring Data of *Escherichia coli* Contamination During Swine-Slaughtering Processes

From the *E. coli* monitoring data of slaughtering processes in slaughterhouses of different sizes (**Figure 1**), it can be

[1] http://mlst.warwick.ac.uk/mlst/mlst/dbs/Ecoli

**TABLE 1 |** *Escherichia coli* exposure assessment model of swine-slaughtering processes.

| Swine slaughterhouses' size | Module | Symbol | Description | Unit | Distribution/model | References |
|---|---|---|---|---|---|---|
| All slaughterhouses | Skinning | m | Surface area of a single pig | $100cm^2$ | RiskUniform (96,180) | Investigation |
| | | Las | Log number of *E. coli* after skinning | $Log_{10}CFU/100cm^2$ | RiskTriang (2.7825, 5.1249, 5.1249) | — |
| | | Pas | Prevalence of *E. coli* after skinning | — | RiskDiscrete ({0,1}, {0.3750, 0.6250}) | Data simulation |
| | | | Output of *E. coli* after skinning | $Log_{10}CFU/100cm^2$ | RiskOutput ("skinning") + IF(Pas = 0, 0, Las) | — |
| | Washing (1) | Lcw1 | Log number of *E. coli* changed through washing (1) | $Log_{10}CFU/100cm^2$ | RiskBetaGeneral (6.5293, 4.0775, −2.5387, 2.4624) | Data simulation |
| | | Law1 | Log number of *E. coli* after washing (1) | $Log_{10}CFU/100cm^2$ | Las-Lcw (1) | — |
| | | Paw1 | Prevalence of *E. coli* after washing (1) | — | RiskDiscrete ({0,1}, {0.4500, 0.5500}) | Data simulation |
| | | | Output of *E. coli* after washing(1) | $Log_{10}CFU/100cm^2$ | RiskOutput ["washing (1)"] + IF [Paw (1) = 0, 0, Law (1)] | — |
| | Eviscerating | Lce | Log number of *E. coli* changed through eviscerating | $Log_{10}CFU/100cm^2$ | RiskBetaGeneral (13.124, 10.199, −5.0443, 5.8443) | Data simulation |
| | | Lae | Log number of *E. coli* after eviscerating | $Log_{10}CFU/100cm^2$ | Law (1) + Lce | — |
| | | Pae | Prevalence of *E. coli* after eviscerating | — | RiskDiscrete ({0, 1}, {0.4182, 0.5818}) | Data simulation |
| | | | Output of *E. coli* after eviscerating | $Log_{10}CFU/100cm^2$ | RiskOutput ("eviscerating") + IF (Pae = 0, 0, Lae) | — |
| | Washing (2) | Lcw2 | Log number of *E. coli* changed through washing (2) | $Log_{10}CFU/100cm^2$ | RiskBetaGeneral (26.609, 19.752, −11.782, 10.133) | Data simulation |
| | | Law2 | Log number of *E. coli* after washing (2) | $Log_{10}CFU/100cm^2$ | Lae-Lcw (2) | — |
| | | Paw2 | Prevalence of *E*. coli after washing (2) | — | RiskDiscrete ({0, 1}, {0.6182, 0.3818}) | Data simulation |
| | | | Output of *E. coli* after washing (2) | $Log_{10}CFU/100cm^2$ | RiskOutput ["washing (2)"] + IF [Paw (2) = 0, 0, Law (2)] | — |
| | Trimming | Lct | Log number of *E. coli* changed through trimming | $Log_{10}CFU/100cm^2$ | RiskBetaGeneral (33.414, 29.533, −17.982, 16.126) | Data simulation |
| | | Lat | Log number of *E. coli* after trimming | $Log_{10}CFU/100cm^2$ | Law (2) + Lct | — |
| | | Pat | Prevalence of *E*. coli after trimming | — | RiskDiscrete ({0, 1}, {0.149, 0.851}) | Data simulation |
| | | | Output of *E. coli* after trimming | $Log_{10}CFU/100cm^2$ | RiskOutput ("trimming") + IF (Pat = 0, 0, Lat) | — |
| | Pre-cooling | Lcp | Log number of *E. coli* changed through pre-cooling | $Log_{10}CFU/100cm^2$ | RiskNormal (0.75094, 3.1227) | Data simulation |
| | | Lap | Log number of *E. coli* after pre-cooling | $Log_{10}CFU/100cm^2$ | Lat-Lcp | — |
| | | Pap | Prevalence of *E. coli* after pre-cooling | — | RiskDiscrete ({0, 1}, {0.3263, 0.6737}) | Data simulation |
| | | | Output of *E. coli* after pre-cooling | $Log_{10}CFU/100cm^2$ | RiskOutput ("pre-cooling") + IF (Pap = 0, 0, Lap) | — |

*(Continued)*

**TABLE 1 |** (Continued)

| Swine slaughterhouses' size | Module | Symbol | Description | Unit | Distribution/model | References |
|---|---|---|---|---|---|---|
| Big slaughterhouses | Skinning | m | Surface area of a single pig | $100cm^2$ | RiskUniform (96, 180) | Investigation |
| | | Las | Log number of *E. coli* after skinning | $Log_{10}CFU/100cm^2$ | RiskUniform (2.8672, 5.2577) | — |
| | | Pas | Prevalence of *E.* coli after skinning | — | RiskDiscrete ({0, 1}, {0.4000, 0.6000}) | Data simulation |
| | | | Output of *E. coli* after skinning | $Log_{10}CFU/100cm^2$ | RiskOutput ("skinning") + IF (Pas = 0, 0, Las) | — |
| | Washing (1) | Lcw (1) | Log number of *E. coli* changed through washing (1) | $Log_{10}CFU/100cm^2$ | RiskBetaGeneral (6.4614, 6.2514, −2.7547, 3.3950) | Data simulation |
| | | Law (1) | Log number of *E. coli* after washing (1) | $Log_{10}CFU/100cm^2$ | Las-Lcw (1) | — |
| | | Paw (1) | Prevalence of *E. coli* after washing (1) | — | RiskDiscrete ({0, 1}, {0.4000, 0.6000}) | Data simulation |
| | | | Output of *E. coli* after washing (1) | $Log_{10}CFU/100cm^2$ | RiskOutput ["washing (1)"] + IF [Paw (1) = 0, 0, Law (1)] | — |
| | Eviscerating | Lce | Log number of *E. coli* changed through eviscerating | $Log_{10}CFU/100cm^2$ | RiskBetaGeneral (8.0576, 7.9915, −4.4410, 6.2900) | Data simulation |
| | | Lae | Log number of *E. coli* after eviscerating | $Log_{10}CFU/100cm^2$ | Law (1) + Lce | — |
| | | Pae | Prevalence of *E. coli* after eviscerating | — | RiskDiscrete ({0, 1}, {0.5143, 0.4857}) | Data simulation |
| | | | Output of *E. coli* after eviscerating | $Log_{10}CFU/100cm^2$ | RiskOutput ("eviscerating") + IF (Pac = 0, 0, Lae) | — |
| | Washing (2) | Lcw (2) | Log number of *E. coli* changed through washing (2) | $Log_{10}CFU/100cm^2$ | RiskBetaGeneral (16.939, 17.633, −9.6107, 11.382) | Data simulation |
| | | Law (2) | Log number of *E. coli* after washing (2) | $Log_{10}CFU/100cm^2$ | Lae-Lcw (2) | — |
| | | Paw (2) | Prevalence of *E.* coli after washing (2) | — | RiskDiscrete ({0, 1}, {0.6857, 0.3143}) | Data simulation |
| | | | Output of *E. coli* after washing (2) | $Log_{10}CFU/100cm^2$ | RiskOutput ["washing (2)"] + IF [Paw (2) = 0, 0, Law (2)] | — |
| | Trimming | Lct | Log number of *E. coli* changed through trimming | $Log_{10}CFU/100cm^2$ | RiskNormal (0.22194, 2.4674) | Data simulation |
| | | Lat | Log number of *E. coli* after trimming | $Log_{10}CFU/100cm^2$ | Law (2) + Lct | — |
| | | Pat | Prevalence of *E. coli* after trimming | — | RiskDiscrete ({0, 1}, {0.133, 0.867}) | Data simulation |
| | | | Output of *E. coli* after trimming | $Log_{10}CFU/100cm^2$ | RiskOutput ("trimming") + IF (Pat = 0, 0, Lat) | — |
| | Pre-cooling | Lcp | Log number of *E. coli* changed through pre-cooling | $Log_{10}CFU/100cm^2$ | RiskNormal (1.0892, 3.6776) | Data simulation |
| | | Lap | Log number of *E. coli* after pre-cooling | $Log_{10}CFU/100cm^2$ | Law (2)-Lcp | — |
| | | Pap | Prevalence of *E. coli* after pre-cooling | — | RiskDiscrete ({0, 1}, {0.2667, 0.7333}) | Data simulation |
| | | | Output of *E. coli* after pre-cooling | $Log_{10}CFU/100cm^2$ | RiskOutput ("pre-cooling") + IF (Pap = 0, 0, Lap) | — |
| Small slaughterhouses | Skinning | m | Surface area of a single pig | $100cm^2$ | RiskUniform (96, 180) | Investigation |
| | | Las | Log number of *E. coli* after skinning | $Log_{10}CFU/100cm^2$ | RiskUniform (4.0586, 5.1097) | — |

*(Continued)*

**TABLE 1 |** (Continued)

| Swine slaughterhouses' size | Module | Symbol | Description | Unit | Distribution/model | References |
|---|---|---|---|---|---|---|
| | | Pas | Prevalence of *E. coli* after skinning | — | RiskDiscrete ({0, 1}, {0.3500, 0.6500}) | Data simulation |
| | | | Output of *E. coli* after skinning | $Log_{10}CFU/100cm^2$ | RiskOutput ("skinning") + IF (Pas = 0, 0, Las) | — |
| | Washing (1) | Lcw (1) | Log number of *E. coli* changed through washing (1) | $Log_{10}CFU/100cm^2$ | RiskTriang (-0.65974, 0.53949, 1.7135) | Data simulation |
| | | Law (1) | Log number of *E. coli* after washing (1) | $Log_{10}CFU/100cm^2$ | Las-Lcw (1) | — |
| | | Paw (1) | Prevalence of *E. coli* after washing (1) | — | RiskDiscrete ({0, 1}, {0.5000, 0.5000}) | Data simulation |
| | | | Output of *E. coli* after washing (1) | $Log_{10}CFU/100cm^2$ | RiskOutput ["washing (1)"] + IF [Paw (1) = 0, 0, Law (1)] | — |
| | Eviscerating | Lce | Log number of *E. coli* changed through eviscerating | $Log_{10}CFU/100cm^2$ | RiskBetaGeneral (6.8886, 7.0685, −1.3571, 3.7428) | Data simulation |
| | | Lae | Log number of *E. coli* after eviscerating | $Log_{10}CFU/100cm^2$ | Law (1) + Lce | — |
| | | Pae | Prevalence of *E. coli* after eviscerating | — | RiskDiscrete ({0, 1}, {0.2500, 0.7500}) | Data simulation |
| | | | Output of *E. coli* after eviscerating | $Log_{10}CFU/100cm^2$ | RiskOutput ("eviscerating") + IF (Pac = 0, 0, Lae) | — |
| | Washing (2) | Lcw (2) | Log number of *E. coli* changed through washing (2) | $Log_{10}CFU/100cm^2$ | RiskBetaGeneral (22.859, 20.081, −6.4813, 7.6571) | Data simulation |
| | | Law (2) | Log number of *E. coli* after washing (2) | $Log_{10}CFU/100cm^2$ | Lae-Lcw (2) | — |
| | | Paw (2) | Prevalence of *E. coli* after washing (2) | — | RiskDiscrete ({0, 1}, {0.5000, 0.5000}) | Data simulation |
| | | | Output of *E. coli* after washing (2) | $Log_{10}CFU/100cm^2$ | RiskOutput ["washing (2)"] + IF [Paw (2) = 0, 0, Law (2)] | — |
| | Trimming | Lct | Log number of *E. coli* changed through trimming | $Log_{10}CFU/100cm^2$ | RiskBetaGeneral (18.039, 16.645, −8.3443, 8.3145) | Data simulation |
| | | Lat | Log number of *E. coli* after trimming | $Log_{10}CFU/100cm^2$ | Law (2) + Lct | — |
| | | Pat | Prevalence of *E. coli* after trimming | — | RiskDiscrete ({0, 1}, {0.214, 0.786}) | Data simulation |
| | | | Output of *E. coli* after trimming | $Log_{10}CFU/100cm^2$ | RiskOutput ("trimming") + IF (Pat = 0, 0, Lat) | — |
| | Pre-cooling | Lcp | Log number of *E. coli* changed through pre-cooling | $Log_{10}CFU/100cm^2$ | RiskNormal (0.12818, 1.9733) | Data simulation |
| | | Lap | Log number of *E. coli* after pre-cooling | $Log_{10}CFU/100cm^2$ | Law (2)-Lcp | — |
| | | Pap | Prevalence of *E. coli* after pre-cooling | — | RiskDiscrete ({0, 1}, {0.5500, 0.4500}) | Data simulation |
| | | | Output of *E. coli* after pre-cooling | $Log_{10}CFU/100cm^2$ | RiskOutput ("pre-cooling") + IF (Pap = 0, 0, Lap) | — |

found that the contamination of *E. coli* decreased significantly after washing and pre-cooling ($P < 0.01$). The number of *E. coli* in slaughterhouses of different sizes increased significantly after eviscerating ($P < 0.01$), and it had particularly obvious impact on small slaughterhouses. The number of *E. coli* after trimming in slaughterhouses of different sizes also increased.

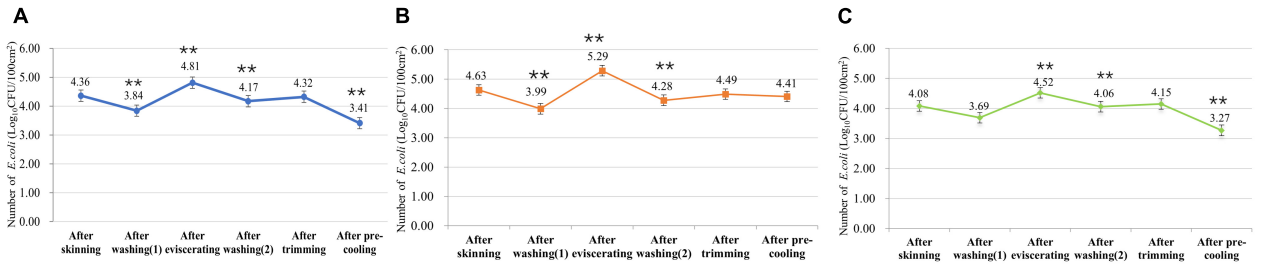


**FIGURE 1 |** *Escherichia coli* contamination of surfaces of swine carcasses in slaughterhouses of different sizes. **(A)** Number of *E. coli* in all swine slaughterhouses. **(B)** Number of *E. coli* in small swine slaughterhouses. **(C)** Number of *E. coli* in big swine slaughterhouses. **means the statistical difference between this process and the previous process is extremely significant ($P < 0.01$).

In addition, the overall *E. coli* contamination of carcass swabs in big slaughterhouses was lower than that in small slaughterhouses, indicating that the sanitary control of large slaughterhouses was better.

## Establishment of an *Escherichia coli* Exposure Assessment Model

### Model Simulation of *Escherichia coli* Contamination on the Swine Carcass Surface After Pre-cooling

Through the established model for slaughterhouses of different sizes, we showed that 90% of the *E. coli* contamination of a single pig in big slaughterhouses after skinning, washing (1), eviscerating, washing (2), trimming, and pre-cooling may be distributed between −4.26 and 10.40 $\log_{10}$ CFU/100 $cm^2$ (**Figure 2C**), with an average of 2.06 $\log_{10}$ CFU/100 $cm^2$. After the above processes, 90% of the *E. coli* contamination of a single pig in a small slaughterhouse may be distributed between 0.00 and 8.34 $\log_{10}$ CFU/100 $cm^2$ (**Figure 2B**), with an average of 2.95 $\log_{10}$ CFU/100 $cm^2$. In general, 90% of the *E. coli* contamination of a single pig after the above processes may be distributed between −2.93 and 9.91 $\log_{10}$ CFU/100 $cm^2$ (**Figure 2A**), with an average of 2.33 $\log_{10}$ CFU/100 $cm^2$.

### *Escherichia coli* Contamination Pattern During Slaughtering Simulated by the Model

Through the established exposure assessment model, the total number of *E. coli* on the surface of swine carcasses in skinning, washing (1), eviscerating, washing (2), trimming, and pre-cooling were further simulated. According to the mean value obtained, the *E. coli* contamination pattern on the surfaces of swine carcasses during slaughtering in slaughterhouses of different sizes were established (**Figure 3**). In general, regardless of slaughterhouse size, the number of *E. coli* after washing decreased to some extent, indicating that the washing process can effectively flush out some *E. coli*. The number of *E. coli* increased obviously after trimming, while it decreased after pre-cooling, especially in big slaughterhouses. Although the *E. coli* contamination pattern of the whole slaughtering processes in large and small slaughterhouses were the same, the *E. coli* contamination of small slaughterhouses was slightly higher than that of large slaughterhouses, indicating that the overall hygiene condition of large slaughterhouses was better. In addition, by comparing the *E. coli* monitoring data of different slaughterhouses (**Figure 1**) with the results of the established model (**Figure 3**), we found that the actual monitoring data fell within the 90% confidence interval of the model simulation results. This indicates that the credibility of the model was quite good.

### Analysis of Key Control Points of Microbial Contamination Risk

The correlation between the *E. coli* contamination of the surface of terminal swine carcasses and the slaughtering processes were discussed through sensitivity analysis of the parameters in the exposure assessment model. The risk contribution of each slaughtering process to the *E. coli* contamination of the surface of terminal swine carcasses can be determined. As shown in **Figure 4**, the sensitivity analysis in slaughterhouses of different sizes showed that the trimming process contributed the most to the risk of *E. coli* contamination (correlation coefficient of all slaughterhouses, small slaughterhouses and large slaughterhouses were 0.49, 0.47, 0.47, respectively), followed by the eviscerating process (correlation coefficient of all slaughterhouses, small slaughterhouses and large slaughterhouses were 0.23, 0.25, 0.25, respectively). Therefore, the trimming process was the most important key control point affecting the *E. coli* contamination on the surfaces of terminal swine carcasses, followed by the eviscerating process. The pre-cooling and washing processes were negatively correlated, indicating that they had a positive effect on reducing the number of *E. coli*.

The skinning process had a slight risk introduction effect to the *E. coli* contamination of the surface of terminal swine carcasses.

## Further Validation of the Exposure Assessment Model

### Contamination State of *Salmonella* in the Slaughtering Processes

Two slaughterhouses were randomly selected to isolate and identify *Salmonella* on the surface of swine carcasses, and then the *Salmonella* isolation rate in the different slaughtering processes were analyzed (**Figure 5**). It can be seen from **Figure 5** that the *Salmonella* isolation rate increased from 4.00% to the maximum of 26.67% after trimming. Therefore, the contamination state of *Salmonella* in different slaughtering processes can support

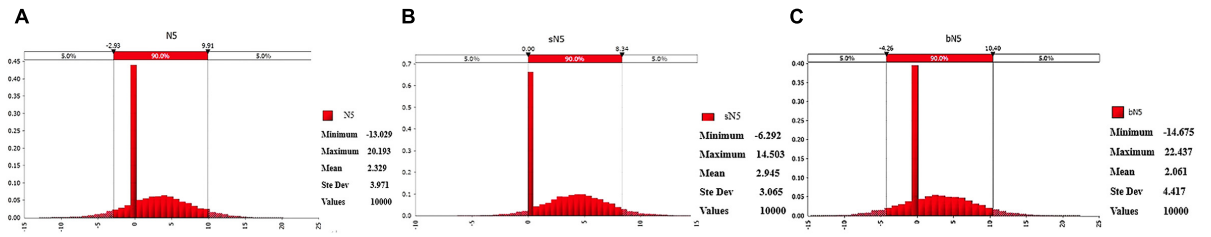


**FIGURE 2 |** Probability distribution of *Escherichia coli* contamination of swine carcasses after pre-cooling in slaughterhouses of different sizes. **(A)** Probability distribution of *E. coli* contamination of swine carcasses after pre-cooling in all swine slaughterhouses. **(B)** Probability distribution of *E. coli* contamination of swine carcasses after pre-cooling in small swine slaughterhouses. **(C)** Probability distribution of *E. coli* contamination of swine carcasses after pre-cooling in big swine slaughterhouses. N5- Log number of *E. coli* on swine carcasses after pre-cooling.

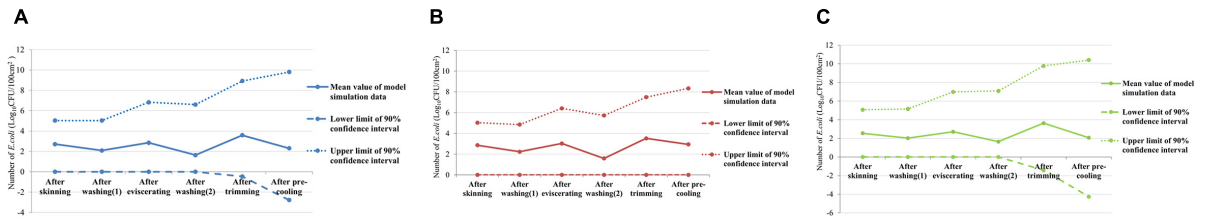


**FIGURE 3 |** *Escherichia coli* contamination simulated by exposure model of slaughterhouses of different sizes. **(A)** *E. coli* contamination simulated by exposure model of all slaughterhouses. **(B)** *E. coli* contamination simulated by exposure model of small swine slaughterhouses. **(C)** *E. coli* contamination simulated by exposure model of big swine slaughterhouses.

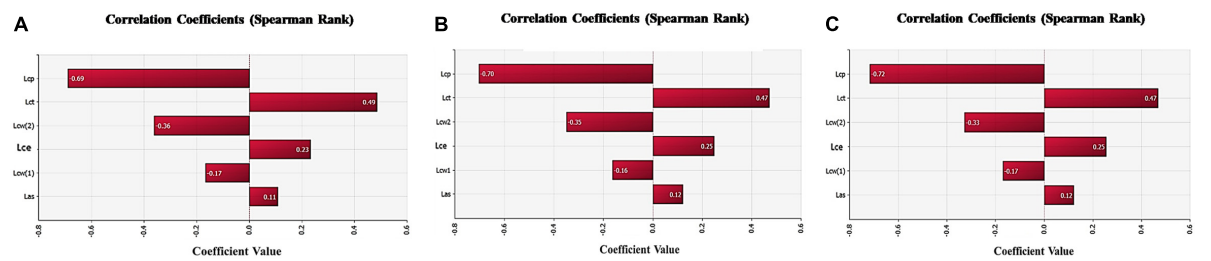


**FIGURE 4 |** Sensitivity analysis of each slaughtering processes in the model. **(A)** Sensitivity analysis of each slaughtering process in all swine slaughterhouses. **(B)** Sensitivity analysis of each slaughtering process in small swine slaughterhouses. **(C)** Sensitivity analysis of each slaughtering process in big swine slaughterhouses. Lcp-Log number of *Escherichia coli* changed through pre-cooling;Lct-Log number of *E. coli* changed through trimming;Lcw-Log number of *E. coli* changed through washing;Lce- Log number of *E. coli* changed through eviscerating;Las- Log number of *E. coli* after skinning.

the most important key control point obtained by the exposure assessment model.

### Results of Multilocus Sequence Typing of *Escherichia coli*

The typing results show that there were various sequence types of *E. coli* in the whole swine-slaughtering chain; 51 *E. coli* strains were divided into 32 ST types. The specific typing results are shown in **Supplementary Table 2**. The minimum spanning tree (**Figure 6**) shows that the most dominant ST type was ST10, and that ST10 strains could be isolated from all slaughtering processes. The ST10 strain was also presented in anal swabs, indicating that ST10 was introduced by the process of swine breeding. The dominant type in skinning, washing (1), eviscerating, and washing (2) processes is ST10. ST1434 began to appear in the trimming process and then became the dominant type in the trimming and pre-cooling processes. It is worth noting that ST1434 also included samples from workers' hands and appliances. Therefore, the trimming process can be regarded as the most important risk process, which further proved the most important key control point obtained by the exposure assessment model.

## DISCUSSION

Risk assessments of food safety are an essential part of the risk analysis process (FAO/WHO, 2005). Microbial risk assessments of livestock and poultry products are of great

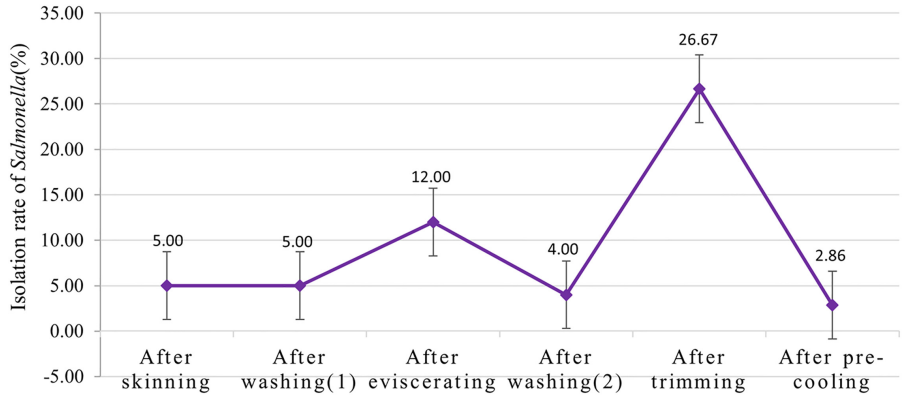


**FIGURE 5 |** Isolation rate of *Salmonella* in different slaughtering processes.

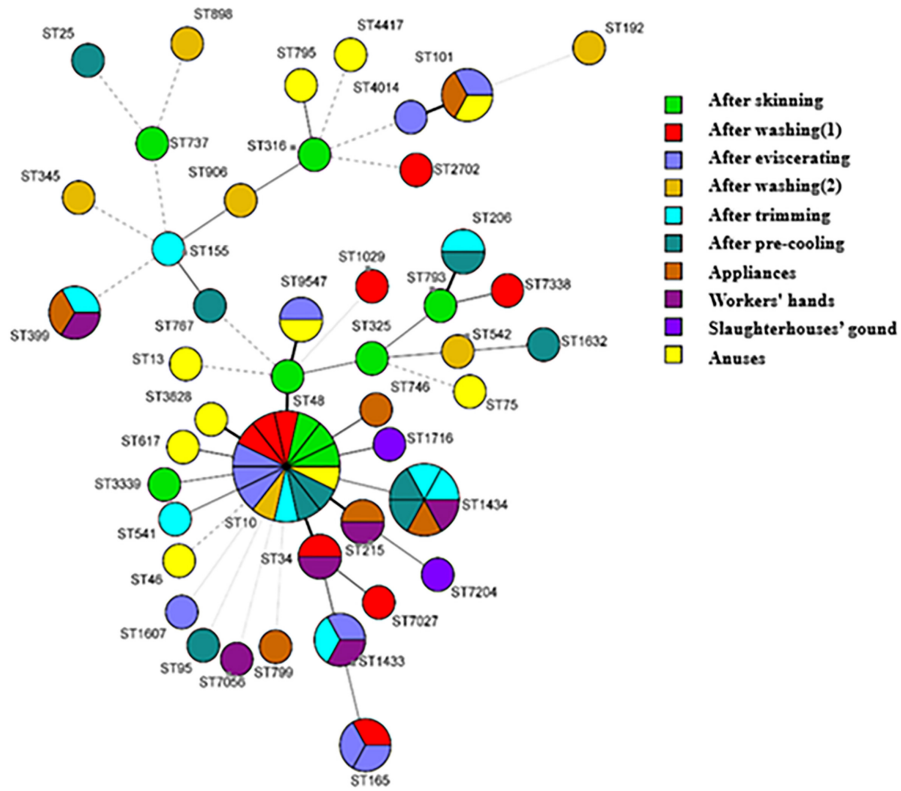


**FIGURE 6 |** The minimum spanning tree of 51 strains of *Escherichia coli*.

significance to improve the quality and safety risk management system of livestock and poultry products, provide high-quality livestock and poultry products for consumers, and guide safety in enterprises' production. In China, most published studies on microbiological risk assessments are qualitative, describing the risk characterization of pathogens in specific foods (Huang et al., 2017b). In addition, risk assessments in China are mostly conducted for pathogenic microorganisms in livestock and poultry products. To our knowledge, there are no studies on establishing models and carrying out risk assessments through data monitoring for contamination using health-indicator bacteria. In other countries, microbial risk assessments of livestock and poultry products have focused on consumption risks (Fares and Rouviere, 2010) or the exposure assessment of chemical hazards and certain toxins in livestock and poultry products (Flores et al., 2019; Sanchez-Montero et al., 2019). Therefore, the *E. coli* exposure assessment model established in this study not only fills the gaps in microbial exposure assessment of swine slaughtering chain to a certain extent, but also further scientifically

supplements the HACCP system of livestock and poultry products in China.

The detection level of *E. coli*, a health-indicator bacterium, can reflect the potential food risk caused by pathogenic bacteria. Therefore, the risk assessment of *E. coli* can strengthen food hygiene control and reduce the risk of disease. In this study, skinning, washing (1), eviscerating, washing (2), trimming, and pre-cooling processes were successively incorporated into the establishment of the exposure assessment model of *E. coli* contamination. The model was used to simulate the *E. coli* contamination on the surface of swine carcasses in differently sized slaughterhouses, and then the contamination pattern of *E. coli* was constructed. We concluded that the contamination level of *E. coli* in the whole slaughtering process of big slaughterhouses is lower than in small slaughterhouses. Therefore, we can illustrate to a certain extent that the health control of big slaughterhouses is better than that of small slaughterhouses. This conclusion is consistent with the monitoring conclusion for *E. coli* in pork by the USDA (United States Department of Agriculture, 2011). Because of the influence of water activity and low temperature of swine carcasses, the pre-cooling process significantly reduced the *E. coli* contamination of the swine carcass surface, especially for big slaughterhouses. The contamination of *E. coli* increased to the maximum after eviscerating, which may be caused by cross-contamination from a broken gut or the environment.

According to the sensitivity analysis of the model, the most important key control point is the trimming process whether in big or small slaughterhouses. ST1434 appeared in the carcass swabs of the trimming process and then became the dominant type in this process. ST1434 also existed in the workers' hands and appliances swabs, illustrating that there was cross-contamination between carcasses and the environment in the trimming process. The contamination carried by environmental factors such as the workers' hands or appliances in the trimming process, spreading downstream along the slaughtering chain. Therefore, the trimming process can be considered as an important risk process. The eviscerating process is another key control point; the reason for the increase of *E. coli* contamination in this process may be the eviscerating operation, which led to visceral rupture and overflow of autologous microorganisms (Huang et al., 2017a; Crotta et al., 2018) and then contaminated appliances and workers' hands and caused cross-contamination. In addition, according to the typing results, ST1433 and ST101 in the eviscerating process also existed in the workers' hands and appliances. Therefore, it can be reasonably speculated that *E. coli* in the environment is closely related to *E. coli* on carcasses in this process. The minimum spanning tree showed that ST10 strains could be isolated from anal swabs and all slaughtering processes, which indicated that the ST10 was widespread in the slaughtering chain and was more likely to be carried by the pigs themselves and spread along the slaughtering chain.

The key control points of swine-slaughtering processes in this study were different from those of other studies, which mainly because the different slaughterhouses chosen for the study and different slaughtering processes. In fact, skinning, eviscerating, trimming, and cutting may all be the key control points for microbial contamination. In addition, the key control points of differently sized slaughterhouses in this study were the same, which maybe because the slaughtering processes of differently sized slaughterhouses in Shandong Province is basically the same, and the appliances and sanitary control measures adopted in slaughtering were also roughly similar. Using the quantitative risk assessment model of *Salmonella* contamination during swine slaughtering, Zhao et al. (2018) obtained three main key control points, which were splitting, eviscerating, and scalding; splitting was also a part of the trimming process. Pearce et al. (2004) discussed the impact of pig slaughter processes on carcass microbiology and their potential use as critical control points (C) during pork production. The main critical control points they concluded were bleeding and eviscerating. Yu et al. (1999) investigated swine slaughtering operations in America to establish their critical control points. Their study indicated that the polishing and eviscerating processes can be identified as critical control points. Liu et al. (2013) applied the HACCP principle to analyze the microbial hazards in chilled-pork processing. The main key control points they obtained were bleeding, eviscerating, pre-cooling, cutting, and packaging, of which the cutting process was the most important key control point. Our study did not involve the cutting process, but the trimming and cutting processes used appliances most frequently. Zhou et al. (2018) considered that eviscerating and polishing were the main key risk points in the study on the key risk points of *Salmonella* during swine slaughtering. Although all kinds of key control points for pathogenic microorganism risk assessments in swine-slaughtering process were different, the eviscerating process and the processes with frequent use of appliances were often regarded as the key control points in slaughtering (Arguello et al., 2013). This is consistent with the conclusion we obtained, that is, that the trimming process is the most important key control point, and the eviscerating process is the second.

The results of *E. coli* multilocus sequence typing and the *Salmonella* contamination monitoring data show that the trimming process was the most important key control point, the same as the conclusion from the exposure assessment model. The credibility of the constructed exposure assessment model was verified. Notably, from the actual monitoring data of *E. coli* during slaughtering in differently sized slaughterhouses, it can be found that *E. coli* contamination is the most serious in the eviscerating process, but the most important key control point of the exposure assessment model is the trimming process. We speculated that this may be because the monitoring data adopted the commonly used deterministic numerical calculation method such as the mean value. However, the model simulation was a statistical analysis of a large number of sample values after thousands of simulations; the results that met a certain accuracy were then obtained. Therefore, the exposure assessment model has more scientific mathematical basis and statistical significance.

Of course, there are some uncertainties in the exposure evaluation of microorganisms due to various factors. The uncertainties of the evaluation model established in this study include firstly the uncertainty of the process and model. The study assumed that *E. coli* did not proliferate throughout the slaughtering processes, but *E. coli* can

proliferate in the actual processes. Second is the uncertainty of slaughtering modes in different slaughterhouses. The model was based on the slaughtering modes of several representative enterprises in Shandong; the slaughtering processes of different enterprises are different, however, because of regional or policy factors. Therefore, the model is not necessarily applicable to slaughterhouses with different slaughtering modes. The uncertainties of the model and the complexities of microorganisms affect the accuracy and effectiveness of the final evaluation results.

In this study, an exposure assessment model of *E. coli* contamination suitable for the general swine-slaughtering process in China was established. The results of correlation analysis showed that the trimming process is the most important key control point, which can be further proved from different aspects by using the contamination state of *Salmonella* and the multilocus sequence typing results of *E. coli*. Therefore, the sanitation control of workers' hands in the trimming process should be strengthened in a more targeted manner to reduce potential cross-contamination as much as possible. Although this model contained limitations and assumptions, as with all QMRA, it provides a scientific basis for microbial hygiene supervision during swine slaughtering, as well as technical support for the prevention and control of pork-derived foodborne diseases in the future.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

## AUTHOR CONTRIBUTIONS

TY was involved in microbial and molecular experiment, data analysis, investigation, and writing—original draft. GZ contributed to conception and design of the study, model construction, data curation, and writing—review and editing. YL, LW, YG, JZ, NL, XH, QZ, JL, and XZ performed investigation. JW was involved in project administration, funding acquisition, and supervision. YX done project administration and supervision. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.828279/full#supplementary-material

## REFERENCES

Arguello, H., Alvarez-Ordonez, A., Carvajal, A., Rubio, P., and Prieto, M. (2013). Role of slaughtering in *salmonella* spreading and control in pork production. *J. Food Prot.* 76, 899–911. doi: 10.4315/0362-028X.JFP-12-404

Crotta, M., Luisi, E., Dadios, N., and Guitian, J. (2018). Probabilistic modelling of events at evisceration during slaughtering of pigs using expert opinion: quantitative data in support of stochastic models of risk of contamination. *Microb. Risk Anal.* 11, 57–65. doi: 10.1016/j.mran.2018.10.001

Dang-Xuan, S., Nguyen-Viet, H., Pham-Duc, P., Unger, F., Tran-Thi, N., Grace, D., et al. (2018). Risk factors associated with *salmonella* spp. prevalence along smallholder pig value chains in Vietnam. *Int. J. Food Microbiol.* 290, 105–115. doi: 10.1016/j.ijfoodmicro.2018.09.030

Dong, Q. L., Barker, G. C., Gorris, L. G. M., Tian, M. S., Song, X. Y., and Malakar, P. K. (2015). Status and future of quantitative microbiological risk assessment in China. *Trends Food Sci. Technol.* 42, 70–80. doi: 10.1016/j.tifs.2014.12.003

FAO/WHO (2005). *Food Safety Risk Analysis. Part I. An overview and Framework Manual*. Available online at: http://www.fsc.go.jp/sonota/foodsafety_riskanalysis.pdf (accessed March 5, 2021).

Fares, M., and Rouviere, E. (2010). The implementation mechanisms of voluntary food safety systems. *Food Policy*. 35, 412–418. doi: 10.1016/j.foodpol.2010.05.008

Flores, M., Mora, L., Reig, M., and Toldrá, F. (2019). Risk assessment of chemical substances of safety concern generated in processed meats. *Food Sci. Hum. Wellness*. 8, 244–251. doi: 10.1016/j.fshw.2019.07.003

GB 50317-2009 (2009). *Code for Design of Pig's Slaughtering and Cutting Rooms*. Beijing: China Planning Press.

Gill, C. O., McGinnis, J. C., and Bryant, J. (1998). Microbial contamination of meat during the skinning of beef carcass hindquarters at three slaughtering plants. *Int. J. Food Microbiol.* 42, 175–184. doi: 10.1016/S0168-1605(98)00074-9

Gurman, P. M., Ross, T., and Kiermeier, A. (2018). Quantitative microbial risk assessment of salmonellosis from the consumption of Australian Pork: minced meat from retail to burgers prepared and consumed at home. *Risk Anal.* 38, 2625–2645. doi: 10.1111/risa.13163

Huang, J. L., Zang, X. Q., Zhai, W. H., Guan, C. N., Lei, T. Y., and Jiao, X. N. (2017a). Quantitative analysis of *Campylobacter* spp. contamination in chicken slaughtering lines by "label tracking method" in eastern China. *Food Control* 80, 67–73. doi: 10.1016/j.foodcont.2017.03.052

Huang, J. L., Zang, X. Q., Zhai, W. H., Guan, C. N., Lei, T. Y., and Jiao, X. N. (2017b). *Campylobacter* spp. in chicken-slaughtering operations: a risk assessment of human campylobacteriosis in East China. *Food Control* 86, 249–256. doi: 10.1016/j.foodcont.2017.11.026

ISO 6579-1:2017 (2007). *Microbiology of the Food Chain-Horizontal Method for the Detection, Enumeration and Serotyping of Salmonella-Part 1: Detection of Salmonella spp.* New York, NY: Information Handling Services.

Kusturov, V. B., Kasyanchuk, V. V., and Bergievich, A. M. (2017). Analysis of microbial contamination of pigs' carcasses during the slaughtering and primary processes. *Sci. Mess. LNU Vet. Med. Biotechnol.* 19, 194–199. doi: 10.15421/nvlvet7742

Liu, S. C., Zhao, C. J., Yang, X. T., Wang, G. L., and Zhong, S. Y. (2013). Microbiological hazard analysis and critical control points in the processing of chilled pork. *Food Sci.* 34, 285–289.

Min, S., Bai, J. F., Seale, J. Jr., and Wahl, T. (2015). Demographics, societal aging, and meat consumption in China. *J. Integr. Agric.* 14, 995–1007. doi: 10.1016/S2095-3119(14)60984-9

Pearce, R. A., Bolton, D. J., Sheridan, J. J., Mcdowell, D. A., Blair, I. S., and Harrington, D. (2004). Studies to determine the critical control points in pork slaughter hazard analysis and critical control point systems. *Int. J. Food Microbiol*. 90, 331–339. doi: 10.1016/S0168-1605(03)00333-7

Rahn, K., De Grandis, S. A., Clarke, R. C., McEwen, S. A., Galán, J. E., Ginocchio, C., et al. (1992). Amplification of an *inv*A gene sequence of *Salmonella typhimurium* by polymerase chain reaction as a specific method of detection of *Salmonella*. *Mol. Cell. Probes* 6, 271–279. doi: 10.1016/0890-8508(92)90002-F

Sanchez-Montero, L., Cordoba, J. J., Peromingo, B., Álvarez, M., and Núñez, F. (2019). Effects of environmental conditions and substrate on growth and ochratoxin A production by *Penicillium verrucosum* and *Penicillium nordicum*: relative risk assessment of OTA in dry-cured meat products. *Food Res. Int.* 121, 604–611. doi: 10.1016/j.foodres.2018.12.025

Setiabuhdi, M., Theis, M., and Norback, J. (1997). Integrating hazard analysis and critical control point(HACCP) and sanitation for verifiable food safety. *J. Am. Diet. Assoc.* 97, 889–891. doi: 10.1016/S0002-8223(97)00217-4

Swart, A. N., Evers, E. G., Simons, R. L., and Swanenburg, M. (2016). Modeling of *Salmonella* contamination in the pig slaughterhouse. *Risk Anal.* 36, 498–515. doi: 10.1111/risa.12514

United States Department of Agriculture (2011). *The Nationwide Microbiological Baseline Data Collection Program: Market Hogs Survey August 2010–August 2011*. Available online at: https://www.fsis.usda.gov/sites/default/files/media_file/2020-07/Baseline_Data_Market_Hogs_2010-2011 (accessed March 2, 2021).

Van Ba, H., Seo, H. W., Seong, P. N., Kang, S. M., Cho, S. H., Kim, Y. S., et al. (2019). The fates of microbial populations on pig carcasses during slaughtering process, on retail cuts after slaughter, and intervention efficiency of lactic acid spraying. *Int. J. Food Microbiol.* 294, 10–17. doi: 10.1016/j.ijfoodmicro.2019.01.015

Wirth, T., Falush, D., Lan, R., Colles, F., Mensa, P., Wieler, L. H., et al. (2006). Sex and virulence in *Escherichia coli*: an evolutionary perspective. *Mol. Microbiol.* 60, 1136–1151. doi: 10.1111/j.1365-2958.2006.05172.x

Wu, Y. N., Liu, P., and Chen, J. S. (2018). Food safety risk assessment in china: past, present and future. *Food Control* 90, 212–221. doi: 10.1016/j.foodcont.2018.03.009

Yu, S. L., Bolton, D., Laubach, C., Kline, P., Oser, A., and Palumbo, S. A. (1999). Effect of dehairing operations on microbiological quality of swine carcasses. *J. Food Prot.* 62, 1478–1481. doi: 10.4315/0362-028X-62.12.1478

Zhang, L., Yin, D. F., Zhang, D. W., and Luo, L. G. (2018). Quantitative microbiological risk assessment of *Salmonella* cross-contamination from fresh pork to ready-to-eat food in domestic kitchen. *Food Sci.* 39, 184–191. doi: 10.7506/spkx1002-6630-201811028

Zhao, G., Liu, N., Zhao, J. M., Wang, J., Huang, X. M., Li, Y. H., et al. (2018). Quantitative risk assessment on *salmonella* contamination during pig slaughtering. *Qual. Saf. Agro Prod.* 2, 21–25.

Zhou, Z. H., Jin, X. C., Zheng, H. J., Li, J. W., Meng, C., Yin, K. Q., et al. (2018). The prevalence and load of *Salmonella*, and key risk points of *Salmonella* contamination in a swine slaughterhouse in Jiangsu province, China. *Food Control* 87, 153–160. doi: 10.1016/j.foodcont.2017.12.026

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# Effect of Bacterial Resistance of *Escherichia coli* From Swine in Large-Scale Pig Farms in Beijing

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With widespread use of antibiotics in the aquaculture industry, bacterial resistance has recently attracted increasing attention. Continuous emergence of multi-resistant bacteria has greatly threatened human and animal health, as well as the quality and safety of livestock products. To control bacterial resistance, the effect of bacterial resistance needs to be well understood. The purpose of this study was to explore the factors influencing *Escherichia coli* (*E. coli*) drug resistance in large-scale pig farms. In this study, 296 strains of *E. coli* isolated and identified from large-scale pig farms in Beijing were used as the research objects. *In vitro* drug sensitivity tests were used to determine the sensitivity to 10 antibiotics of pig-derived *E. coli*. SPSS logistic regression was employed to analyze the effects of the season, pig type, sampling point (medication type) and sampling location on resistance and multi-drug resistance of *E. coli* from pigs. The degrees of drug resistance to 10 antibiotics of the 296 strains of pig-derived *E. coli* were varied, their resistance rates were between 4.05 and 97.64%, and their multi-drug resistance was appalling, with the highest resistance to six antibiotics being 26.35%. The isolated strains were proven more resistant to tetracyclines, penicillin and chloramphenicol, which are commonly used for disease prevention in pig farms, and less resistant to quinolones and aminoglycosides, which are not used in pig farms. The resistance of the isolated strains in spring and summer was generally higher than that in winter. *E. coli* resistance in piglets, fattening pigs and sows was more serious than that in nursery and sick pigs. The results showed that the season, type of medication and type of pig had an influence on the pig-derived *E. coli* resistance, among which the type of medication was the most influencing factor.

**Keywords: pig farm, *Escherichia coli*, multi-drug resistance, factor, antibiotic resistance**

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## INTRODUCTION

Antibiotic resistance (AR) affects treatment of bacterial diseases in humans and animals and will become one of the major threats facing the world in the future (World Health Organization, 2012; Centner, 2016; Tornimbene et al., 2018; Borgmann et al., 2020). The main reasons for bacterial resistance development are the abuse of antibiotics and scarce measures to control the spread of

**Abbreviations:** *E. coli*, *Escherichia coli*; AR, antibiotic resistance; MDR, multi-drug resistance; OR, odds ratio; CLSI, Clinical and Laboratory Standards Institute.

resistant bacteria. Antibiotic use generates selective pressure that is conducive to resistant bacteria development and allows the bacteria to spread among bacterial populations in humans, animals and the environment (Baquero, 2011; World Health Organization, 2012). The research showed that the number of deaths due to drug-resistant bacterial diseases was estimated to be 50,000 each year in Europe and the United States. By 2050, it is estimated that the number of such deaths in the world will reach 10 million each year, posing a threat to global economy and biosecurity (Zhang et al., 2017; Courtenay et al., 2019). In the aquaculture industry and others, antibiotics are employed to promote animal growth and to prevent or treat diseases (Cabello, 2006; Sarmah et al., 2006; Kuang et al., 2020). However, due to antibiotic abuse, bacteria have increased occurrence of drug resistance as they increased selective pressure and generated drug resistance pools (van den Bogaard, 2000; Smith et al., 2002), which not only diminished treatment options for farmers, but also led to harder-to-treat animal diseases resulted from multidrug-resistant (MDR) bacteria (Ramchandani et al., 2005; Michael et al., 2012). Magiorakos et al. (2012) have claimed that enterobacteriaceae strains are MDR if they are non-susceptible to more than one agent in over 3 antimicrobial categories, extensively drug-resistant (XDR) if they are non-susceptible to more than one agent in all but over 2 categories and pandrug-resistant (PDR) if they are non-susceptible to all the listed antimicrobial agents.

Swine production accounts for a large proportion of global meat production (Osterberg et al., 2016), and the use of antibiotics in food animals may lead to the spread of drug-resistant bacteria from livestock to human through the food chain (Meemken et al., 2008; Chantziaras et al., 2014). *Escherichia coli* (*E. coli*) is an intestinal symbiotic bacterium that exists in animals and humans and is susceptible to high selection pressure from antimicrobial agents in contact with the host. Therefore, *E. coli* is often used as a carrier for monitoring antimicrobial resistance in human or livestock groups (European Food Safety Authority, and European Centre for Disease Prevention and Control, 2018). In addition, although *E. coli* is generally harmless to humans and animals, it may constitute a resistant gene pool for transmission of pathogenic bacteria (Marshall et al., 2009), threatening animal and human health. At present, the prevalence of *E. coli* extended-spectrum β-lactamases (ESBLs) is increasing worldwide, which has attracted extensive attention (Pitout and Laupland, 2008). ESBLs can destroy β-lactam antibiotics (Malloy and Campos, 2011), causing these antibiotics to lose their antimicrobial activity and thus making *E. coli* resistant to antibiotics. *E. coli* producing ESBLs in farms may affect public health through environmental pollution and contaminated animal products (Horton et al., 2011). Currently, large-scale pig production generally relies on antibiotics to maintain livestock health and productivity (Robinson et al., 2017). Antibiotic use in pig production may be the major reason for AR in pigs, but it may not solely determine the level of resistance in the pig population. The season, pig type, herd size, and animal exposure may also serve as the factors for emergence and persistence of drug-resistant bacteria.

To understand the factors important for antimicrobial resistance of *E. coli* derived from pigs in large-scale pig farms, a total of 296 strains of *E. coli* isolated and identified from large-scale pig farms were chosen, and their susceptibility to 10 antibiotics were determined by the Kirby-Bauer disk diffusion method in this study. SPSS logistic regression was applied to analyze the effects of multiple factors on the resistance and multi-drug resistance (MDR) of pig-derived *E. coli*, including the season, pig type, sampling position (medication type) and sampling location. This study explored the main factors affecting AR of *E. coli* in pigs and provided a scientific basis for rational use of drugs in large-scale pig farms to control growing bacterial drug resistance.

# MATERIALS AND METHODS

## Farms

Large-scale pig farms in Shunyi District of Beijing were selected for a visit. The herds were farrow-to-finish herds with at least 800 sows, generating 12,000 heads every year. Herds were visited three times to collect samples for antimicrobial resistance profiling of (nasal and anal) swabs from *E. coli* derived from sick pigs, piglets, sows, nursery pigs, and fattening pigs.

## Isolation and Identification of Pig-Derived *Escherichia coli*

After collection, the swab samples were transported to the laboratory where they were processed within a few hours after arrival (collected sample information shown in **Table 1**). The swab samples were cultured in trypticase soy broth, followed by overnight incubation at 37°C, while being shaken at 150 rpm. Next, 1 μL loopfuls were used to streak onto MacConkey (MAC) agar plates and incubated for 18–24 h at 36 ± 1°C. Typical brick red to peach red single colonies were selected from MAC and inoculated onto Eosin Methylene Blue (EMB) agar plates (18–24 h, 36 ± 1°C). The isolates were examined according to standard methods of bacteriology and biochemistry. All bacteriological media were acquired from Beijing Land Bridge Technology Co., Ltd (Beijing, China). Isolates were confirmed by 16S rRNA gene sequences, which were 90–100% homologous to *E. coli* by BLAST [Sangon Biotech (Shanghai) Co., Ltd.]. All isolates confirmed as *E. coli* were tested for antibiotic resistance.

## Test of *Escherichia coli* Susceptibility to Antimicrobial Agents

Susceptibility of *E. coli* isolates was tested on a panel of 10 antimicrobial agents at the 0.5 McF concentration using Kirby-Bauer disk diffusion method. The Clinical and Laboratory Standards Institute (CLSI) standards were followed for inoculum standardization, incubation conditions and internal quality control organisms (Watts et al., 2013). For some antimicrobial agents, susceptibility was tested at two or more concentrations, but resistance was declared only by CLSI-defined resistance

**TABLE 1 |** Specific sampling volume of a large-scale pig farm in Shunyi District of Beijing (strain).

| Type of pig | Spring | | Summer | | | | Winter | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Southern area | | Southern area | | Northern area | | Southern area | | Northern area | |
| | Nasal swab | Anal swab | Nasal swab | Anal swab | Nasal swab | Anal swab | Nasal swab | Anal swab | Nasal swab | Anal swab |
| Piglet | — | — | 5 | 5 | 5 | 5 | 7 | 5 | 5 | 6 |
| Nursery pig | — | — | 5 | 5 | 5 | 5 | 9 | 9 | — | — |
| Sow | 12 | 12 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 |
| Sick pig | 10 | — | 6 | 4 | 5 | 6 | — | — | — | — |
| Fattening pig | 40 | — | 20 | — | 10 | 10 | 10 | — | 13 | 12 |
| Total | 74 | | 125 | | | | 100 | | | |

**TABLE 2 |** Interpretation criteria for the diameter of the inhibition zone of *E. coli.*

| Class | Antimicrobial | Antibiotic disc (μg/piece) | Interpretation criteria (mm) | | |
|---|---|---|---|---|---|
| | | | *S* | *I* | *R* |
| Chloramphenicol | Florfenicol | 30 | ≥19 | 15–18 | ≤14 |
| Semisynthetic penicillin broad-spectrum β-lactam | Amoxicillin | 20 | ≥18 | 14–17 | ≤13 |
| Tetracycline | Doxycycline | 30 | ≥16 | 13–15 | ≤12 |
| Quinolone | Enrofloxacin | 10 | ≥17 | 13–16 | ≤12 |
| Macrolide | Erythromycin | 15 | ≥23 | 14–22 | ≤13 |
| Sulfonamide synergist | Trimethoprim | 5 | ≥16 | 11–15 | ≤10 |
| Aminoglycoside | Gentamicin | 10 | ≥15 | 13–14 | ≤12 |
| Quinolone | Ciprofloxacin | 5 | ≥21 | 16–20 | ≤15 |
| Tetracycline | Tetracycline | 30 | ≥15 | 12–14 | ≤11 |
| Chloramphenicol | Chloramphenicol | 30 | ≥18 | 13–17 | ≤12 |

breakpoints (**Table 2**). *E. coli* ATCC25922 was used for quality control.

## Data Management and Statistical Analysis

Microsoft Excel was used to include all data, and all the data were classified and a library was constructed using Statistical Product and Service Solutions (SPSS, IBM SPSS Statistics version 23) software. Single-factor binary logistic regression was utilized to analyze the effects of four factors, including the season, type of pig, sampling position and sampling location, on resistance of *E. coli*, and the specific assignment instructions were shown in **Supplementary Table 1**. Based on the odds ratio (OR) value, 95% confidence interval and *P*-value of the OR value were employed to identify possible influencing factors. Then, these influencing factors were added for multivariate binary logistic regression analysis, and significant factors affecting *E. coli* drug resistance were analyzed with $P < 0.05$ (statistically significant) and OR $\neq 1$.

By single-factor multiple logistic regression, the influence of four factors on MDR of *E. coli* was analyzed, and specific assignment instructions were listed in **Supplementary Table 2**. The 95% confidence interval and *P*-value of the OR value helped screen out possible associations with MDR of *E. coli*. Significant factors that affect MDR of *E. coli* were analyzed by adding the selected influencing factors to multi-factor multiple logistic regression analysis based on the magnitude of the OR value with $P < 0.05$ as the significant difference and OR $\neq 1$, and then the main way that could affect MDR of *E. coli* was analyzed.

# RESULTS

## Impact Factors of Antimicrobial Resistance to 10 Antibiotics of Pig-Derived *Escherichia coli*

A total of 296 strains identified as *E. coli* were tested for their resistance to 10 antibiotics by drug susceptibility. From **Supplementary Table 3**, the strains were known highly sensitive to quinolones (enrofloxacin and ciprofloxacin), with the drug resistance rates of only about 4%. In contrast, the tested bacteria showed poor sensitivity to tetracycline drugs including doxycycline, tetracycline and amoxicillin, with the resistance rates being 91.55, 97.64, and 93.24%, respectively. The rate of AR to the banned drug chloramphenicol was 54.39%, which was similar to the special drug florfenicol (68.92%). However, the intermediate sensitivity of the tested bacteria was 0.34% to florfenicol, in contrast with 21.96% to chloramphenicol. The rates of resistance of the tested bacteria to trimethoprim and gentamicin were 68.92 and 15.54%, respectively, and that to

macrolide antibiotic erythromycin was 28.72%, which mainly showed an intermediate result (65.54%).

## Single-Factor Binary Logistic Regression Analysis of Pig-Derived *Escherichia coli* Resistance in Different Seasons

Herein, pig-derived *E. coli* resistance in different seasons was evaluated, and the results were shown in **Figure 1A** and **Supplementary Table 4**. The resistance to the 10 antibiotics of the tested bacteria isolated from the samples in spring and summer was higher than in winter. The tested bacteria exhibited high resistance to amoxicillin, tetracycline, and doxycycline despite the season, and their resistance rates were all higher than 90%. The distribution of the tested bacteria's susceptibility to enrofloxacin and ciprofloxacin was concentrated in spring, summer and winter, and the AR rates were lower than 4% in spring and winter and slightly higher than 8% in summer. The resistance to erythromycin of the tested bacteria was relatively low in spring, summer and winter, with the highest rate of 35.77% in summer and the average rate of 27.74% in these three seasons. It was mainly distributed near the intermediate value, and the results indicated that the tested bacteria might have potential resistance to erythromycin. The tested bacteria were higher resistant to trimethoprim, with an average resistance rate of 69.42%. The drug resistance rate in spring was higher than that in summer and winter, not being an intermediate value. The tested bacteria were more sensitive to gentamicin, with an average resistance rate of 14.55%, and their rates were arranged in descending order in summer (22.76%), in spring (12.94%) and in winter (7.95%).

In order to better understand the influence of seasons on *E. coli* resistance, binary logistic regression analysis was employed. AR of *E. coli* isolated from the samples collected in winter was used as a reference and the results were given in **Supplementary Table 5**. It could be seen from the table that there was a significant increase in resistance of *E. coli* sampled in spring and summer to all the 10 antibiotics except tetracycline. Compared with *E. coli* sampled in winter, what was sampled in spring and summer showed a significant difference in resistance to florfenicol, and the OR value of florfenicol (2.963) was higher than other antibiotics in spring. Moreover, *E. coli* resistance to gentamicin and erythromycin in summer was significantly different from what was in winter, and the OR values were 3.411 and 1.894, respectively.

## Single-Factor Binary Logistic Regression Analysis of Resistance of *Escherichia coli* Derived From Different Types of Pigs

Resistance of *E. coli* isolated from different types of pigs (sick pigs, piglets, sows, nursery pig and fattening pigs) was evaluated, and the results were shown in **Figure 1B** and **Supplementary Table 6**. Overall, the rates of resistance to amoxicillin, tetracycline and doxycycline of the tested bacteria isolated from all types of pigs were high (>86%), and resistance to two quinolones (enrofloxacin and ciprofloxacin) was very low, as the tested bacteria isolated from all pig types showed low AR. The resistance to the two antibiotics of piglets, nursery pigs and sick pigs was higher than that of sows and fattening pigs, with the resistance of nursery pigs shifting from sensitive to intermediate. The resistance to erythromycin of the tested bacteria isolated from piglets, sows and fattening pigs was mainly within the intermediate range, and their resistance rates were low. However, the resistance was higher than that of nursery pigs and sick pigs, especially for that isolated from nursery pigs, for which the resistance rate was 66.67%, and the sensitivity to erythromycin of the tested bacteria began to shift from intermediate to resistant. The tested bacteria isolated from different pig types showed high resistance to trimethoprim, with an average AR rate of 69.88%, and there was no great difference in these pig types. The tested bacteria isolated from piglets and nursery pigs showed high resistance to gentamicin, with the resistance rates of 38.89 and 22.22%, respectively, while the AR rates of the tested bacteria isolated from sows, fattening pigs and sick pigs were low. The tested bacteria isolated from nursery pigs and sick pigs showed high resistance to florfenicol, reaching resistance rates of more than 90%, while the resistance of the tested bacteria isolated from other pig types was relatively low. Similar to florfenicol, the tested bacteria isolated from nursery pigs and sick pigs showed higher resistance to chloramphenicol, with the AR rates reaching 75%. The resistance to chloramphenicol of the tested bacteria isolated from piglets, sows and fattening pigs was low, but the susceptibility mainly fell into the intermediate range, and there was a tendency of changing to resistant.

To understand the influence of pig types on resistance of *E. coli*, binary logistic regression analysis was exploited in this study. AR to 10 antibiotics of *E. coli* isolated from piglets was used as a reference, and all the results were shown in **Supplementary Table 7**. The AR of *E. coli* in nursery pigs and sick pigs was relatively serious. Compared with piglets, significant differences existed in resistance to florfenicol of *E. coli* from nursery pigs, fattening pigs and sick pigs, and the resistance to florfenicol was very serious in nursery pigs (OR = 12.294). Compared with piglets, resistance to chloramphenicol of *E. coli* from nursery pigs, fattening pigs and sick pigs was also significantly different, and resistance of *E. coli* in nursery pigs was more serious than that in fattening pigs and sick pigs (OR = 6). Similarly, compared with piglets, resistance to erythromycin of *E. coli* from nursery pigs was significantly different (OR = 7). Compared with piglets, there were considerable differences in gentamicin resistance among fattening pigs, sows and sick pigs. By comparing the OR values, it could be found that resistance of piglets to gentamicin was more serious. The results of resistance to florfenicol, chloramphenicol, erythromycin, and gentamicin of *E. coli* isolated from different pig types obtained by binary logistic regression analysis were consistent with those obtained by drug resistance analysis. In short, a significant correlation was presented between pig types and *E. coli* resistance.

## Single-Factor Binary Logistic Regression Analysis of Resistance of *Escherichia coli* Derived From Different Sampling Positions of Pigs

The influence of *E. coli* on antibiotic sensitivity was analyzed according to different sampling sites, and the results were shown

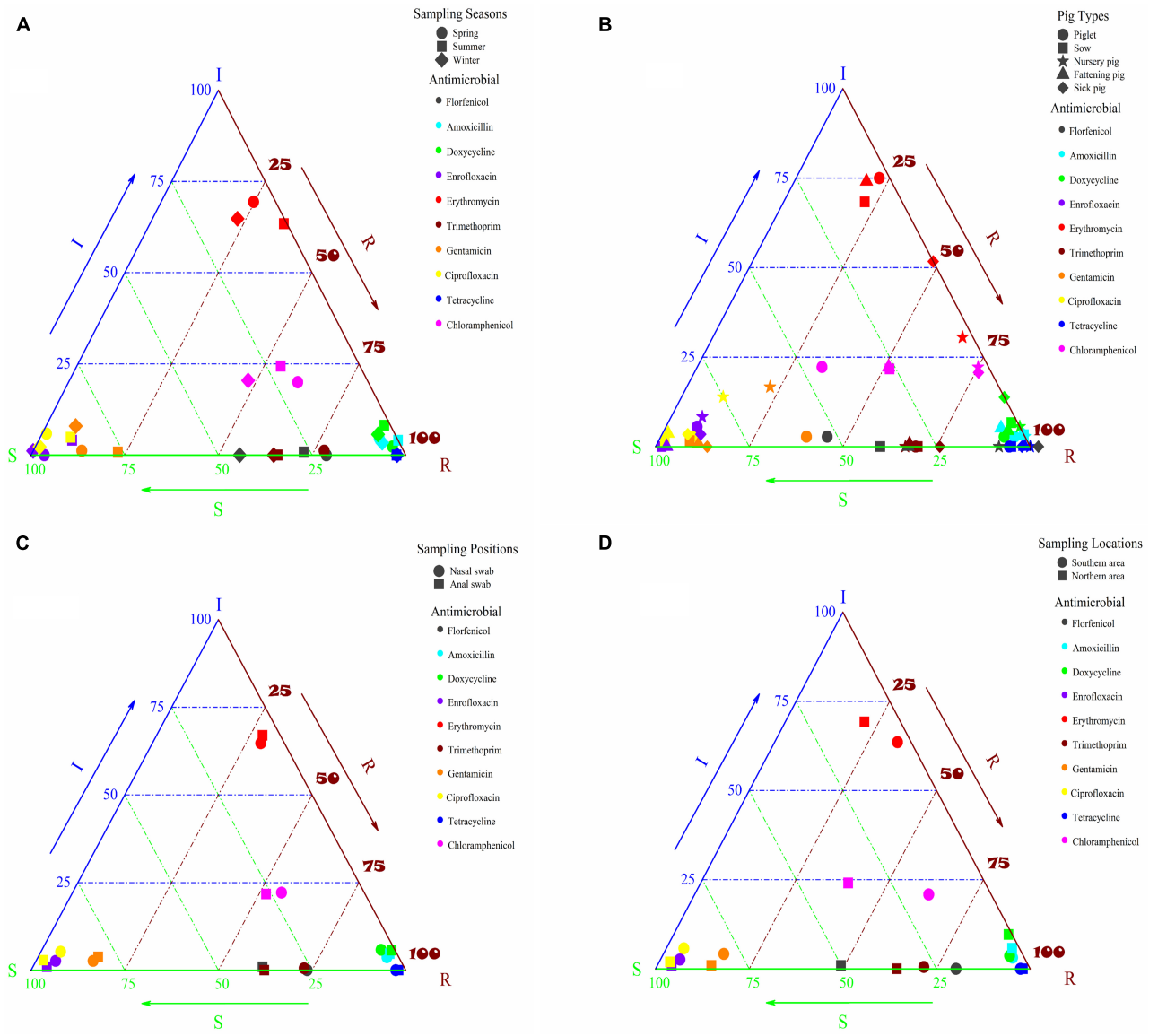


**FIGURE 1 | (A)** Susceptibility of pig-derived *E. coli* to different antibiotics in different seasons; **(B)** susceptibility of *E. coli* derived from different types of pigs to different antibiotics; **(C)** susceptibility of *E. coli* derived from different sampling positions of pigs to different antibiotics; **(D)** susceptibility of *E. coli* derived from pigs in different sampling locations to different antibiotics.

in **Figure 1C** and **Supplementary Table 8**. The overall resistance to antibiotics other than florfenicol and trimethoprim of the bacteria isolated from nasal swabs and anal swabs basically remained the same. The resistance to amoxicillin, tetracycline and doxycycline of the tested bacteria was divided into two parts, and their resistance rates were all above 90%. The resistance to enrofloxacin and ciprofloxacin was low, despite the resistance of the tested bacteria from nasal swabs was slightly higher than from anal swabs, especially to florfenicol and trimethoprim.

To understand the influence of sampling positions on resistance of *E. coli*, binary logistic regression analysis was utilized. Resistance to 10 antibiotics of *E. coli* isolated from nasal swabs was used as a reference and the results were listed in **Supplementary Table 9**. There was a significant difference in resistance to florfenicol of *E. coli* from anal and nasal swabs, and the OR values were 0.589, showing that *E. coli* from nasal swabs was more resistant to florfenicol.

## Single-Factor Binary Logistic Regression Analysis of Resistance of Pig-Derived *Escherichia coli* From Different Sampling Locations

The antibiotic sensitivity of *E. coli* from pigs was statistically analyzed according to different sampling locations, and the

results were shown in **Figure 1D** and **Supplementary Table 10**. The resistance to amoxicillin, tetracycline and doxycycline of the tested bacteria in the southern and northern areas was concentrated (above 90%) except for the tested bacteria's AR to doxycycline in the northern area (89.42%). It may be attributed to the fact that amoxicillin, tetracycline and doxycycline were used in both the northern and southern areas, which caused high rates of resistance to these three antibiotics. In the northern and southern areas, the tested bacteria were more sensitive to enrofloxacin and ciprofloxacin, and the resistance rates were less than 6%. The tested bacteria in the southern area were slightly more resistant to erythromycin than in the northern area, and they were mainly distributed around the intermediate area, indicating that the tested bacteria in the northern and southern areas are potentially resistant to erythromycin. The tested bacteria had high resistance to trimethoprim and the average resistance rate was 67.89%. The sensitivity to gentamicin of the isolates from the northern and southern areas was relatively high, and the average resistance rate was 15.28%.

The tested bacteria isolated from the southern area had higher resistance to florfenicol and chloramphenicol, reaching the resistance rates of 80.21 and 62.5%, respectively, compared with those from the northern area, with their respective resistance rates of 49.04 and 39.42%. The differences in drug use in the northern and southern areas may be the reason for disparate *E. coli* resistance. Chloramphenicol, banned for veterinary use, possessed a high drug resistance rate for *E. coli* from pigs, similar to florfenicol. The possible reason is that both drugs belong to the class of chloramphenicol and have been affected by the spread of resistance genes.

To understand the influence of sampling locations on resistance of *E. coli*, binary logistic regression analysis was utilized. The resistance to 10 antibiotics of *E. coli* isolated from the northern area was used as a reference and the results were listed in **Supplementary Table 11**. It could be seen from the table that there was a significant increase in resistance of *E. coli* sampled from the southern area to all the 10 antibiotics except tetracycline. Moreover, resistance to florfenicol, chloramphenicol and erythromycin of *E. coli* from the southern area was significantly different from what was from the northern area, and the OR values were 4.035, 2.561, and 1.82, respectively.

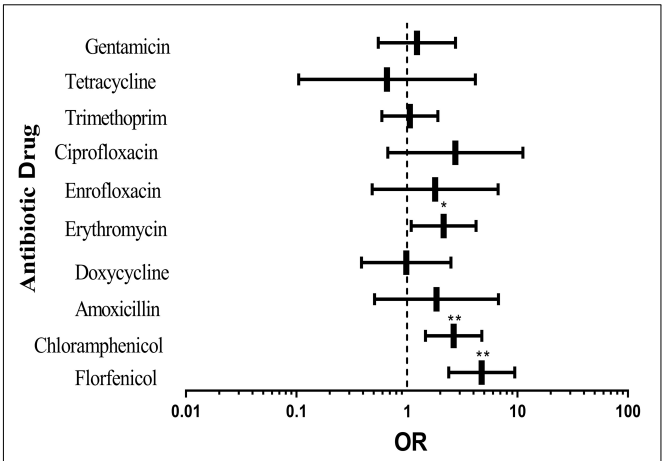


**FIGURE 2 |** Effects of different sampling locations on pig-derived *E. coli* resistance. The middle box is the OR value representing the odds ratio of *E. coli* resistance to antibiotics in the southern area compared with that in the northern area, the horizontal line is 95% confidence interval, $^{*}p < 0.05$, and $^{**}p < 0.001$.

## Multi-Factor Binary Logistic Regression Analysis of Pig-Derived *Escherichia coli* Resistance

Single-factor binary logistic regression analysis was applied to analyze the relationship between the four factors and pig-derived *E. coli* resistance. There was a significant correlation between AR of *E. coli* and the sampling season, pig type and sampling location. The three factors were introduced into the logistic regression equation for multivariate logistic regression analysis. It could be seen from **Figure 2** that resistance to florfenicol, chloramphenicol and erythromycin of the tested bacteria in the southern area was significantly higher than that in the northern area. The results demonstrated that although some antibiotics did not show a tremendous difference, the overall rate of *E. coli* resistance to all antibiotics except tetracycline in the southern area was higher than that in the northern area.

From **Figure 3A**, it can be seen that gentamicin resistance of the experimental bacteria isolated in spring was significantly higher than that in winter, and other antibiotics did not show any significant difference. In summer (**Figure 3B**), resistance to florfenicol, erythromycin, ciprofloxacin and gentamicin of the tested bacteria was significantly higher than that in winter, and the rates of resistance to other antibiotics except tetracycline were higher than that in winter, although there was no substantial difference. The results implied that resistance to antibiotics of the tested bacteria in spring and summer was more serious than that in winter. The causes may be a higher incidence rate of pigs in spring and summer and more serious transmission of pathogens.

**Figure 4** shows the effects of different pig types on drug resistance of *E. coli* from pigs. The rates of resistance to all antibiotics except gentamicin in nursery pigs were higher than that in piglets (**Figure 4A**). Resistance to erythromycin, chloramphenicol, and florfenicol was vastly different. It can be seen from **Figure 4B** that the rates of resistance to all antibiotics except erythromycin, chloramphenicol and florfenicol of the experimental bacteria in sick pigs were lower than that in piglets. It was also obvious in **Figure 4C** that the resistance rate of sows was low, indicating that sows and piglets have similar drug resistance to antibiotics and may possess a vertical transmission risk. It can be seen from **Figure 4D** that the rates of resistance to all antibiotics except chloramphenicol, florfenicol and tetracycline of the tested bacteria in fattening pigs were lower than that in piglets.

According to the above results, *E. coli* isolated from nursery pigs was more drug-resistant than others. In terms

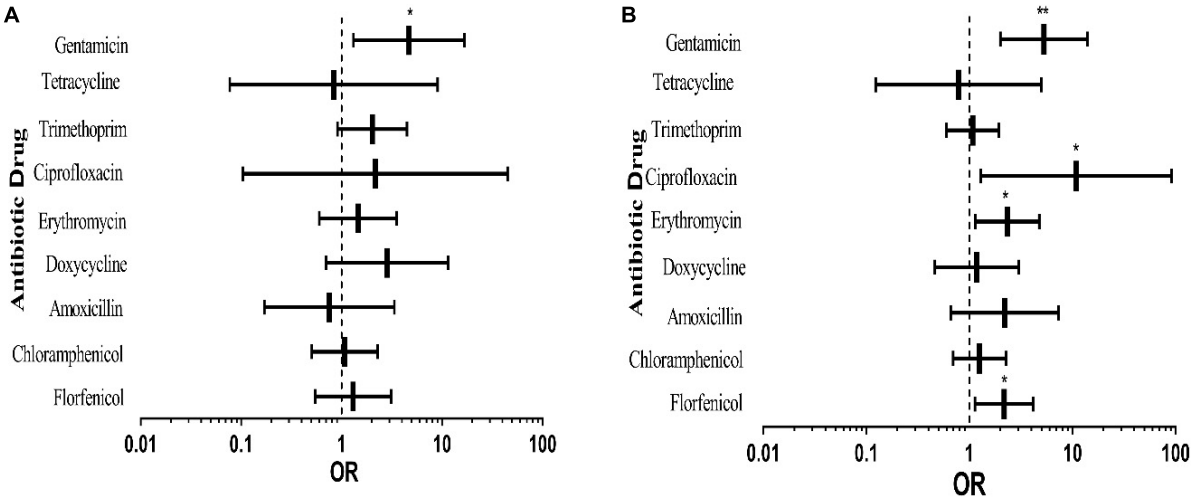


**FIGURE 3 |** Effects of different sampling seasons on pig-derived *E. coli* resistance. The middle box is the OR value representing the odds ratio of *E. coli* resistance to antibiotics in spring **(A)** and summer **(B)** compared to that in winter, the horizontal line is 95% confidence interval, $*p < 0.05$, and $**p < 0.001$.

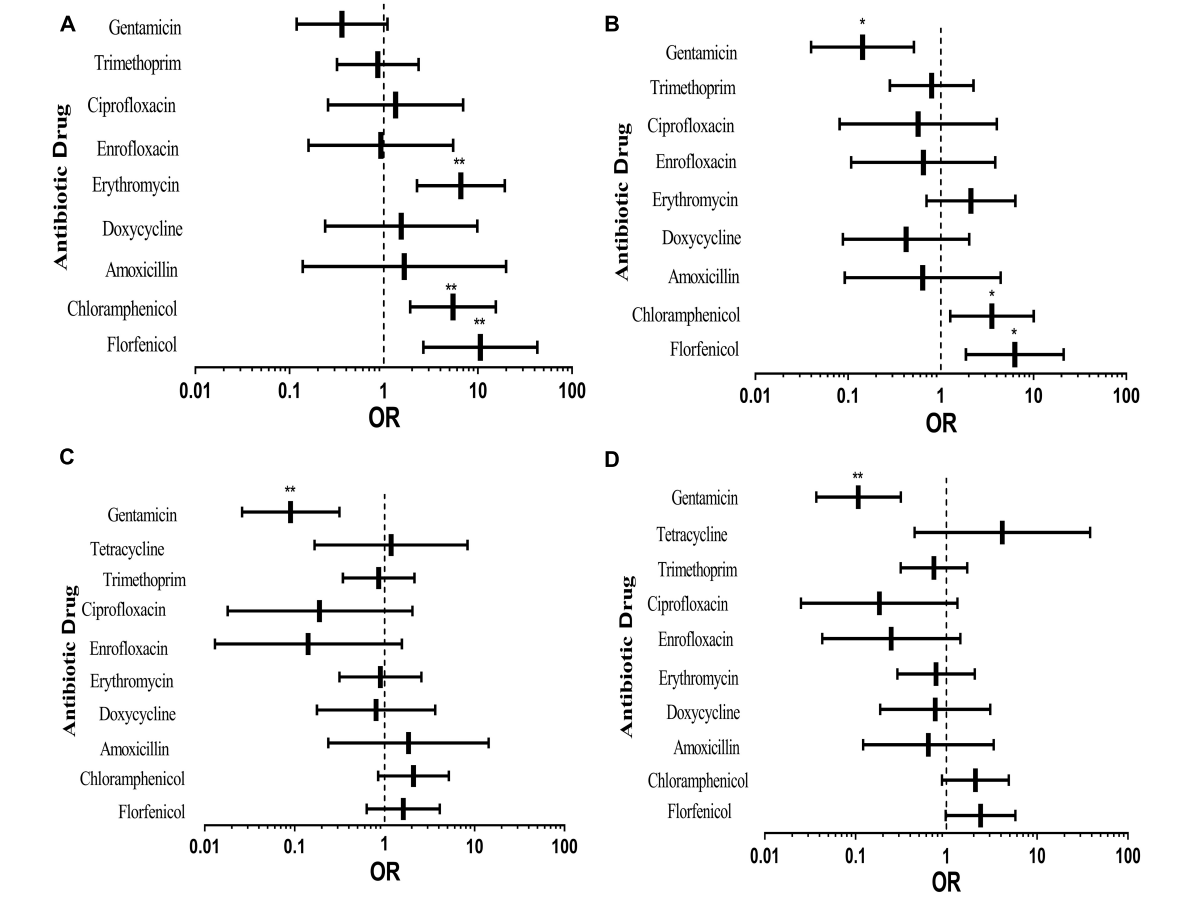


**FIGURE 4 |** Effects of different pig types on pig-derived *E. coli* resistance. The middle box is the OR value representing the odds ratio of *E. coli* resistance to antibiotics in nursery pigs **(A)**, sick pigs **(B)**, sows **(C)** and fattening pigs **(D)** compared to piglets, the horizontal line is 95% confidence interval, $*p < 0.05$, and $**p < 0.001$.

of antibiotics, resistance to gentamicin of piglets was higher than that of other pig types; compared with piglets, *E. coli* from other types of pigs had higher resistance to florfenicol and chloramphenicol. Among them, AR of nursery pigs was the most serious, which may be caused by different growth environments of these pigs. In the process of breeding, pigs are nurtured in different environments at different growth stages, and piglets and nursery pigs grow intensively. Moreover, pigs at this age have low immunity, being prone to diseases, which may lead to *E. coli* AR enhancement. Through multivariate binary logistic regression analysis, it can be seen that antibiotic use, pig growth status and environment have impacts on transmission of *E. coli* drug resistance; meanwhile, there may also be vertical transmission of AR from sows to piglets.

## Analysis of Multiple Factors Influencing Resistance to 10 Antibiotics of Pig-Derived *Escherichia coli* Isolates

A total of 296 isolates of *E. coli* were cultured and tested for susceptibility using a panel of 10 antimicrobials, and the results were listed in **Table 3**. All the isolates were resistant to the antibiotics, among which 14.53% were resistant to less than 3 antibiotics, and 26.35% were resistant to 6 antibiotics, accounting for the largest proportion. There was even one strain of *E. coli* resistant to all the antibiotics tested.

## Single-Factor Multiple Logistic Regression Analysis of Multidrug-Resistant of *Escherichia coli* Derived From Pigs in Different Sampling Seasons

The *E. coli* MDR results were statistically analyzed according to different sampling seasons, as shown in **Supplementary Table 12**. The analysis focused on resistance to 1–8 antibiotics in spring and winter and to 1–10 antibiotics in summer. One strain of *E. coli* was resistant to all the tested antibiotics. In all the three seasons, 5–6 kinds of antibiotics were predominant in MDR analysis, but the median of MDR in winter was lower than that in spring and summer, indicating that the prevalence of MDR in spring and summer was more serious than that in winter. In all, extreme drug resistance of *E. coli* in summer was the most serious. According to Brower et al. (2017) since several antimicrobials did not follow a normal distribution, a categorical variable describing multidrug resistance was constructed with four levels [0 = susceptible, 1 = singly resistant, 2–4 = moderately multidrug-resistant (MDR), > 4 = extremely MDR].

According to **Supplementary Table 13**, compared with extreme drug resistance, MDR of *E. coli* in spring was 0.293 times that in winter (CI: 0.143–0.599; $P < 0.001$), revealing that *E. coli* in winter tended to be more multi-drug resistant than that in spring. Meanwhile, MDR of *E. coli* in summer was 0.629 times higher than that in winter. Although there was no significant difference, *E. coli* tended to be more extremely resistant in summer than in winter. In spring and summer, the prevalence of *E. coli* resistance was more serious.

## Single-Factor Multiple Logistic Regression Analysis of Multidrug-Resistant of *Escherichia coli* Derived From Different Types of Pigs

The *E. coli* MDR results were statistically analyzed according to different pig types, as shown in **Supplementary Table 14**. MDR of sick pigs, fattening pigs, sows and piglets was mainly concentrated in 4–5 antibiotics, while that of nursery pigs was mainly concentrated in 7–8 antibiotics, demonstrating that the prevalence of extreme AR of nursery pigs was more serious.

According to **Supplementary Table 15**, with extreme drug resistance considered, the MDR odds of *E. coli* from nursery pigs were 0.229 times that of piglets (CI: 0.065–0.804; $P < 0.05$), indicating that *E. coli* from nursery pigs was more likely to obtain extreme drug resistance than piglets. It also showed that the distribution of *E. coli* resistance in piglets and sows was similar. The comparison of the OR values showed that there was little difference in MDR between piglets and sows (OR = 1.013). From the analysis of different pig types, the extreme drug resistance of *E. coli* in nursery pigs was more serious, and there was a possibility of vertical transmission from sows to piglets.

## Single-Factor Multiple Logistic Regression Analysis of Multidrug-Resistant of *Escherichia coli* Derived From Different Sampling Positions of Pigs

*E. coli* MDR was statistically analyzed according to different sampling sites of *E. coli* from pigs, and the results were displayed in **Supplementary Table 16**. MDR of *E. coli* from nasal swabs and anal swabs was mainly concentrated in 5–6 antibiotics and the results were listed in **Supplementary Table 17**. Compared with those from anal swabs, *E. coli* from nasal swabs was mostly concentrated in the MDR category, and extreme resistance of *E. coli* from anal swabs was more serious. Meanwhile, there was no significant difference between the two. Therefore, the sampling position was not used as a dependent variable for multi-factor multinomial logistic analysis.

## Single-Factor Multiple Logistic Regression Analysis of Multidrug-Resistant of *Escherichia coli* Derived From Pigs in Different Sampling Locations

MDR of *E. coli* from pigs was analyzed according to different sampling locations, and the results were shown in **Supplementary Table 18**. MDR in the southern area was mainly concentrated in 5–7 antibiotics, while that in the northern area was mainly concentrated in 3–5 antibiotics, suggesting that the prevalence of extreme drug resistance of *E. coli* was more serious in the southern area.

**TABLE 3 |** Multi-drug resistance results of isolated pig-derived *E. coli.*

| Multi-drug resistance | Number (%) of drug-resistant strains *n* = 296 | Multi-drug resistance | Number (%) of drug-resistant strains *n* = 296 |
|---|---|---|---|
| 0 | 0 | 6 | 78 (26.35) |
| 1 | 3 (1.01) | 7 | 46 (15.54) |
| 2 | 10 (3.38) | 8 | 13 (4.39) |
| 3 | 30 (10.14) | 9 | 5 (1.69) |
| 4 | 45 (15.2) | 10 | 1 (0.34) |
| 5 | 65 (21.96) | | |

According to **Supplementary Table 19**, compared with extreme drug resistance, the MDR odds of *E. coli* in the northern area were 4.851 times higher (CI: 2.831–8.312; $P < 0.001$) than those in the southern area. Resistance of *E. coli* in the southern area was more prone to extreme drug resistance than that in the northern area. Analysis from different sampling locations demonstrated that the prevalence of MDR and resistance to 10 antibiotics of *E. coli* in the southern area was higher than those in the northern area, indicating that the sampling location was an important factor affecting *E. coli* drug resistance.

## Multi-Factor Multiple Logistic Regression Analysis of Multidrug-Resistant to 10 Antibiotics of Pig-Derived *Escherichia coli*

Single-element multiple logistic regression was used to analyze the relationship between the four factors and MDR of *E. coli* from pigs. Among them, the sampling season, pig type and sampling location were significantly associated with MDR of pig-derived *E. coli*. The three factors were introduced into logistic regression equation to carry out multi-factor multiple logistic regression analysis. According to **Supplementary Table 20**, compared with extreme drug resistance, MDR of *E. coli* in the northern area was 4.113 times that in the southern area (CI: 2.144–7.89; $P < 0.001$), which indicated that *E. coli* in the southern area was more likely to produce extreme drug resistance than that in the northern area. After logistic regression analysis of the three factors at the same time, only the sampling location was significant, revealing that it was the main factor affecting MDR of *E. coli* from pigs. The biggest difference between the two sampling locations was caused by the type of drug used. The use of antibiotics was an important factor affecting extreme AR of *E. coli* from pigs.

## Detection and Analysis of Extended Spectrum β-Lactamases in Isolated Pig-Derived *Escherichia coli*

The expression of extended spectrum β-lactamases (ESBLs) in 85 strains of *E. coli* isolated from pig farms in spring was detected by paper agar amplification. There was only one strain (1.18%) of *E. coli* that can produce ESBLs, and the results were given in **Supplementary Table 21**.

# DISCUSSION

By exploring the differences in resistance of pig-derived *E. coli* based on types of drugs, pig types, seasons and sampling locations, it is possible to find out the risk factors that trigger bacterial resistance in the pig breeding process and to analyze the factors affecting bacterial resistance, which provides a theoretical basis for mitigating the transmission of drug resistance of *E. coli*.

Comprehensive analysis of AR and MDR distribution of pig-derived *E. coli* revealed that *E. coli* is especially sensitive to quinolones and aminoglycoside drugs, highly resistant to tetracyclines and penicillin, and relatively resistant to sulfonamides and chloramphenicol. The rate of *E. coli* resistance to erythromycin is low, mainly concentrated between susceptibility and resistance, indicating that *E. coli* is potentially resistant to erythromycin. This warns farmers that use of macrolide drugs should be controlled to reduce the risk of *E. coli* resistance to macrolide drugs. Pig-derived *E. coli* is mainly resistant to 5–6 kinds of antibiotics, and the ratio of resistance to more than 3 kinds of drugs is 85.47%, indicating that resistance of *E. coli* in farms is relatively serious. Similar *E. coli* resistance results have been found in the study that tested pig farms in France, Italy, Denmark and Sweden (Burow et al., 2019). A study has also shown that pig-derived *E. coli* is highly resistant to tetracyclines and penicillin and relatively more sensitive to aminoglycoside drugs in pig farms in Thailand (Strom et al., 2017), which is similar to our results. Zhang et al. (2017) studied the resistance trend of *E. coli* in food animals in China from 2008 to 2015 and discovered that pig-derived *E. coli* has high MDR in the country, and the rates of resistance to several earlier drugs such as ampicillin and tetracycline are high (> 80%), suggesting that pig-derived *E. coli* in global pig farms is highly resistant to tetracyclines, penicillin, and sulfonamides, which may be related to long-term, widespread use of these three types of drugs in the pig-feeding industry.

Infections caused by MDR Gram-negative bacteria could be treated by colistin, which has been utilized as the last-resort antimicrobial. Colistin resistance, which is especially associated with mobile genetic elements, such as (mcr) genes, emerged as a major threat to the control of infections caused by Gram-negative bacteria. Dadashi et al. (2021) investigated the global prevalence of mcr-mediated colistin-resistant *E. coli* clinical isolates in Oceania, Africa, America, Europe, and Asia, which were 0.32, 2.27, 5.19, 25.49, and 66.72, respectively. Their research included 79% of the works published between 2014 and 2020 (Dadashi et al., 2021). Shen et al. (2018) investigated 774 non-duplicate MCR-1-positive *E. coli* (MCRPEC) isolates from 774 stool samples collected from 5,159 healthy individuals in 30 provinces and municipalities in 2016, with MCRPEC prevalence ranging from 3.7 to 32.7% (average: 15.0%)—substantially higher than previously reported (Shen et al., 2018).

As human aplastic anemia and certain reproductive/hepatotoxic issues in animals could be induced by chloramphenicol, its use in food, feed, medicine and aquaculture production has been banned for at least 20 years in different parts of the world (Sathya et al., 2020; Wang et al., 2021). Since 2002,

China has clearly stipulated that chloramphenicol is prohibited in animal-source foods, as described in Announcement No. 235, "Maximum Residue Limits of Veterinary Drugs in Foods of Animal Origin." Similarly, concerns about the use of chloramphenicol were expressed and related regulations were set by different countries and regions including the US, Canada, European Union, Australia, Japan, and Brazil, because of bacterial resistance to chloramphenicol and associated clinical toxicity (Barreto et al., 2016). When it is exposed to antimicrobial organisms that are multidrug resistant, its resistance to unrelated drugs will increase. According to the relationship between aminoglycoside and chloramphenicol resistance genes indicated by many previous studies, existence of aminoglycosides and macrolides in lactating piglets may lead to persistence of chloramphenicol resistance in growing pigs (Bischoff et al., 2005; Travis et al., 2006).

Among all types of pigs, nursery pigs and sick pigs had more serious *E. coli* resistance than piglets, fattening pigs and sows, and the epidemic situation of extreme drug resistance of *E. coli* was more serious in nursery pigs. SPSS logistic regression equation was used to analyze the effects of pig types on AR and MDR of pig-derived *E. coli*. It was found that *E. coli* resistance for piglets and nursery pigs in pig farms was more serious, which was similar to the situation in pig farms in Liaoning and other provinces, indicating that most pig farms were facing high *E. coli* AR in piglets and nursery pigs across the country. This may be ascribed to younger ages of piglets and nursery pigs, underdevelopment of their bodies, low immunity and susceptibility to diseases. In the treatment process, resistance of *E. coli* is further enhanced due to the influence of selective pressure. The spread of infectious diseases in pig farms is affected by many factors such as the feeding mode and disease control, herd size, biosecurity and sanitation level (Alvarez-Ordonez et al., 2013; Laanen et al., 2013). These factors may vary between herds and countries and indirectly lead to resistance.

In addition, the feeding mode in pig farms is influential in spreading pathogens and resistant bacteria (Liebana et al., 2013; Tobias et al., 2014). Limited transmission between pig herds reduces the spread of infectious diseases and drug-resistant bacteria, which in turn mitigates bacterial resistance in pigs and ensures healthy growth of pigs. A study has shown that colonization of bacteria in the intestinal tract of newborns is affected by environmental microbiota of mothers and early delivery rooms (Chen et al., 2018). Belloc et al. (2005) found that piglets from sows treated with quinolones after delivery showed quinolone-resistant *E. coli*, while pigs treated with quinolones 2 months after delivery had less quinolone-resistant *E. coli*. It has also been found that sows treated with lincomycin/macrolides have a higher risk of carrying enrofloxacin-resistant bacteria in their piglets, suggesting that development of bacterial resistance is not limited to used antibiotics (Callens et al., 2015). The results of this investigation showed that distribution of *E. coli* resistance in piglets and sows was similar, and there was a possibility of vertical transmission of *E. coli* resistance from sows to piglets.

In terms of sampling locations, multi-drug resistance in the southern area was mainly concentrated in 5–7 antibiotics, while that in the northern area was mainly concentrated in 3–5 antibiotics. SPSS logistic regression equation was employed to analyze the effects of sampling locations on drug resistance and MDR of pig-derived *E. coli*. It was found that compared with the northern area, *E. coli* in the southern area was associated with a significant increase in resistance to all antibiotics except tetracycline, and the epidemic situation of extreme drug resistance of *E. coli* in the southern area was more serious. The results suggested that *E. coli* resistance in the southern part of the country was more serious than the northern part.

In the preliminary investigation of pig farms, it was found that there were differences in the types of drugs used in the two areas. Amoxicillin, doxycycline, florfenicol, and tilmicosin were used in the southern area, while amoxicillin, doxycycline and Yuanlanjing were mainly used in the northern area. The results demonstrated that resistance of *E. coli* to amoxicillin and doxycycline was relatively high in these two areas, indicating that frequent use of these two drugs in pig farms may impose pressure on bacterial selection and lead to AR. It is worth noting that resistance of *E. coli* to florfenicol in the southern area is significantly higher than that in the northern area. By studying drug use in the two areas, it can be found that drug use is significantly related to formation of bacterial resistance. A total ban on certain antibiotics used for growth promotion (tetracyclines, glycopeptides, and macrolides) and for therapeutic purposes (cephalosporins) in the Netherlands revealed a relationship between antimicrobial use and antimicrobial resistance (Speksnijder et al., 2015), resembling the results of this study.

A study has discovered that there is no resistance to cefotaxime of pig-derived *E. coli* in France, Italy, Denmark and Sweden, which is similar to the results of this test, indicating that resistance to the third-generation cephalosporin of *E. coli* obtained from pig farms is low. However, this does not rule out resistance to the third-generation cephalosporin of certain *E. coli* in swine intestinal flora. Therefore, unless cefotaxime-resistant strains are selectively cultured, the actual degree of this low-level resistance cannot be guaranteed. Zhang et al. (2017) found that the rate of resistance to cefotaxime of *E. coli* from chickens and pigs in China increased from 25.60% in 2008 to 46.91% in 2012, and then dropped to 35.08% in 2015. Compared with EU countries and the United States, the overall rate of resistance to the third-generation cephalosporin in pig farms in China is relatively high (Osterberg et al., 2016), implying that new drugs should be used rationally in livestock and poultry farms in China to prolong the usage of new drugs.

## CONCLUSION

A total of 85 isolated pig-derived *E. coli* in spring were detected, and there was one strain that could produce ESBLs, while 296 strains of identified *E. coli* were tested for resistance to 10 antibiotics by the drug susceptibility method. The rates of resistance to quinolone, amoxicillin and tetracycline, chloramphenicol and sulfonamides, gentamicin and

erythromycin of the tested bacteria were, respectively, about 4%, > 90%, between 50 and 70%, 15.54, and 28.72%. According to the results of resistance to 10 antibiotics of 296 pig-derived *E. coli*, the proportion of resistance was 10.14% to 3 antibiotics, 15.2% to 4 antibiotics, 21.96% to 5 antibiotics, 26.35% to 6 antibiotics, 15.54% to 7 antibiotics, 4.39% to 8 antibiotics and 1.69% to 9 antibiotics. There was only one strain (sample 88) of *E. coli* resistant to all tested antibiotics, defining its relatively high resistance.

In this paper, the effects of seasons, pig types, sampling positions (medication type) and sampling locations on drug resistance and MDR of *E. coli* isolated from pigs were analyzed and it was found that there were significant differences in AR and MDR of *E. coli* from pigs. Among these factors, drug types significantly affected AR and MDR of *E. coli* isolated from pigs. In conclusion, the seasonal change, selective drug use and type of pig have impacts on AR of *E. coli* from pigs according to SPSS logistic regression analysis. The experimental comparison results implied that the medication type was the main factor affecting AR of *E. coli*.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

## AUTHOR CONTRIBUTIONS

SY conceived and designed the experiments. QL and YC provided the detection samples, statistical and data analysis in this research. RZ and JW contributed to the reagents and materials. XL prepared the draft of this manuscript. RL and YW collected the samples. AC revised the article and checked the figures. All authors have read and agreed to the published version of the manuscript.

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## REFERENCES

Alvarez-Ordonez, A., Martinez-Lobo, F. J., Arguello, H., Carvajal, A., and Rubio, P. (2013). Swine dysentery: aetiology, pathogenicity, determinants of transmission and the fight against the disease. *Int. J. Environ. Res. Public Health* 10, 1927–1947. doi: 10.3390/ijerph10051927

Baquero, F. (2011). The 2010 garrod lecture: the dimensions of evolution in antibiotic resistance: ex unibus plurum et ex pluribus unum. *J. Antimicrob. Chemother.* 66, 1659–1672. doi: 10.1093/jac/dkr214

Barreto, F., Ribeiro, C., Barcellos Hoff, R., and Dalla Costa, T. (2016). Determination of chloramphenicol, thiamphenicol, florfenicol and florfenicol amine in poultry, swine, bovine and fish by liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A* 1449, 48–53. doi: 10.1016/j.chroma.2016.04.024

Belloc, C., Lam, D. N., Pellerin, J. L., Beaudeau, F., and Laval, A. (2005). Effect of quinolone treatment on selection and persistence of quinolone-resistant *Escherichia coli* in swine faecal flora. *J. Appl. Microbiol.* 99, 954–959. doi: 10.1111/j.1365-2672.2005.02667.x

Bischoff, K. M., White, D. G., Hume, M. E., Poole, T. L., and Nisbet, D. J. (2005). The chloramphenicol resistance gene cmlA is disseminated on transferable plasmids that confer multiple-drug resistance in swine *Escherichia coli*. *FEMS Microbiol. Lett.* 243, 285–291. doi: 10.1016/j.femsle.2004.12.017

Borgmann, S., Riess, B., Meintrup, D., Klare, I., and Werner, G. (2020). Long-lasting decrease of the acquisition of *Enterococcus faecium* and gram-negative bacteria producing extended spectrum beta-lactamase (ESBL) by transient application of probiotics. *Int. J. Environ. Res. Public Health* 17:6100. doi: 10.3390/ijerph17176100

Brower, C. H., Mandal, S., Hayer, S., Sran, M., Zehra, A., Patel, S. J., et al. (2017). The prevalence of extended-spectrum beta-lactamase-producing multidrug-resistant *Escherichia Coli* in poultry chickens and variation according to farming practices in Punjab, India. *Environ. Health Perspect.* 125:077015. doi: 10.1289/EHP292

Burow, E., Rostalski, A., Harlizius, J., Gangl, A., Simoneit, C., Grobbel, M., et al. (2019). Antibiotic resistance in *Escherichia coli* from pigs from birth to slaughter and its association with antibiotic treatment. *Prev. Vet. Med.* 165, 52–62. doi: 10.1016/j.prevetmed.2019.02.008

Cabello, F. C. (2006). Heavy use of prophylactic antibiotics in aquaculture: a growing problem for human and animal health and for the environment. *Environ. Microbiol.* 8, 1137–1144. doi: 10.1111/j.1462-2920.2006.01054.x

Callens, B., Faes, C., Maes, D., Catry, B., Boyen, F., Francoys, D., et al. (2015). Presence of antimicrobial resistance and antimicrobial use in sows are risk factors for antimicrobial resistance in their offspring. *Microb. Drug Resist.* 21, 50–58. doi: 10.1089/mdr.2014.0037

Centner, T. J. (2016). Efforts to slacken antibiotic resistance: labeling meat products from animals raised without antibiotics in the United States. *Sci. Total Environ.* 563-564, 1088–1094. doi: 10.1016/j.scitotenv.2016.05.082

Chantziaras, I., Boyen, F., Callens, B., and Dewulf, J. (2014). Correlation between veterinary antimicrobial use and antimicrobial resistance in food-producing animals: a report on seven countries. *J. Antimicrob. Chemother.* 69, 827–834. doi: 10.1093/jac/dkt443

Chen, X., Xu, J., Ren, E., Su, Y., and Zhu, W. (2018). Co-occurrence of early gut colonization in neonatal piglets with microbiota in the maternal and surrounding delivery environments. *Anaerobe* 49, 30–40. doi: 10.1016/j.anaerobe.2017.12.002

Courtenay, M., Castro-Sanchez, E., Fitzpatrick, M., Gallagher, R., Lim, R., and Morris, G. (2019). Tackling antimicrobial resistance 2019-2024 – The UK's five-year national action plan. *J. Hosp. Infect.* 101, 426–427. doi: 10.1016/j.jhin.2019.02.019

Dadashi, M., Sameni, F., Bostanshirin, N., Yaslianifard, S., Khosravi-Dehaghi, N., Nasiri, M. J., et al. (2021). Global prevalence and molecular epidemiology of mcr-mediated colistin resistance in *Escherichia coli* clinical isolates: a systematic review. *J. Glob. Antimicrob. Resist.* doi: 10.1016/j.jgar.2021.10.022

European Food Safety Authority, and European Centre for Disease Prevention and Control (2018). The European Union summary report on antimicrobial resistance in zoonotic and indicator bacteria from humans, animals and food in 2016. *EFSA J.* 16:e05182. doi: 10.2903/j.efsa.2018.5182

Horton, R. A., Randall, L. P., Snary, E. L., Cockrem, H., Lotz, S., Wearing, H., et al. (2011). Fecal carriage and shedding density of CTX-M extended-spectrum {beta}-lactamase-producing *escherichia coli* in cattle, chickens, and pigs: implications for environmental contamination and food production. *Appl. Environ. Microbiol.* 77, 3715–3719. doi: 10.1128/AEM.02831-10

Kuang, Y., Guo, X., Hu, J., Li, S., Zhang, R., Gao, Q., et al. (2020). Occurrence and risks of antibiotics in an urban river in northeastern Tibetan Plateau. *Sci. Rep.* 10:20054. doi: 10.1038/s41598-020-77152-5

Laanen, M., Persoons, D., Ribbens, S., de Jong, E., Callens, B., Strubbe, M., et al. (2013). Relationship between biosecurity and production/antimicrobial treatment characteristics in pig herds. *Vet. J.* 198, 508–512. doi: 10.1016/j.tvjl.2013.08.029

Liebana, E., Carattoli, A., Coque, T. M., Hasman, H., Magiorakos, A. P., Mevius, D., et al. (2013). Public health risks of enterobacterial isolates producing extended-spectrum beta-lactamases or AmpC beta-lactamases in food and food-producing animals: an EU perspective of epidemiology, analytical methods, risk factors, and control options. *Clin. Infect. Dis.* 56, 1030–1037. doi: 10.1093/cid/cis1043

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., et al. (2012). Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clin. Microbiol. Infect.* 18, 268–281. doi: 10.1111/j.1469-0691.2011.03570.x

Malloy, A., and Campos, J. M. (2011). Extended-spectrum beta-lactamases a brief clinical update. *Pediatr. Infect. Dis. J.* 30, 1092–1093. doi: 10.1097/INF.0b013e31823c0e9d0

Marshall, B. M., Ochieng, D. J., and Levy, S. B. (2009). Commensals: underappreciated reservoir of antibiotic resistance. *Microbe Mag.* 4, 231–238. doi: 10.1128/microbe.4.231.1

Meemken, D., Cuny, C., Witte, W., Eichler, U., and Blaha, T. J. (2008). [Occurrence of MRSA in pigs and in humans involved in pig production–preliminary results of a study in the northwest of Germany]. *DTW* 115, 132–139. doi: 10.2377/0341-6593-115-132

Michael, G. B., Kadlec, K., Sweeney, M. T., Brzuszkiewicz, E., Liesegang, H., Daniel, R., et al. (2012). ICEPmu1, an integrative conjugative element (ICE) of *Pasteurella multocida*: analysis of the regions that comprise 12 antimicrobial resistance genes. *J. Antimicrob. Chemother.* 67, 84–90. doi: 10.1093/jac/dkr406

Osterberg, J., Wingstrand, A., Nygaard Jensen, A., Kerouanton, A., Cibin, V., Barco, L., et al. (2016). Antibiotic resistance in *Escherichia coli* from pigs in organic and conventional farming in four european countries. *PLoS One* 11:e0157049. doi: 10.1371/journal.pone.0157049

Pitout, J. D. D., and Laupland, K. B. (2008). Extended-spectrum β-lactamase-producing *Enterobacteriaceae*: an emerging public-health concern. *Lancet Infect. Dis.* 8, 159–166. doi: 10.1016/s1473-3099(08)70041-0

Ramchandani, M., Manges, A. R., DebRoy, C., Smith, S. P., Johnson, J. R., and Riley, L. W. (2005). Possible animal origin of human-associated, multidrug-resistant, uropathogenic *Escherichia coli*. *Clin. Infect. Dis.* 40, 251–257. doi: 10.1086/426819

Robinson, T. P., Bu, D. P., Carrique-Mas, J., Fevre, E. M., Gilbert, M., Grace, D., et al. (2017). Antibiotic resistance: mitigation opportunities in livestock sector development. *Animal* 11, 1–3. doi: 10.1017/S1751731116001828

Sarmah, A. K., Meyer, M. T., and Boxall, A. B. (2006). A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere* 65, 725–759. doi: 10.1016/j.chemosphere.2006.03.026

Sathya, A., Prabhu, T., and Ramalingam, S. (2020). Structural, biological and pharmaceutical importance of antibiotic agent chloramphenicol. *Heliyon* 6:e03433. doi: 10.1016/j.heliyon.2020.e03433

Shen, Y., Zhou, H., Xu, J., Wang, Y., Zhang, Q., Walsh, T. R., et al. (2018). Anthropogenic and environmental factors associated with high incidence of mcr-1 carriage in humans across China. *Nat. Microbiol.* 3, 1054–1062. doi: 10.1038/s41564-018-0205-8

Smith, D. L., Harris, A. D., Johnson, J. A., Silbergeld, E. K., and Morris, J. G. Jr. (2002). Animal antibiotic use has an early but important impact on the emergence of antibiotic resistance in human commensal bacteria. *Proc. Natl. Acad. Sci. U.S.A.* 99, 6434–6439. doi: 10.1073/pnas.082188899

Speksnijder, D. C., Mevius, D. J., Bruschke, C. J., and Wagenaar, J. A. (2015). Reduction of veterinary antimicrobial use in the Netherlands. The dutch success model. *Zoonoses Public Health* 62 (Suppl. 1), 79–87. doi: 10.1111/zph.12167

Strom, G., Halje, M., Karlsson, D., Jiwakanon, J., Pringle, M., Fernstrom, L. L., et al. (2017). Antimicrobial use and antimicrobial susceptibility in *Escherichia coli* on small- and medium-scale pig farms in north-eastern Thailand. *Antimicrob. Resist. Infect. Control* 6:75. doi: 10.1186/s13756-017-0233-9

Tobias, T. J., Bouma, A., van den Broek, J., van Nes, A., Daemen, A. J., Wagenaar, J. A., et al. (2014). Transmission of *Actinobacillus pleuropneumoniae* among weaned piglets on endemically infected farms. *Prev. Vet. Med.* 117, 207–214. doi: 10.1016/j.prevetmed.2014.07.017

Tornimbene, B., Eremin, S., Escher, M., Griskeviciene, J., Manglani, S., and Pessoa-Silva, C. L. (2018). WHO global antimicrobial resistance surveillance system early implementation 2016–17. *Lancet Infect. Dis.* 18, 241–242. doi: 10.1016/s1473-3099(18)30060-4

Travis, R. M., Gyles, C. L., Reid-Smith, R., Poppe, C., McEwen, S. A., Friendship, R., et al. (2006). Chloramphenicol and kanamycin resistance among porcine *Escherichia coli* in Ontario. *J. Antimicrob. Chemother.* 58, 173–177. doi: 10.1093/jac/dkl207

van den Bogaard, A. (2000). Epidemiology of resistance to antibiotics links between animals and humans. *Int. J. Antimicrob. Agents* 14, 327–335. doi: 10.1016/s0924-8579(00)00145-x

Wang, Y., Zhang, W., Mhungu, F., Zhang, Y., Liu, Y., Li, Y., et al. (2021). Probabilistic risk assessment of dietary exposure to chloramphenicol in guangzhou, china. *Int. J. Environ. Res. Public Health* 18:8805. doi: 10.3390/ijerph18168805

Watts, J. L., Shryock, T. R., Apley, M., Bade, D. L., Brown, S. D., Gray, J. T., et al. (2013). *Performance Standards for Antimicrobial Disk And Dilution Susceptibility Test For Bacteria Isolated From Animals; Approved Standard*. Wayne, PA: NCCLS.

World Health Organization (2012). *The Evolving Threat Of Antimicrobial Resistance: Options For Action*. Geneva: World Health Organization.

Zhang, P., Shen, Z., Zhang, C., Song, L., Wang, B., Shang, J., et al. (2017). Surveillance of antimicrobial resistance among *Escherichia coli* from chicken and swine, China, 2008-2015. *Vet. Microbiol.* 203, 49–55. doi: 10.1016/j.vetmic.2017.02.008

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# Assessments of Probiotic Potentials of *Lactiplantibacillus plantarum* Strains Isolated From Chinese Traditional Fermented Food: Phenotypic and Genomic Analysis

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The lack of rapid and effective approaches to determine the health benefits of strains is one of the main challenges affecting the selection of probiotics from large numbers of candidates. In this study, the probiotic potential of 44 *Lactiplantibacillus plantarum* strains isolated from different Chinese traditional fermented foods was evaluated, including acid and bile salt resistance, adhesion ability, survival in simulated human gastrointestinal transit, antioxidant activity, bile salt hydrolase (BSH) activity, and antibacterial activity. All tested *L. plantarum* strains showed high antioxidant capacity, BSH activity, and antibacterial activity. Among the strains, B652, C232, D444, and E932 were identified as the best comprehensive performed strains, which were selected for whole-genome sequencing, in order to provide clear information and identify key genes responsible for functional characteristics *in vitro*. It demonstrated that the antioxidant activity, adhesion activity, and ability to survive in the simulated gastric environment were found to be closely correlated with antioxidant enzyme encoding genes, cell-surface protein-encoding genes, and stress response genes, respectively. The numbers of functional genes present in strains might decide their performance in probiotic profile evaluation. The outcome of the study could support the development of a novel approach for the screening and identification of probiotics.

**Keywords: fermented food, probiotic profile, whole genome sequencing, functional genes, *Lactiplantibacillus plantarum***

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## INTRODUCTION

The history of fermented food can be traced back to thousand years ago. In ancient times, fermentation was a common way for food preservation and processing. At present, fermented food plays an important role in human diets due to its special taste and unique aroma (Marco et al., 2021; Soto-Giron et al., 2021). In addition, the health-related properties of fermented food significantly promote their consumption around the world (Castellone et al., 2021).

However, the health-related properties are mostly determined by the microorganisms present in fermented food. Microorganisms can release nutrients and bioactive compounds, which could confer antioxidant and antibacterial properties on food, reduce cholesterol levels, and regulate intestinal flora (Park et al., 2016; Xiang et al., 2019). Therefore, fermented food has been

considered a good source for discovering probiotics. Among the various potential probiotic strains, *Lactiplantibacillus plantarum*, a kind of lactic acid bacteria (LAB), represents one of the predominant genera related to health-promoting and fermentative properties in fermented food. *L. plantarum* has one of the largest microbial genomes known, of which 222 *L. plantarum* have completed the whole genome sequencing according to the NCBI database (Bintsis, 2018; Huang et al., 2021). In addition, *L. plantarum* was found in various food fermentations, such as *Paojiao*, pickled chayote, and yogurt, in conjunction with healthy beneficial properties (Pan et al., 2020; Shang et al., 2022; Ye et al., 2022).

Probiotic therapy is becoming increasingly popular nowadays. To satisfy customers' needs for probiotic products, it is necessary to develop fast and reliable strategies to evaluate the safety and health benefits of probiotic candidates. The available functional evaluation methods mainly include *in vitro* tests, *in vivo* tests, and human clinical studies (Koirala and Anal, 2021). As high-throughput sequencing technology is becoming more affordable, genomic analysis is an expected method for accurate strain identification and functional prediction for probiotics (Wang Y. et al., 2021). The combination of characteristic experiments and genetic information of candidate strains has the potential to promote the evaluation system of probiotics (Huang et al., 2021).

The aim of the study is to develop an approach based on the phenotype and genomic analysis to evaluate the probiotic properties of *L. plantarum* strains isolated from Chinese traditional fermented foods. Acid and bile salt resistance, adhesion ability, survival in a simulated human gastrointestinal tract, antioxidant activity, antibacterial activity, bile salt hydrolase (BSH) activity, and hemolytic activity were carried out to evaluate the potential probiotic properties of these strains. Furthermore, the whole genome sequencing analysis was conducted on selected strains to further identify the relevant functional genes.

# MATERIALS AND METHODS

## Isolation and Identification of *Lactiplantibacillus plantarum* Strains From Fermented Foods

The homemade fermented food samples were collected from different cities in Yunnan Province, China. Each sample was stored in a sterilized centrifuge tube at 4°C and transported to the laboratory once collected. Each sample (0.50 g) was mixed with 4.50 ml of 0.85% (w/v) saline and crushed using a mortar. After a series of dilutions with 0.85% (w/v) saline, these samples were spread on de Man, Rogosa, and Sharpe (MRS) agar plates and incubated in an anaerobic incubator (YY-S, Maworde, China) at 37℃ for 2–3 days. Next, single colonies were picked from plates and cultivated in a liquid MRS medium under the same conditions. To identify the obtained strains, the morphology of strains was initially observed using a microscope (DM500, Leica, China). The strains were then further identified using 16S rDNA sequencing. During the isolation of LAB, 44 strains identified as *L. plantarum* were selected and maintained in 40% (v/v) glycerol stock at −80℃ for further experiments, namely, *L. plantarum* (strains A182, A232, B652, etc.). Strain's number, nomenclature, source of isolation, and location of samples collection are listed in **Supplementary Table 1**.

## Preparation of Cell-Free Supernatant and Intact Cell Solution of *Lactiplantibacillus plantarum*

For further tests, a cell-free supernatant (CFS) and an intact cell solution (ICS) were prepared. *L. plantarum* strains were first cultivated in the MRS broth under an anaerobic condition at 37℃ for 20 h. The CFS was obtained by centrifuging MRS broth and filtering it with a 0.22 μm membrane. ICS of *L. plantarum* was obtained by centrifugation at 3,800 rpm for 5 min and washed cells three times with sterilized phosphate-buffered saline (PBS, pH 7.40). In the following steps, these cells were resuspended in the same PBS to reach an optical density of cells at 600 nm ($OD_{600\ nm}$) of 1.00 ± 0.05.

## Probiotic Potential Evaluation of *Lactiplantibacillus plantarum* Strains

### Acid and Bile Salt Resistance

The MRS medium adjusted to different pH values (5.50, 4.90, 4.30, 3.60, and 3.30) and the MRS medium supplemented with bile salt (0.10–0.60%, w/v) were prepared for the tests of acid and bile salt tolerance of strains, respectively. Cells from the overnight culture of *L. plantarum* strains were collected and resuspended in acid MRS medium and bile salt medium to reach an $OD_{600\ nm}$ of 0.10 ± 0.05. Then, a 200 μl volume of each culture was transferred to 96-well plates covered with lids. The plates were incubated in a Logphase 600 (BioTek, Agilent, United States) at 37℃, 500 rpm for 24–96 h. Control experiments containing only MRS medium and cell inoculum were also performed. The $OD_{600\ nm}$ of cultures was read at 10 min intervals. The lag phase for each strain to adapt to the inhibition conditions was used to indicate the resistance of strains to acid and bile salt. All assays were conducted in triplicate.

### Adhesion Ability

The adhesion ability of strains was evaluated using cells' hydrophobicity and auto-aggregation index. Protocols developed by Lee and Kim (2019) were adopted to measure these two indexes. All experiments were conducted in triplicate. Briefly, 1 ml of xylene was added to 3 ml of ICS and mixed well. Then the mixtures were kept at 37℃ until a phase separation was observed. The bottom phase was used to measure the hydrophobicity of strains. For the assay of the hydrophobicity of strains, the absorbance of bottom phases was read at 600 nm, which was defined as the value of $A_t$. The hydrophobicity of cells was expressed using the following equation:

$$\text{Hydrophobicity (\%)} = \left(1 - \frac{A_t}{A_0}\right) \times 100 \quad (1)$$

where $A_0$ is the initial $OD_{600\ nm}$ of samples measured at $t$ = 0 h.

Briefly, 4 ml of ICS was incubated at 20℃ for 24 h. Next, 1 ml of the upper layer solution was read at $OD_{600\ nm}$ to determine

the auto-aggregation of strains. The auto-aggregation index was defined as follows:

$$\text{Auto}-\text{aggregation}(\%) = \left(1-\frac{A_{24}}{A_0}\right) \times 100 \quad (2)$$

where $A_{24}$ is the $OD_{600\ nm}$ measured of the upper layer at $t = 24$ h and $A_0$ is the initial $OD_{600\ nm}$ measured at $t = 0$ h.

### Survival in Simulated Human Gastrointestinal Transit

The simulated gastric juice (SGJ) was composed of 27 mg/ml of pepsin (Solarbio, Beijing, China) dissolved in 0.50% (w/v) saline solution (pH 2.50), whereas the simulated small intestinal juice (SSIJ) was composed of 0.27 mg/ml of pancreatin (Solarbio, Beijing, China) in 0.50% of saline at pH 8.00. Cells from the pre-cultivation of *L. plantarum* strains were harvested and resuspended in SGJ with an initial cell density of $10^8$ CFU/ml and incubated at 37℃ for 3 h. Then, 0.50 ml of incubated SGJs was added to 4.50 ml SSIJ and cultivated at 37℃ for another 3 h. The survival rates of SGJ and SSIJ were determined by counting viable cells on MRS agar plates after inoculation with cultures from simulating gastrointestinal transit tests (Fei et al., 2018). The analyses for each strain were performed in triplicate. The survival rate was expressed using the following equation:

$$\text{Survival rate}\ (\%) = \frac{\textit{Viable cells after incubating}\ (\textit{Log CFU/mL})}{\textit{Initial viable cells}\ (\textit{Log CFU/mL})} \quad (3)$$

### Antioxidant Activity

The antioxidant activity of strains was measured with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay and 2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical cation scavenging activity assay as described by Son et al. (2017) and Tang et al. (2021), respectively. The experiment for each strain was performed in triplicate. In brief, 0.50 ml of ICS (sample) or PBS (control) was mixed with 0.50 ml of 0.20 mmol/L of DPPH solution. An equal volume of methanol was used to replace the DPPH solution as the blank. Then the mixtures were incubated at dark for 30 min at 37℃. After centrifugation (12,000 rpm, 10 min), the absorbance of mixtures was measured at 517 nm using a microplate reader (M5, SpectraMax, United States). The antioxidant activity was calculated as follows:

$$\text{DPPH radical scavenging activity}\ (\%) = \left[1-\frac{\left(A_{sample}-A_{blank}\right)}{A_{control}}\right] \times 100 \quad (4)$$

For the ABTS radical cation scavenging assay, a working solution composed of 7.40 mmol/L of $ABTS^+$ and 40 mmol/L of potassium persulfate was prepared. After reacting for 12 h in the dark at room temperature, the working solution was diluted with absolute ethanol to an absorbance of $0.70 \pm 0.02$ at 734 nm and stored at 30℃. Next, 0.25 ml of ICS (sample) or PBS (control) was mixed with 4 ml of working solution. The blank samples were prepared with 4 ml of absolute ethanol to replace the working solution. All the samples were kept at 30℃ for 6 min of incubation. Absorbance against the blank samples was read at 734 nm. Results were calculated as follows:

$$\text{ABTS radical cation scavenging activity}\ (\%) = \left[1-\frac{\left(A_{sample}-A_{blank}\right)}{A_{control}}\right] \times 100 \quad (5)$$

### Antibacterial Activity

The antibacterial activity of selected strains was determined using the agar diffusion test based on the method previously reported by Wang et al. (2018). Briefly, 100 μl of CFS was added into the holes with a 7-mm diameter in Luria Bertani (LB) agar plates that had been spread with indicator bacteria (*Escherichia coli*, *Staphylococcus aureus*, *Shigella flexneri*, and *Salmonella typhimurium*). The plates were cultured at 30℃ or 37℃ for 48 h. The diameter of the inhibitory zone formed on the plates was considered an indication of antibacterial activity.

### Bile Salt Hydrolase Activity

As for BSH activity, 100 μl of ICS from cultures of different strains were added into the holes (7 mm diameter) in the MRS agar plates supplemented with 0.37 g/L of $CaCl_2$ and 5 g/L of sodium taurocholate (Park et al., 2016). Precipitation observed in the holes can be determined as capable strains with BSH activity.

### Hemolytic Activity

One loop of pre-culture of each strain was streaked on Columbia blood agar plates containing 5% of sheep blood (Sharma et al., 2018). If transparent cycles formed around strain colonies, it meant strains hold the hemolytic activity.

## Genomic Analysis of Selected Strains

### Whole-Genome Sequencing of *Lactiplantibacillus plantarum* Strains

To further identify the probiotic properties of the best-performed strains (B652, C232, D444, and E932), the whole genome sequencing analysis was performed. The overnight culture of *L. plantarum* strains was collected by centrifugation at 3,800 rpm for 5 min and submitted to Shanghai Majorbio Bio-Pharm Tech Co., Ltd., (China) for DNA extraction and sequencing analysis using the Illumina HiSeq2000 sequencing platform at 2 × 150 paired-end reads.

### Analysis of Sequencing Data

For genome assembly, after removing low-quality data obtained from the sequencing platform, the clean reads of each strain were assembled using SOAPdenovo 2.0 and SPAdes 3.11. Glimmer version 3.02 was used for the prediction of coding sequence and GC content. Functional annotation of the genome was performed by blasting genes with multiple public databases including Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups of proteins (COG), Non-redundant protein database (NR), and Gene Ontology (GO) under an

*E*-value cutoff of 1e-5. A certificated probiotic *L. plantarum* strain ST-III (accession number CP002222 in NCBI) was used as a reference to identify the probiotic potentials of candidate strains on genomic levels (Huang et al., 2021). Antismash software was used to predict the gene clusters of secondary metabolite synthesis. The core and pan genome of selected strains were analyzed using PGAP 1.2.1 and CG-HIT 4.6.1, respectively. Based on genome mapping and sequencing, the MUMmer 3.23 with default parameters was used to compare known genes and genome structures of each strain at the nucleotide level (Li et al., 2016a).

## Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics 26. The results were calculated as the mean ± standard deviation (SD) of three replicates. Significant differences at $P < 0.05$ between the means of parameters were calculated with Duncan's multiple range test.

# RESULTS AND DISCUSSION

## Probiotic Potential Evaluation of *Lactiplantibacillus plantarum* Strains

### Acid and Bile Salt Resistance

The acid resistance assay showed that the lag phase of 44 *L. plantarum* strains increased significantly ($P < 0.05$) as pH decreased (**Figure 1A**). Although most tested strains proliferated within 48 h at pH 3.60, C232 started an exponential phase at pH 3.30. Similarly, the lag phase duration of the strains was prolonged as the increase in bile salt concentration in the cultivation medium (**Figure 1A**). As shown, 75% of the 44 strains could tolerate 0.30% bile salt, the average bile concentration in the human gastrointestinal tract, but only 23% of tested strains could enter the exponential phase when the concentration of bile salt reached up to 0.60% in the medium. No significant differences were observed in terms of the lag phase of D531, E932, and L117 at this condition ($P > 0.05$). This result indicated that *L. plantarum* had a considerable tolerance toward bile salt. Lin et al. (2020) also found that three *L. plantarum* strains isolated from kimchi have a stronger resistance against bile salt than those of gastric acid.

### Adhesion Ability

As demonstrated in **Figure 1B**, the cell surface hydrophobicity of all tested strains ranged from 12.97 to 83.63%. Strains, whose cell surface hydrophobicity index was higher than 70%, were classified as hydrophobic ones with good adhesion activity (Shangpliang et al., 2017). Among the tested strains, the hydrophobicity of B652, D2510, and K125 was all above 70%. Specifically, the number for B652 reached up to 83.63%, making it an ideal candidate for probiotics. In addition, auto-aggregation activity is a crucial index, reflecting the ability of strains to colonize in gastrointestinal environments. In the study, the

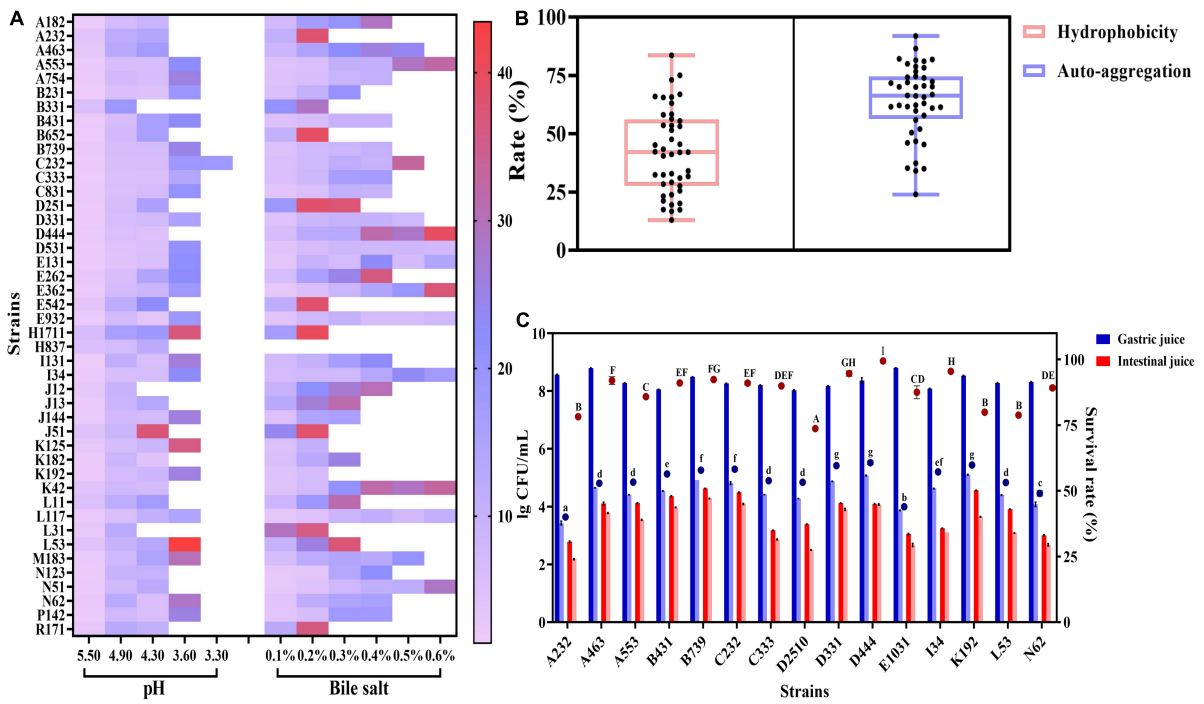


**FIGURE 1 |** Adaptation of *L. plantarum* strains to the gastrointestinal environment: **(A)** lag phase of strains growth in different concentrations of acid and bile salt environments; **(B)** adhesion ability of strains by hydrophobicity (light pink box plot) and auto-aggregation index (light blue box plot); **(C)** survival of strains in simulated gastric juice and simulated small intestinal juice. The bar charts show the viable cell number of the strains at 0 and 3 h and the dots indicate the survival rates of strains. The letters a–g and A–I indicate statistically significant differences ($p < 0.05$) within each dot.

auto-aggregation capability of tested strains showed significant differences ($P < 0.05$), ranging from 24.03 to 91.88%. The highest auto-aggregation value (91.88%) was found in L11, followed by A232 with 86.54%. A recent study showed that *L. plantarum* MYSRD 71, isolated from fermented Vellappam, exhibited a high auto-aggregation value (88.5%) and good adhesion performance (Divyashree et al., 2021).

## Survival in Simulated Human Gastrointestinal Transit

The survival rate of *L. plantarum* in gastrointestinal transit has been evaluated (**Figure 1C**). Among the tested 44 strains, 15 strains survived in artificial gastrointestinal transit. All strains showed an obvious reduction in viable cells after 3 h exposure to artificial gastric juice (pH 2.50). The loss of viability ranged from 3 log to 5 log and the survival rate was about 50%. However, it was found that 3 h incubation in simulated intestinal juice did not result in a significant decrease (73.71–99.46%) in the survival rate of test strains ($P > 0.05$). The result was aligned with our observation that *L. plantarum* had a stronger tolerance to bile salt than that of acid. After a total of 6 h of digestion in the simulated gastrointestinal tract, D444 and C232 still keep high viable counts (around 4 log CFU/ml). In general, C232 had better performance in tolerant acid and bile salt than other strains.

## Antioxidant Activity

The DPPH and ABTS scavenging ability of 44 *L. plantarum* is summarized in **Figure 2**. All strains showed antioxidant activity. The DPPH radical scavenging ability of the strains ranged from 0.88 to 28.50%, while a much higher number for ABTS radical cation scavenging was found from 16.02 to 54.24%. The highest DPPH scavenging (28.02%) was found in strain D444, followed by 20.33% found in B652. As for ABTS radical scavenging activity, B652 held the highest value at 54.24% among all tested strains.

## Antibacterial Property

The antibacterial activity of 44 *L. plantarum* strains against 4 pathogens (*Escherichia coli*, *Staphylococcus aureus*, *Shigella flexneri*, and *Salmonella typhimurium*) was evaluated (**Table 1**). A total of 14 *L. plantarum* strains were capable of inhibiting the growth of more than 3 kinds of pathogens. L117 could inhibit the growth of the 4 pathogens, especially *Shigella flexneri* and *Salmonella typhimurium*. A182 and A463 displayed the highest inhibitory to *Salmonella typhimurium*, with inhibitory zone diameters of 6.01–10.00 mm. As the CFS was directly used in the study without any pretreatment, the antibacterial compounds present in CFS should be further identified. Wang et al. (2018) treated the CFS with catalase and 1 mol/L NaOH to eliminate the influence of organic acids and hydrogen peroxide, which resulted in an inhibition zone with a diameter of 29.60 mm. The antibacterial capacity of *L. plantarum* is possibly related to the antibacterial substances generated by strains, such as organic acids, hydrogen peroxide, and antibacterial peptides (Sikorska and Smoragiewicz, 2013).

**TABLE 1 |** Inhibitory spectrum of *L. plantarum* strains.

| Strains | Inhibition zone (mm) | | | |
|---|---|---|---|---|
| | *Escherichia coli* | *Staphylococcus aureus* | *Shigella flexneri* | *Salmonella typhimurium* |
| L117 | + | + | ++ | ++ |
| H10711 | + | ++ | + | − |
| R171 | ++ | + | + | − |
| B331 | ++ | + | ++ | − |
| J102 | ++ | ++ | + | − |
| K182 | + | + | ++ | − |
| D531 | ++ | ++ | − | + |
| E1031 | + | − | + | + |
| B431 | ++ | − | ++ | + |
| B739 | ++ | − | + | ++ |
| J144 | ++ | − | + | ++ |
| K125 | − | + | + | + |
| E262 | − | + | ++ | ++ |
| D444 | − | + | ++ | ++ |
| D2510 | ++ | ++ | − | − |
| I34 | + | + | − | − |
| E542 | ++ | ++ | − | − |
| P142 | ++ | ++ | − | − |
| J51 | ++ | − | + | − |
| A463 | ++ | − | − | +++ |
| K192 | ++ | − | − | ++ |
| B231 | − | + | − | + |
| L31 | − | ++ | − | ++ |
| B652 | − | − | ++ | ++ |
| A232 | − | − | + | + |
| A553 | − | − | + | ++ |
| L53 | − | − | ++ | + |
| A182 | − | − | ++ | ++ |
| E932 | − | − | ++ | +++ |
| C831 | − | − | + | − |
| C331 | − | − | + | − |
| C232 | − | − | ++ | − |
| C333 | ++ | − | − | − |
| N62 | + | − | − | − |
| N51 | + | − | − | − |
| M183 | ++ | − | − | − |
| K42 | − | ++ | − | − |
| E362 | − | − | − | ++ |
| L11 | − | − | − | + |
| N123 | − | − | − | + |
| P142 | − | − | − | + |
| I131 | − | − | − | ++ |

*The different scores reflect the different degrees of growth inhibition expressed in mm. Inhibition zone (mm): no inhibition: –; 0–3.00 mm: +; 3.01–6.00 mm: ++; 6.01–10.00 mm: +++.*

## Bile Salt Hydrolase Activity

The generation of BSH in strains is suggested as one of the factors to screen probiotics for lowering serum cholesterol (Pereira et al., 2003). A BSH screening medium was used to characterize the BSH activity of strains, in which insoluble unbound bile salt derived from the taurine bile salt hydrolyzing under the catalysis of BSH can react with $CaCl_2$ in the culture medium to form precipitation circles (Meng et al., 2021). The precipitation observed in the BSH screening medium was used to indicate

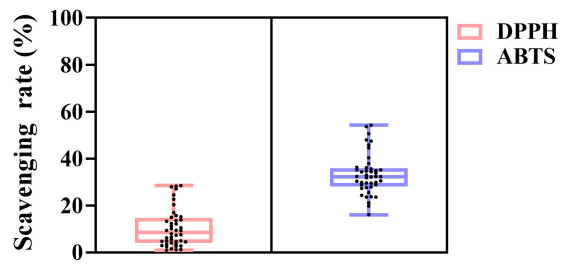


**FIGURE 2 |** Antioxidant activity of *L. plantarum* strains. The light pink box plot and light blue box plot represent DPPH radical scavenging activity and ABTS radical cation scavenging activity, respectively. The black dots indicate the distribution of 44 strains.

the generation of BSH in strains. The precipitate circles were observed on 44 *L. plantarum* strains (data not shown), implying that these 44 *L. plantarum* strains might be promising probiotic candidates to decrease cholesterol levels.

### Hemolytic Activity

Hemolytic activity is a decisive index to evaluate the safety of probiotic candidates. If strains show a positive hemolytic activity, it is forbidden to be used as probiotics. In the study, the Columbia blood agar plates were used to assay the hemolytic activity of the strains. The red blood cells in the Columbia blood agar plates could be dissolved by the hemolysin produced by strains and form a transparent hydrolysis circle around colonies of strains. If there are no hydrolysis circles formed around colonies, the strain is defined as γ-hemolytic and considered a safe one (Sharma et al., 2018). All the tested strains displayed negative results for this experiment (data not shown).

## Genomic Analysis of Selected Strains

According to *in vitro* experiments shown in previous sections, it was found that *L. plantarum* C232 demonstrated considerable tolerance to gastric acid, E932 showed the strongest resistance toward bile salt, D444 had a good performance to survive in synthetic gastrointestinal environments and scavenge DPPH, and B652 was the best-performed strain for antioxidant activity. To link the functional phenotype of these 4 strains and their genetic characteristics and further evaluate their probiotic potential at genetic levels, whole-genome sequencing was performed. In addition, a certificated probiotic *L. plantarum* ST-III was used as a reference to compare the genomic similarity and variations of the strains. The strain has probiotic traits of antibacterial activity, strong adhesion, and cholesterol-lowering effect (Wang et al., 2011).

### Genomic Overview of Selected Strains

The basic genomic features of B652, C232, D444, and E932 compared with ST-III are illustrated in **Figure 3**. It was found that the genomic size of 5 strains ranged from 3.25 to 3.78 Mb and the number of coding genes was about 3,013–3,488 with the GC content of 44.58–47.31%. Notably, the genome size and GC content of B652 were obviously higher than those of others (**Figure 3A**). Furthermore, from the distribution of common

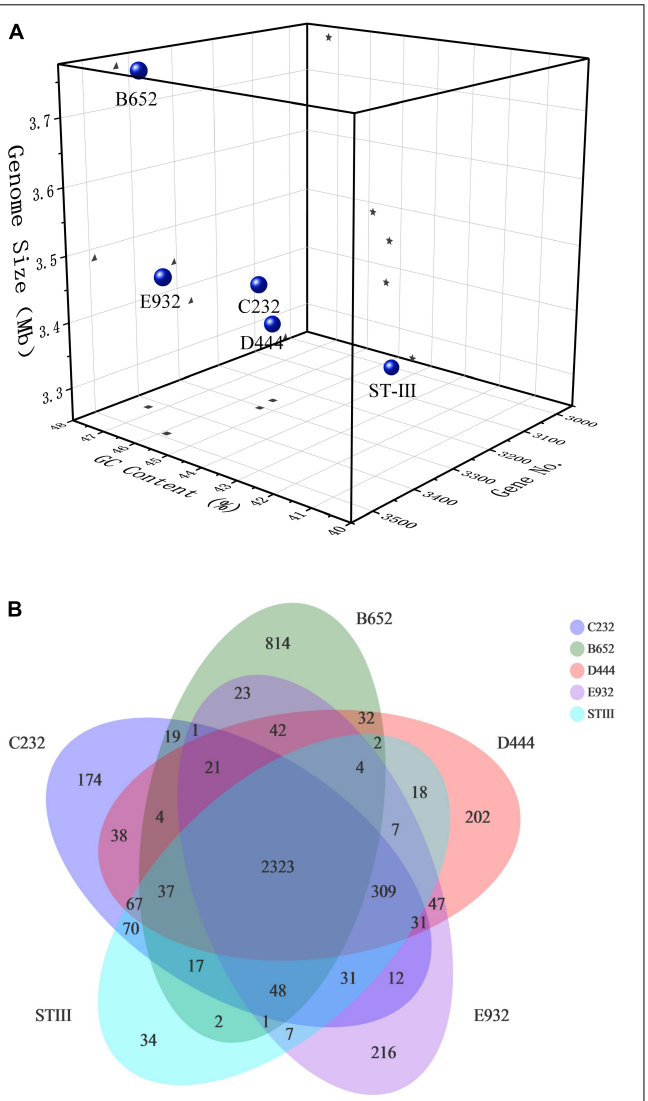


**FIGURE 3 |** Genomic analysis of the selected *L. plantarum* strains: **(A)** basic genomic features; **(B)** common and unique genes distribution. In the Venn diagram **(B)**, the number in the center shows the common genes shared by contained all strains and the outermost edge show the number of unique genes for each strain.

and unique genes of the 5 strains (**Figure 3B**), it could be seen that there were 2,323 genes shared by the 5 strains and B652 had the most unique genes (814). The result was aligned with the bigger genome size found in B652. The unique genes occupied by B652 might be related to its special properties, such as good antioxidant activity. The higher GC content may indicate that microorganisms consume more energy during reproduction, while microorganisms with a low GC content may be easier to maintain genomic stability due to low energy metabolism (Rocha and Danchin, 2002).

### Genomic Annotation of Selected Strains

According to the annotation result of KEGG, the genomic function enrichment of 5 strains is illustrated in **Figure 4A**. It

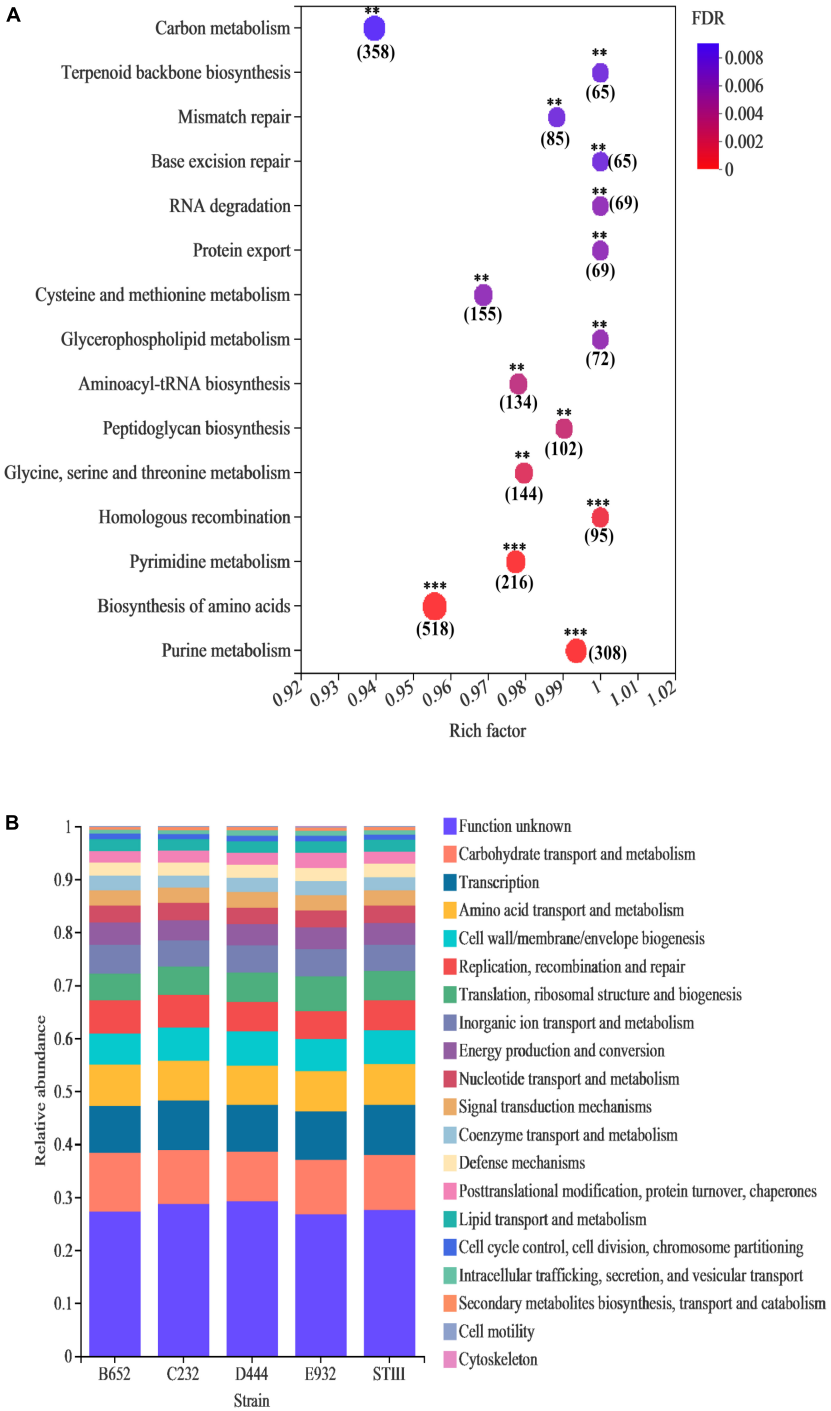


**FIGURE 4 |** Genomic annotation of the selected *L. plantarum* strains: **(A)** enriched genes in KEGG database; **(B)** genes distribution in core genomes based on COG database. In bubble map **(A)**, the vertical axis and horizontal axis represent pathways and enrichment rate, respectively. The number indicates the number of genes in this pathway, and the color of the dot indicates the significance level of enrichment. The darker color indicates the more significant enrichment of this pathway, where the false discovery rate (FDR) < 0.001 is marked with ***, FDR < 0.01 is marked with **, and FDR < 0.05 is marked with *.

was noted that 15 pathways of the 5 strains were significantly enriched in nucleotide and amino acid metabolism. Among them, genes responsible for replication and repair accounted for the highest proportion. **Figure 4B** illustrates that, except for unknown functions, most of the annotated genes were enriched in metabolism, especially carbohydrate transport and metabolism, amino acid transport and metabolism, and energy production and conversion, which was consistent with the

previous study reported by Huang et al. (2021). The result indicated that tested strains could metabolize different types of carbon sources and had good propagation ability. In addition, genes of all 5 strains were equally distributed in several COG categories, such as transcription, cell wall/membrane/envelope biogenesis, replication, recombination and repair, translation, ribosomal structure, and biogenesis. The results implied that the strains had a high similarity on genomic levels.

## Correlations Between Functional Phenotype and Genomic Property

The potential probiotic properties possessed by the 5 strains were verified at genomic levels. **Supplementary Table 2** shows the probiotic property-related genes that were identified in ST-III and selected strains.

According to the sequencing data analysis, it was found that B652, C232, D444, and E932 contained various antioxidant enzyme encoding genes including catalase encoding gene *katE* (K03781), glutathione peroxidase encoding gene *gpx* (K00432), NADH dehydrogenase encoding gene *ndh* (K03885), and *npr* (K05910) gene encoding for NADH peroxidase. In addition, genes related to the synthesis of thioredoxin were identified in the strains, including *nrdH* (K06191) encoding for glutaredoxin, *trxA* (K03671) encoding for thioredoxin, *trxB* (K00384) encoding for thioredoxin reductase, and *tpx* (K11065) encoding for thiol peroxidase (Zhang et al., 2021). These genes have also been found in the *L. plantarum* ST-III genome. The result indicated the strains might have a similar functional property.

The adhesion of microorganisms to epithelial cells is a complex process, as it largely depends on the chemical composition and physical properties of the cell surface of the probiotic strains (de Melo Pereira et al., 2018). Probiotics can specifically bind to intestinal mucosal epithelial cell receptors through adhesin and then colonize in the intestine. For instance, a potential surface exposure (PSE) protein involved in colonization on the intestinal mucosa can act as an adhesin to adhere to the intestinal surface (Jia et al., 2017). The 4 selected strains contained a variety of cell-surface proteins encoding genes, such as *mapA* encoding for maltose phosphorylase (K00691), *lspA* encoding for lipoprotein signal peptidase (K03101), *tuf* encoding for elongation factor Tu (K02358), *gpr* encoding for glyceraldehyde 3-phosphate dehydrogenase (K19265), and *tpi* encoding for triosephosphate isomerase (K01803). The *L. plantarum* ST-III had strong adhesion to epithelial cells' *in vitro* experiment, which might be related to these genes found in its genome (Wang et al., 2011). B652 had the largest number of genes encoding for surface proteins and performed well in terms of adhesion activity.

Probiotic strains capable of surviving in the gastric environment tend to contain stress response genes which are beneficial to bacterial strains to adapt to the intestinal environment and promote health effects (Liu et al., 2015; de Melo Pereira et al., 2018). In previous studies, $F_0F_1$-ATPase was the main regulator of intracellular pH and could improve the resistance of *Lactobacillus* strains at low pH conditions (Angmo et al., 2016; Wu et al., 2022). The 4 selected strains had seven genes encoding for the $F_0F_1$ ATP synthase subunit, including *atpC* (K02114), *atpD* (K02112), *atpG* (K02115), *atpA* (K02111), *atpH* (K02113), *atpF* (K02109), and *atpB* (K02108). In addition, the *ppaC* (K15986) gene encoding for inorganic pyrophosphatase plays an important role in keeping the membrane intact and tolerance to bile salt (Zhang et al., 2021), which was also identified in 4 selected strains.

The utilization of probiotics for lowering cholesterol levels is becoming increasingly popular, and several related gene clusters have also been identified in these stains. A gene cluster *ssuACB* related to ABC-typetransporter for aliphatic sulfonates has been identified in the genome of *L. plantarum* ST-III. These aliphatic sulfonates could be used as sulfur sources for strains and increase the cholesterol removal capability of LAB (Wang et al., 2011). B652 strain contained a complete *ssuACB* gene cluster, while C232, D444, and E932 strains only had partial genes of this cluster. These results indicated that the B652 strain might be a good probiotic candidate for lowering cholesterol levels.

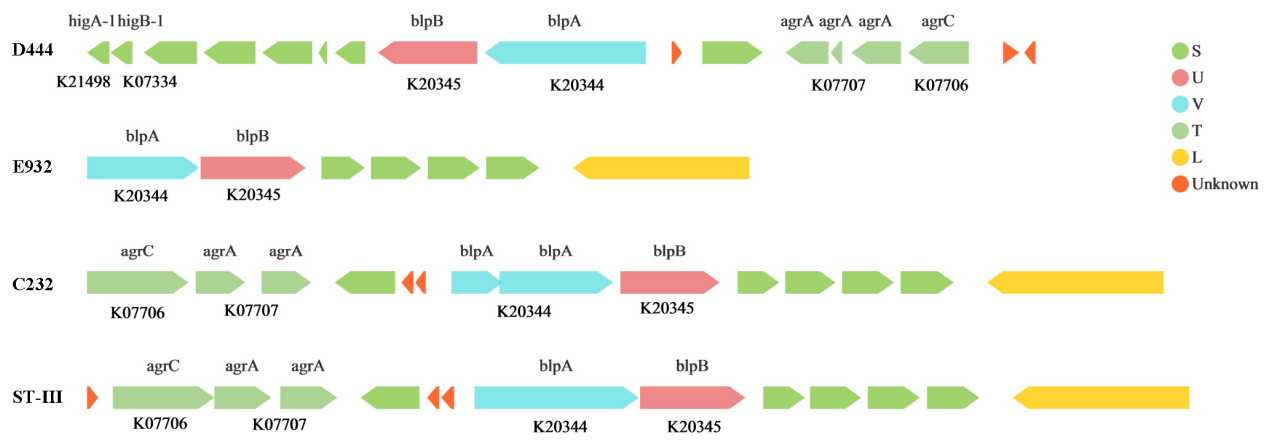


**FIGURE 5 |** Bacteriocin synthesis gene clusters The arrow represents a gene, above the arrow is the name of the gene, and below the arrow is the Ko number of the gene. The length of the arrow represents the length of the gene. The direction indicates whether the gene is encoded by the justice or antisense chain. The different color represents its function in the COG classification.

Moreover, BSH plays an important role in the resistance of *L. plantarum* against bile salt (Hamon et al., 2011). The 4 strains contained genes related to bile stress like *cbh* (K01442) encoding for choloylglycine hydrolase. Additionally, it was previously reported that *bsh* genes were closely related to the formation of precipitate circles observed in the study for the BSH activity assay (Wang G. et al., 2021). The 4 predicted bile salt hydrolase (*bsh*) genes have been identified in *L. plantarum* ST-III. However, gene annotation results indicated that none of the 4 selected strains contained any *bsh* genes. The relationship between the *bsh* gene and BSH activity should be further identified.

The expression of genes encoding for antibacterial peptides and bacteriocin could confer antibacterial activity to LAB strains. Peptides including PlnJ/K, PlnE/F, and PlnA, classified as Class II LAB bacteriocin, were detected in the genomes of the strains. Such peptides can inhibit the growth of both Gram-positive and Gram-negative bacteria (Li et al., 2016b). In addition, D444, E932 and C232 strains contained bacteriocin synthesis gene clusters (**Figure 5**) and demonstrated antibacterial activities according to the *in vitro* experiments. The bacteriocin synthesis gene cluster of the C232 strain was similar to that of *L. plantarum* ST-III. It was reported that *L. plantarum* ST-III could reduce *salmonella* infection in mice (Liu et al., 2019). Therefore, the C232 strain might be a potential probiotic against *salmonella* infection in the body.

## CONCLUSION

The potential probiotic properties of 44 *L. plantarum* obtained from Chinese traditional fermented foods were evaluated. All the tested strains showed high antioxidant capacity, BSH activity, and antibacterial activity, which might be the common characteristics of *L. plantarum*. The negative result of the hemolytic activity for all strains indicated the safety of the strains to be used in food fermentation. Among the strains, B652, C232, D444, and E932 were identified as the best comprehensive performed strains, which were selected for the whole genome sequencing. Except for B652, the selected strains had high similarity in the genome compared with the certificated probiotic strain ST-III. In addition, several identified gene clusters (i.e., genes related to the synthesis of thioredoxin, cell-surface proteins encoding genes, and $F_0F_1$ ATP synthase subunit) were found to be aligned with strains' probiotic properties, such as antioxidant activity, adhesion activity, and survival ability in artificial gastric environments. However, different performances of the probiotic capacities among the selected strains were figured out, which might be due to differences in gene expression and regulation. In general, the four selected *L. plantarum* strains showed the potential to be probiotic candidates that could be applied to fermented food developments. In addition, the outcome of the study could be helpful in linking the probiotic phenotype and genomic characteristics of potential strains, thereby providing knowledge and references to discover and evaluate probiotic candidates.

## DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI repository, accession numbers SUB11199305, SUB11199286, SUB11199241, and SUB11199182.

## AUTHOR CONTRIBUTIONS

YS: visualization, methodology, data curation, and writing—original draft. SZ and HL: methodology and investigation. JZ: data curation. ZL: methodology and writing—review and editing. XH: supervision. JY: conceptualization, supervision, and writing—review and editing. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.895132/full#supplementary-material

## REFERENCES

Angmo, K., Kumari, A., Savitri, and Bhalla, T. C. (2016). Probiotic characterization of lactic acid bacteria isolated from fermented foods and beverage of Ladakh. *LWT Food Sci. Technol.* 66, 428–435. doi: 10.1016/j.lwt.2015.10.057

Bintsis, T. (2018). Lactic acid bacteria as starter cultures: an update in their metabolism and genetics. *AIMS Microbiol.* 4, 665–684. doi: 10.3934/microbiol.2018.4.665

Castellone, V., Bancalari, E., Rubert, J., Gatti, M., Neviani, E., and Bottari, B. (2021). Eating fermented: health benefits of lab-fermented foods. *Foods* 10, 1–22. doi: 10.3390/foods10112639

de Melo Pereira, G. V., de Oliveira Coelho, B., Magalhães Júnior, A. I., Thomaz-Soccol, V., and Soccol, C. R. (2018). How to select a probiotic? A review and update of methods and criteria. *Biotechnol. Adv.* 36, 2060–2076. doi: 10.1016/j.biotechadv.2018.09.003

Divyashree, S., Anjali, P. G., Somashekaraiah, R., and Sreenivasa, M. Y. (2021). Probiotic properties of *Lactobacillus casei* – MYSRD 108 and *Lactobacillus plantarum* – MYSRD 71 with potential antimicrobial activity against *Salmonella* paratyphi. *Biotechnol. Rep.* 32:e00672. doi: 10.1016/j.btre.2021.e00672

Fei, Y., Li, L., Zheng, Y., Liu, D., Zhou, Q., and Fu, L. (2018). Characterization of *Lactobacillus amylolyticus* L6 as potential probiotics based on genome sequence

and corresponding phenotypes. *LWT Food Sci. Technol.* 90, 460–468. doi: 10.1016/j.lwt.2017.12.028

Hamon, E., Horvatovich, P., Izquierdo, E., Bringel, F., Marchioni, E., Aoudé-Werner, D., et al. (2011). Comparative proteomic analysis of *Lactobacillus plantarum* for the identification of key proteins in bile tolerance. *BMC Microbiol.* 11:63. doi: 10.1186/1471-2180-11-63

Huang, Y., Liu, D., Jia, X., Liang, M., Lu, Y., and Liu, J. (2021). Whole genome sequencing of *Lactobacillus plantarum* DMDL 9010 and its effect on growth phenotype under nitrite stress. *LWT Food Sci. Technol.* 149:111778. doi: 10.1016/j.lwt.2021.111778

Jia, F., Zhang, L., Pang, X., Gu, X., Abdelazez, A., Liang, Y., et al. (2017). Complete genome sequence of bacteriocin-producing *Lactobacillus plantarum* KLDS1.0391, a probiotic strain with gastrointestinal tract resistance and adhesion to the intestinal epithelial cells. *Genomics* 109, 432–437. doi: 10.1016/j.ygeno.2017.06.008

Koirala, S., and Anal, A. K. (2021). Probiotics-based foods and beverages as future foods and their overall safety and regulatory claims. *Future Foods* 3:100013. doi: 10.1016/j.fufo.2021.100013

Lee, S., and Kim, M. (2019). *Leuconostoc mesenteroides* MKSR isolated from kimchi possesses α-glucosidase inhibitory activity, antioxidant activity, and cholesterol-lowering effects. *LWT Food Sci. Technol.* 116:108570. doi: 10.1016/j.lwt.2019.108570

Li, P., Gu, Q., and Zhou, Q. (2016a). Complete genome sequence of *Lactobacillus plantarum* LZ206, a potential probiotic strain with antimicrobial activity against food-borne pathogenic microorganisms. *J. Biotechnol.* 238, 52–55. doi: 10.1016/j.jbiotec.2016.09.012

Li, P., Li, X., Gu, Q., Lou, X., Zhang, X., Song, D., et al. (2016b). Comparative genomic analysis of *Lactobacillus plantarum* ZJ316 reveals its genetic adaptation and potential probiotic profiles. *J. Zhejiang Univ. Sci. B* 17, 569–579. doi: 10.1631/jzus.B1600176

Lin, X., Xia, Y., Yang, Y., Wang, G., Zhou, W., and Ai, L. (2020). Probiotic characteristics of *Lactobacillus plantarum* AR113 and its molecular mechanism of antioxidant. *LWT Food Sci. Technol.* 126:109278. doi: 10.1016/j.lwt.2020.109278

Liu, C., Wang, R., Gong, F., Liu, X., Zheng, H., Luo, Y., et al. (2015). Complete genome sequences and comparative genome analysis of *Lactobacillus plantarum* strain 5-2 isolated from fermented soybean. *Genomics* 106, 404–411. doi: 10.1016/j.ygeno.2015.07.007

Liu, J., Gu, Z., Zhang, H., Zhao, J., and Chen, W. (2019). Preventive effects of *Lactobacillus plantarum* ST-III against *Salmonella* infection. *LWT Food Sci. Technol.* 105, 200–205. doi: 10.1016/j.lwt.2019.02.043

Marco, M. L., Sanders, M. E., Gänzle, M., Arrieta, M. C., Cotter, P. D., De Vuyst, L., et al. (2021). The international scientific association for probiotics and prebiotics (ISAPP) consensus statement on fermented foods. *Nat. Rev. Gastroenterol. Hepatol.* 18, 196–208. doi: 10.1038/s41575-020-00390-5

Meng, L., Zhu, X., Tuo, Y., Zhang, H., Li, Y., Xu, C., et al. (2021). Reducing antigenicity of β-lactoglobulin, probiotic properties and safety evaluation of *Lactobacillus plantarum* AHQ-14 and *Lactobacillus bulgaricus* BD0390. *Food Biosci.* 42:101137. doi: 10.1016/j.fbio.2021.101137

Pan, Y., Wang, H., Tan, F., Yi, R., Li, W., Long, X., et al. (2020). *Lactobacillus plantarum* KFY02 enhances the prevention of CCl4-induced liver injury by transforming geniposide into genipin to increase the antioxidant capacity of mice. *J. Funct. Foods* 73:104128. doi: 10.1016/j.jff.2020.104128

Park, S., Ji, Y., Park, H., Lee, K., Park, H., Beck, B. R., et al. (2016). Evaluation of functional properties of *Lactobacilli* isolated from Korean white kimchi. *Food Control* 69, 5–12. doi: 10.1016/j.foodcont.2016.04.037

Pereira, D. I. A., McCartney, A. L., and Gibson, G. R. (2003). An in vitro study of the probiotic potential of a bile-salt-hydrolyzing *Lactobacillus fermentum* strain, and determination of its cholesterol-lowering properties. *Appl. Environ. Microbiol.* 69, 4743–4752. doi: 10.1128/AEM.69.8.4743-4752.2003

Rocha, C., and Danchin, A. (2002). Base composition bias might result from competition for metabolic resources. *Trends Genet.* 18, 291–294. doi: 10.1016/S0168-9525(02)02690-2

Shang, Z., Ye, Z., Li, M., Ren, H., Cai, S., Hu, X., et al. (2022). Dynamics of microbial communities, flavor, and physicochemical properties of pickled chayote during an industrial-scale natural fermentation: correlation between microorganisms and metabolites. *Food Chem.* 377:132004. doi: 10.1016/j.foodchem.2021.132004

Shangpliang, H. N. J., Sharma, S., Rai, R., and Tamang, J. P. (2017). Some technological properties of lactic acid bacteria isolated from Dahi and Datshi, naturally fermented milk products of Bhutan. *Front. Microbiol.* 8:116. doi: 10.3389/fmicb.2017.00116

Sharma, S., Kandasamy, S., Kavitake, D., and Shetty, P. H. (2018). Probiotic characterization and antioxidant properties of *Weissella confusa* KR780676, isolated from an Indian fermented food. *LWT Food Sci. Technol.* 97, 53–60. doi: 10.1016/j.lwt.2018.06.033

Sikorska, H., and Smoragiewicz, W. (2013). Role of probiotics in the prevention and treatment of meticillin-resistant Staphylococcus aureus infections. *Int. J. Antimicrob. Agents* 42, 475–481. doi: 10.1016/j.ijantimicag.2013.08.003

Son, S. H., Jeon, H. L., Jeon, E. B., Lee, N. K., Park, Y. S., Kang, D. K., et al. (2017). Potential probiotic Lactobacillus plantarum Ln4 from kimchi: evaluation of β-galactosidase and antioxidant activities. *LWT Food Sci. Technol.* 85, 181–186. doi: 10.1016/j.lwt.2017.07.018

Soto-Giron, M. J., Kim, J.-N., Schott, E., Tahmin, C., Ishoey, T., Mincer, T. J., et al. (2021). The edible plant microbiome represents a diverse genetic reservoir with functional potential in the human host. *Sci. Rep.* 11, 1–14. doi: 10.1038/s41598-021-03334-4

Tang, S., Cheng, Y., Wu, T., Hu, F., Pan, S., and Xu, X. (2021). Effect of *Lactobacillus plantarum*-fermented mulberry pomace on antioxidant properties and fecal microbial community. *LWT Food Sci. Technol.* 147:111651. doi: 10.1016/j.lwt.2021.111651

Wang, G., Yu, H., Feng, X., Tang, H., Xiong, Z., Xia, Y., et al. (2021). Specific bile salt hydrolase genes in *Lactobacillus plantarum* AR113 and relationship with bile salt resistance. *LWT Food Sci. Technol.* 145:111208. doi: 10.1016/j.lwt.2021.111208

Wang, L., Zhang, H., Rehman, M. U., Mehmood, K., Jiang, X., Iqbal, M., et al. (2018). Antibacterial activity of *Lactobacillus plantarum* isolated from Tibetan yaks. *Microb. Pathogen.* 115, 293–298. doi: 10.1016/j.micpath.2017.12.077

Wang, Y., Chen, C., Ai, L., Zhou, F., Zhou, Z., Wang, L., et al. (2011). Complete genome sequence of the probiotic *Lactobacillus plantarum* ST-III. *J. Bacteriol.* 193, 313–314. doi: 10.1128/JB.01159-10

Wang, Y., Liang, Q., Lu, B., Shen, H., Liu, S., Shi, Y., et al. (2021). Whole-genome analysis of probiotic product isolates reveals the presence of genes related to antimicrobial resistance, virulence factors, and toxic metabolites, posing potential health risks. *BMC Genom.* 22:210. doi: 10.1186/s12864-021-07539-9

Wu, Y., Liu, D., Zhao, S., Huang, Y., Yu, J., and Zhou, Q. (2022). Assessing the safety and probiotic characteristics of *Bacillus coagulans* 13002 based on complete genome and phenotype analysis. *LWT Food Sci. Technol.* 155:112847. doi: 10.1016/j.lwt.2021.112847

Xiang, H., Sun-Waterhouse, D., Waterhouse, G. I. N., Cui, C., and Ruan, Z. (2019). Fermentation-enabled wellness foods: a fresh perspective. *Food Sci. Human Wellness* 8, 203–243. doi: 10.1016/j.fshw.2019.08.003

Ye, Z., Shang, Z., Zhang, S., Li, M., Zhang, X., and Ren, H. (2022). Dynamic analysis of flavor properties and microbial communities in Chinese pickled chili pepper (*Capsicum frutescens* L.): a typical industrial-scale natural fermentation process. *Food Res. Int.* 153:110952. doi: 10.1016/j.foodres.2022.110952

Zhang, L., Ma, H., Kulyar, M. F. e. A, Pan, H., Li, K., Li, A., et al. (2021). Complete genome analysis of *Lactobacillus fermentum* YLF016 and its probiotic characteristics. *Microb. Pathogen.* 162:105212. doi: 10.1016/j.micpath.2021.105212

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# Exploring the Dynamic of Bacterial Communities in Manila Clam (*Ruditapes philippinarum*) During Refrigerated Storage

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Microorganism contamination is one of the most important factors affecting the spoilage and food safety of Manila clams. This study aimed to gain insights into bacterial composition and the dynamic change of bacterial communities on retailed Manila clam during refrigerated storage within the edible period. High-throughput sequencing was conducted to monitor the bacterial population with the prolongation of storage time of Day 0, Day 1, and Day 3. Result demonstrated that phyla of Proteobacteria,Actinobacteriota, Acidobacteriota, and Chloroflexi composed the majority of bacterial communities during the whole observation process. Furthermore, the increase of Proteobacteria showed a positive correlation with the storage time, whereas Acidobacteriota and Chloroflexi continued to decline in storage. For genus annotation, none of genus obtained dominant population in storage. From Day 0 to Day 1, the genera of *Streptomyces*, *Bradyrhizobium*, and *Mycobacterium* significantly increased; meanwhile, 12 genera significantly decreased. Compared with samples at Day 0, a total of 15 genera significantly decreased with the reduced proportion ranging from 0.50 to 4.40% at Day 3. At the end of the storage, the genus *Crossiella* became the most redundant population. Both the richness and diversity decreased at the start of storage at Day 1, and then slightly increased at Day 3 was observed. Based on the result in this study, strategy targeting the increased bacteria could be tested to improve the consumption quality and safety of refrigerated clam.

**Keywords: Manila clam, microbiota, bacterial diversity, high-throughput sequencing, refrigerated storage**

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## INTRODUCTION

The Manila clam, *Ruditapes philippinarum*, is one of the important and most exploited marine bivalves around the world; it is nutritious containing different kinds of proteins, vitamins, and essential elements (Zhao and Zhang, 2016). Due to the great ability on adapting environment, Manila clam reproduced rapidly and distributed widely. Nowadays, Manila clam has become the world's second most important commercially cultured bivalve mollusk (Tan et al., 2020). China is the largest producer of Manila clam, about 3 million tons of clams were produced each year, which is almost 90% of the world's production (Nie et al., 2016). Nevertheless, just like the most shellfish, Manila clam always suffer from the contamination of diverse microorganisms, due to the abundant

water and nutrient contents (Chen et al., 2019). The proliferation of microorganisms may lead to the short shelf-life and unacceptable qualities of clam (Boziaris and Parlapani, 2017). During the transportation and storage, low-temperature storage is the most common approach to preserve the fresh clam. Thus, in order to improve the consumption quality and safety of refrigerated clams, it is essential to investigate the dynamics of contaminating bacterial communities in clam during refrigerated storage.

Among the approaches in microorganism identification, two types of methods including culture-dependent microbial cultivation and culture-independent high-throughput sequencing method have been applied. For culture-based methods, since only 0.1–3% of the bacteria could be cultivated (Amann et al., 1995; Cocolin et al., 2013), the experiment data obtained from cultivating bacteria are insufficient to clarify the microbiota of food (Caporaso et al., 2012). With the development of high-throughput sequencing technology, molecular methods, which based on sequence characterization, could provide a comprehensive description of the whole microbiota. The variable region on 16S rRNA gene was used as the indicator for taxonomy identification. Polymerase chain reaction (PCR) cloning, combined with 16S rRNA gene sequencing could provide high-throughput bacterial identification with high efficacy and low cost (Klindworth et al., 2013), and the technology was applied widely on evaluating the bacterial composition of seafood, during the storage period (La Valley et al., 2009; Trabal et al., 2012; Chen et al., 2016, 2019), with different processing technology (Cao et al., 2018) and with different package (Pan et al., 2018). For example, the microbiome of the fresh clam from different geographic origin was identified using 16S rRNA gene sequencing, and the abundance of Proteobacteria with Acidobacteria could be used as a marker in distinguishing different geographic origin of fresh clam (Liu et al., 2020). Through 16S rRNA gene sequencing, genera *Psychrobacter* and *Psychromonas* were identified as the most important spoilage organisms of oysters during refrigerated storage (Chen et al., 2019). The 16S rRNA gene amplicon sequencing of bacteria on mussel revealed that Proteobacteria, Cyanobacteria, and Firmicutes were the three major phyla in modified atmosphere packed mussel (Odeyemi et al., 2019). Apart from detecting the microbial diversity in seafood, high-throughput sequencing has a wide application in several food products, revealing the bacterial or fungal composition. For example, the bacterial community in zha-chili, fermented by different rice varieties, was detected using high-throughput sequencing (Cai et al., 2021a). It was found that rice variety would lead to different microbiota in fermented zha-chili sample, in which lactic acid bacteria obtained the most dominant abundance of 77.09% (Cai et al., 2021a). The microorganism structure, including bacterial richness and fungal diversity, in low-temperature Daqu for Baijiu-making was also been explored (Cai et al., 2021b). The core microbiota influencing the flavor of Daqu were dominated by *Thermoactinomyces*, *Lactobacillus*, *Saccharopolyspora*, along with *Bacillus*, *Streptomyces*, *Saccharomycopsis*, and *Thermoascus* (Cai et al., 2021b). Pit mud is the important carrier for various microorganism in the production of Chinese Baijiu (Cai et al., 2022). Through Illumina MiSeq sequencing, the fungal community was analyzed, and diverse high-abundance fungi in pit mud at different depths was revealed, which could be useful in improving the quality of pit mud (Cai et al., 2022). All of the above studies illustrated that 16S rRNA gene sequencing could provide a rapid illustration of whole bacterial communities. The identified bacterial profile could provide a theoretical basis on improving food safety and maintain food quality. Nevertheless, few studies have been reported to identify the bacterial change in refrigerated clam, especially during edible shelf-life storage.

In this study, the V3–V4 regions of bacterial 16S rRNA gene on retailed Manila clam were sequenced to monitor the dynamic change of microorganism composition during refrigerated storage. The bacterial abundance at different taxonomy annotation level was investigated, and the change of bacterial diversity was identified. The result in this study could provide a hint on risk assessment for clams during process and storage.

# MATERIALS AND METHODS

## Raw Material Acquisition and Sample Preparation

Philippine clams originated from Dandong City, Liaoning Province, refrigerated and transported from terminal to the Shanghai distributor, and delivered to the school laboratory *via* the online purchase platform of Dingdong in 0.5–1 h, during which the clams were transported in low-temperature brine to ensure quality. The clams were cleaned in the laboratory with sterilized water for surface debris such as mud, sand, and dirt, after which they were drained and stored in the refrigerator at $4 \pm 1°C$ sealed with food wrap. Based on previous microbial early experiments and sensory analysis, the clams with similar size at Day 0, Day 1, and Day 3 were divided into three groups (D 0, D 1, and D 3). Each group is divided into three parallel samples (D 0_1, D 0_2, and D 0_3), (D 1_1, D 1_2, and D 1_3), and (D 3_1, D 3_2, and D 3_3), respectively, and the shell meat was removed aseptically in a sterile ultra-clean table and cut with a sterilized knife to ensure the samples were evenly placed into 10-ml centrifuge tubes and frozen at $-80°C$.

## DNA Extraction, Amplification, and Sequencing

The total genomic DNA of microbial colonies from clams was extracted by E.Z.N.A. Soil DNA Kit (Omega Bio-tek, Norcross, GA, United States). The 1% agarose gel electrophoresis was used to detect DNA samples. The purity of DNA was assessed by NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE, United States).

The V3–V4 regions of bacterial 16S rRNA genes in clams were amplified by PCR with a primer pair of 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The amplification

**TABLE 1 |** Effective sequence numbers, OTUs, and Good's coverage of clam meat during refrigerated storage at 0, 1, and 3 days.

| Sample group | Sample name | Reads | OTU | Good's coverage (%) |
|---|---|---|---|---|
| Day 0 | D 0_1 | 30,341 | 2,060 | 98.18 |
| | D 0_2 | 30,433 | 2,021 | 98.28 |
| | D 0_3 | 28,130 | 2,098 | 98.08 |
| Day 1 | D 1_1 | 44,576 | 519 | 99.46 |
| | D 1_2 | 31,422 | 2,299 | 97.89 |
| | D 1_3 | 30,347 | 2,225 | 98.02 |
| Day 3 | D 3_1 | 33,282 | 2,246 | 97.75 |
| | D 3_2 | 28,498 | 1,541 | 98.89 |
| | D 3_3 | 49,646 | 1,300 | 99.04 |

procedure included an initial denaturation step (95°C for 3 min) followed by 27 cycle reactions composing denaturation (95°C for 30 s), annealing (55°C for 30 s), extension (72°C for 30 s), and continuous extension at 72°C for 10 min. PCR products were extracted from 2% agarose gel electrophoresis and purified using AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States).

A DNA library was constructed using NEXTflex Rapid DNA-Seq Kit (Bioo Scientific, United States) according to the following steps: (1) adding adapter sequences; (2) removing the fragments connected by different adapters; (3) enrichment of library templates using PCR amplification; and (4) collecting PCR products.

Purified amplicons were pooled in equimolar, and paired-end sequencing was conducted on an Illumina MiSeq PE300 Platform (Illumina, San Diego, CA, United States) according to the standard protocols by Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

## Processing of Sequence Data

At first, the raw sequencing reads were filtered by fastp version 0.20.0 (Chen et al., 2018). Using a sliding window of 50 base pair (bp), the 300-bp reads for which the average quality score were less than 20 were truncated. After truncating, sequences longer than 50 bp were remained. Sequencing reads which included ambiguous characters were also abandoned.

Second, filtered sequences with overlapping fragments longer than 10 bp were merged by FLASH version 1.2.7 (Magoc and Salzberg, 2011). In overlapping fragment, the maximum proportion of mismatch ratio was set as 0.2. Reads that could not be assembled were also discarded.

The barcodes and primers added in library construction were used to distinguish different samples, with the following criteria: (1) exact barcode matching and (2) less than 2 mismatches in primer matching.

Operational taxonomic units (OTUs) were clustered by UPARSE version 7.1 (Edgar, 2013) to represent sequences with similarity greater than or equal to 97% (Stackebrandt and Goebel, 1994; Edgar, 2013). The taxonomy assignment of representative sequences for each OTU was annotated by RDP Classifier version 2.2 (Wang et al., 2007) at different levels, using confidence threshold of 0.7.

## Statistical Analysis

To test sequence quantity, Shannon rarefaction curves (Caporaso et al., 2010) were applied to test whether the amount of sequenced reads were enough to reflect the bacterial community in each sample. Different indices, including Sobs, Shannon, Simpson, Ace, and Chao1 (Amato et al., 2013), were calculated to estimate the diversity of microbial communities in each sample. Index of Good's coverage was calculated to evaluate sequencing depth. In order to detect OTU distribution, a Venn diagram was used to reflect the overlapped and unique species in samples at different storage time (Li et al., 2017). Based on the bacterial composition in each sample, hieratical clustering analysis were conducted to test the bacterial similarities. Principal component analysis (PCA) was used to select the discriminated composition variables. Between two different sample groups, bacterial genus with significantly different ($p < 0.05$) proportion were selected by Student's *t*-test (Al Ashhab et al., 2014). To detect the change of bacterial community abundance among multiple groups, linear discriminant analysis effect size (LEfSe) analysis was used to screen the differentially distributed features. At first, the non-parametric factorial Kruskal–Wallis (KW) test rank-sum test was used to screen genera with significant abundance difference. And then linear discriminant analysis (LDA) score of each bacterial genus was calculated as the quantitative estimator of effects on sample grouping; the cutoff of LDA was set as 2 (Segata et al., 2011).

# RESULTS

## Microbial Communities Detected by 16S rRNA Gene Sequencing

To describe the character of bacterial community of clams at different refrigerated storage time, V3–V4 regions on 16S rRNA gene were sequenced by MiSeq high-throughput sequencing. After quality filtering, a total number of 306,675 reads were obtained in all samples. The detailed information of sequencing is shown in **Table 1**. Among them, each sample contained 34,075 ± 7,648.08 (mean ± SD) sequences. The sample of D 3_3 contained the maximum sequence number of 49,646. To avoid the bias caused by different sequence numbers in each sample, effective tags were randomly selected to reach the same amount of reads. The sequence number is set as 28,130 in D 0_3 sample, which is the minimum sequence number among all samples. For all samples, the average length of sequenced reads reached 415 bp. Then 4,883 OTUs were clustered in all samples, each cluster represented a group of similar sequences, and these OTUs were annotated to 40 phyla, 131 classes, 313 orders, 489 families, 893 genera, and 1,815 species. During refrigerated storage, the average number of OTUs decreased from 2,059.67 at Day 0 samples to 1,681 and 1,695.67 at Day 1 and Day 3 samples, respectively. The change of OTUs illustrated the variance in bacterial composition in different samples. The results of rarefaction analysis showed that with the increase of randomly sampled sequences, Shannon index curves for each sample all resulted in a platform stage (**Figure 1**). The result demonstrated that the sequencing quantity

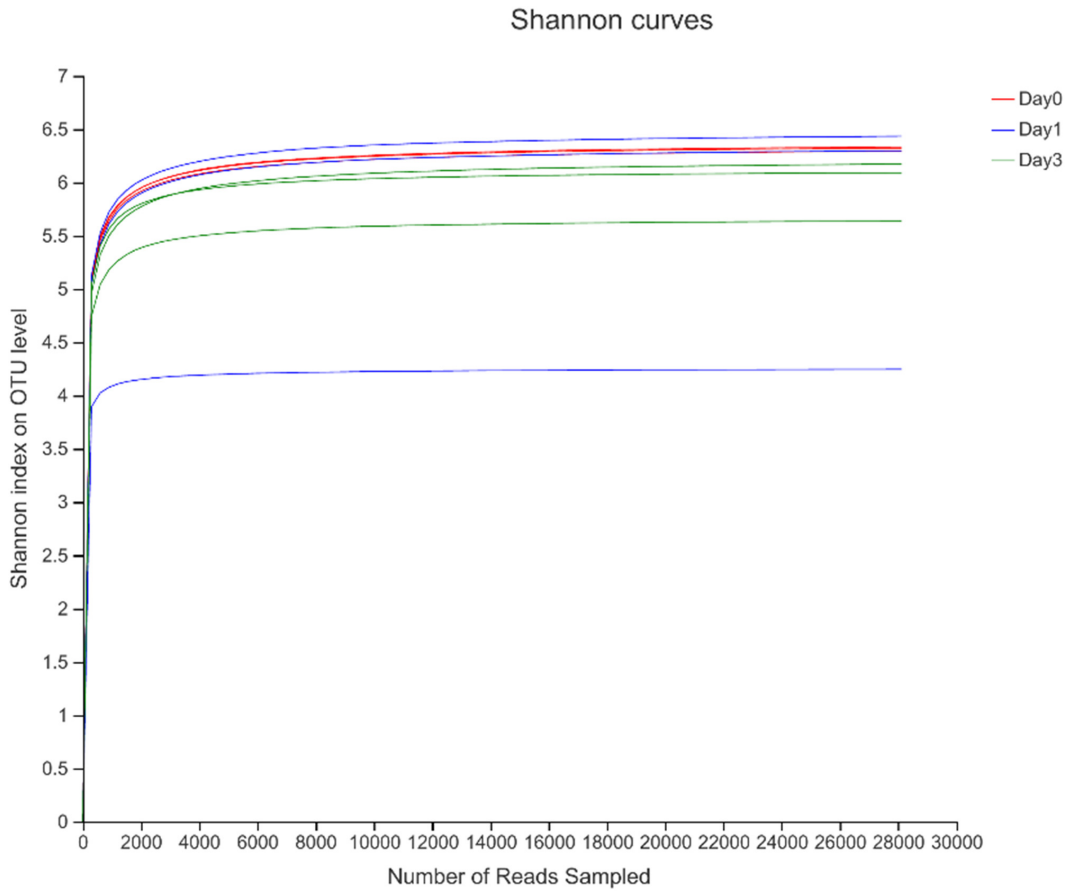


**FIGURE 1 |** Rarefaction analysis of high-throughput sequencing reads in samples at different storage time.

**TABLE 2 |** Alpha diversity analysis at genus level.

| Diversity estimator | Day 0 | Day 1 | Day 3 |
|---|---|---|---|
| Sobs | 513.00 ± 9.64 | 386.67 ± 136.62 | 470.33 ± 78.65 |
| Ace | 568.11 ± 16.49 | 422.03 ± 137.44 | 505.86 ± 74.81 |
| Chao1 | 573.94 ± 28.86 | 430.57 ± 137.20 | 506.26 ± 70.00 |
| Shannon | 4.72 ± 0.04 | 4.52 ± 0.58 | 4.84 ± 0.17 |
| Simpson | 0.018 ± 0.00 | 0.023 ± 0.01 | 0.016 ± 0.00 |

in each sample could well reflect the structure and diversity of bacterial communities. The Good's coverage values calculated in each sample were all higher than 99.68%, which also indicated that the sequence obtained in the current study is sufficient to represent the actual bacteria species.

## Analysis of Bacterial Community Diversity and Richness

To illustrate the diversity of bacterial communities, different estimators were introduced. The index of Sobs, Ace, and Chao1 at OTU level (**Supplementary Table S1**) and genus level (**Table 2**) for each sample was calculated to illustrate the community richness. In **Table 2**, it is found that all samples achieved the highest values for all these three measures at Day 0, indicating that samples at Day 0 obtained the most abundant bacteria. The richness estimator decreased at Day 1 and slightly increased at Day 3. It could also be seen that bacteria at Day 1 contained great variance within the group, which was caused by the data bias at D 1_1 sample. Shannon and Simpson indices were used to indicate the alpha diversity of bacterial communities in clam samples at different refrigeration time; the greater of Shannon index and the smaller Simpson index all referred to the great community diversity. From these two indices, it is demonstrated that during storage, the diversity started to decrease at Day 1 and then increased at Day 3, indicating the communities obtained a more even distribution with less dominant bacteria.

## Bacterial Composition

Further, the Venn diagram was used to reveal the bacterial distribution of clam at different storage time (**Figure 2**). In total, 599, 528, and 776 genera were obtained from different samples at Day 0, Day 1, and Day 3, respectively. Among the annotated genera, there were 417 common genera found in all sample groups during the whole storage process. From Day 0 to Day 1, a set of 96 new bacterial genera were emerged, and with storage time increasing, 196 genera were uniquely found at Day 3. The

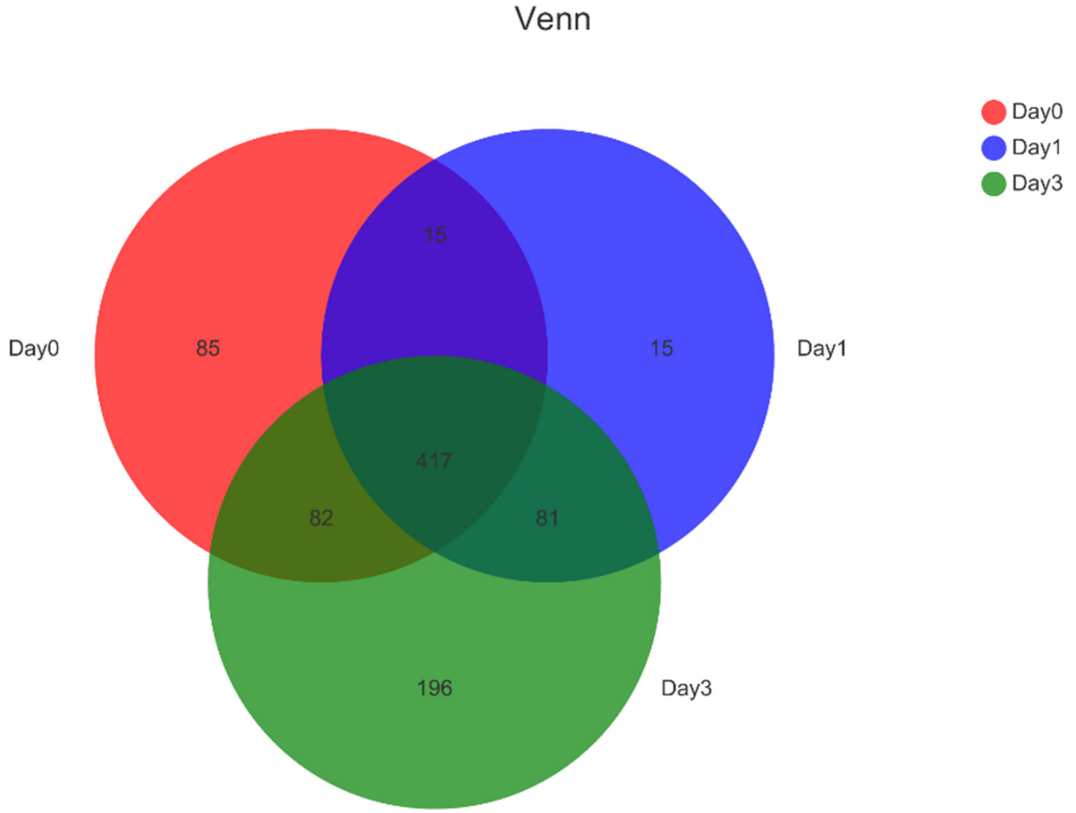


**FIGURE 2 |** Venn diagram for bacterial genera composition in clam at different storage time. Numbers in different region refers to the number of specific or common genera in different sample group. The sum of numbers in each circle refers to the total genera annotated at each storage time.

result indicated that even Day 1 shared an 81.82% (432/528) overlap with Day 0, the bacterial community started to change with increased specific species. The communities continued to vary during storage; in the end, samples at Day 3 obtained 35.70% (277/776) specific genera compared to the initial state of Day 0.

The composition of bacterial communities during storage process was used to analyze the relative abundance at phylum and genus level (**Figure 3**). According to **Figure 3A**, 12 bacterial phyla were found in clam at different storage time. The majority component of phyla were Proteobacteria, Actinobacteriota, Acidobacteriota, and Chloroflexi, composing 77.96, 87.66, and 77.51% of bacterial communities at Day 0, Day 1, and Day 3, respectively. Among them, Proteobacteria accounted for the largest proportion, and the percentage increased during storage, which increased from 27.10% at Day 0 to 31.91% at Day 3, and followed by the phylum of Actinobacteriota, which achieved the abundance of 18.87% at Day 0 and quickly increased to 37.77% at Day 1. On the other hand, Acidobacteriota decreased continuously from 19.27 to 8.81% during storage. Chloroflexi showed a decrease at Day 1 (12.72–8.76%) and maintained a steady abundance afterward. Other phyla were less dominant and included Patescibacteria (0.45–2.05%), Cyanobacteria (0.06–2.48%), and Verrucomicrobiota (0.70–1.43%). Bacteria with a relative abundance less than 1% were labeled as others at phylum and genus levels.

At the genus level (**Figure 3B**), a total number of 39 genera were observed in clam samples. From the relative abundance of bacterial genera, it should be noted that there was not a dominant genus in each sample group, and even the proportion of the most abundant genus was less than 10%. Then, the detected genera abundance changed greatly during storage. The top five most abundant genera at Day 0 group were *Vicinamibacterales* (6.34%), *Pseudolabrys* (3.97%), *Vicinamibacteraceae* (3.87%), *Xanthobacteraceae* (3.20%), and *Gaiella* (3.18%). At Day 1, the community distribution changed to a different set of genera, including *Variovorax* (4.58%), *Crossiella* (3.76%), *Streptomyces* (3.01%), *Bradyrhizobium* (2.74%), *Vicinamibacterales* (2.57%), and so on. Compared to Day 0, the composition rank changed greatly with different genera at Day 1. At Day 3, bacterial distribution continued to change, for example, the top-ranked bacteria included *Crossiella* (2.94%), *Arthrobacter* (2.60%), *Gaiellales* (2.18%), *Azospirillum* (2.17%), and *Bradyrhizobium* (2.04%). It is demonstrated that the previous top-ranked *Vicinamibacterales* (6.34%) at Day 0 proportioned less than 1% at Day 3, whereas the abundance of *Crossiella* continued to increase during storage. The abundance of the most abundant genus of *Variovorax* (4.58%) at Day 1 decreased to less than 1% at Day 3. From Day 0 to Day 3, the bacterial distribution became more even and more variable, with the primary genus continued to decrease.

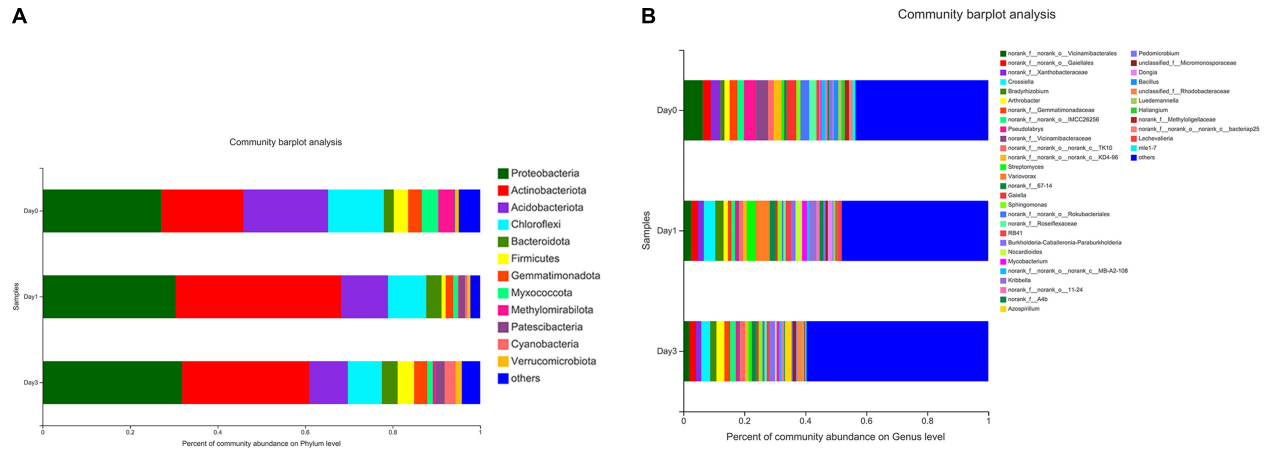


**FIGURE 3 |** Relative abundance of bacterial composition at phylum and genus levels for samples at different storage time. Each color represents specific phylum **(A)** or genus **(B)**, and the length of each band represents the proportion of particular bacteria.

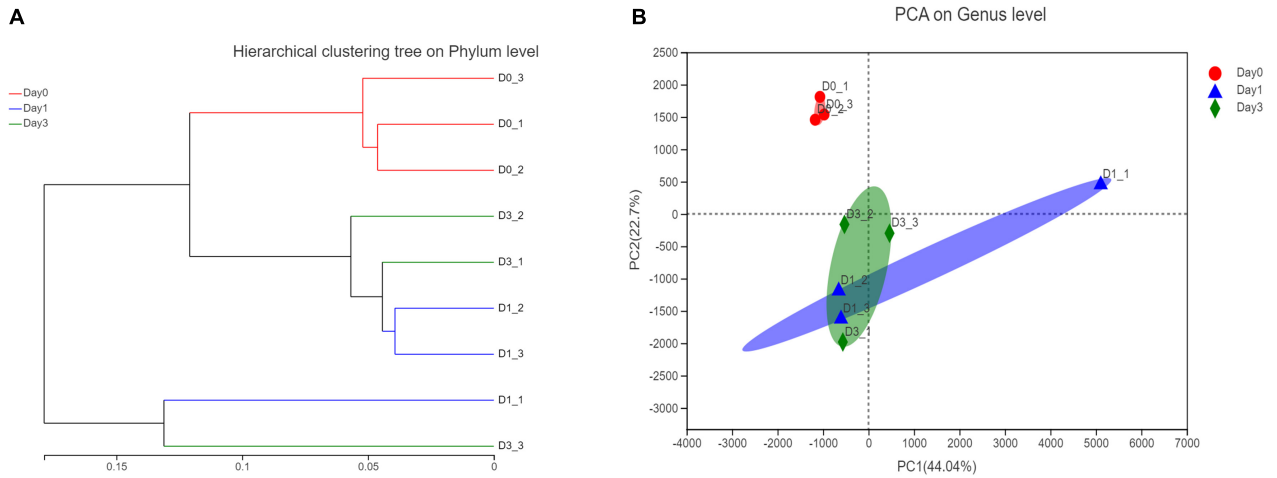


**FIGURE 4 |** Hierarchical clustering and principle component analysis of bacterial composition in different storage time. **(A)** Hierarchical clustering at phylum level. **(B)** PCA analysis at genus level.

Based on the relative abundance of bacterial genera, a hierarchical clustering tree could be constructed to illustrate the relative similar relationship between different samples. It could be found that samples from Day 0 all clustered together, this result showed the similarity within the group. However, samples from Day 1 to Day 3 showed a close relationship in the tree, indicating the similar bacterial community structure (**Figure 4A**). To figure out the principal components contributing on sample classification, PCA was performed. In **Figure 4B**, the cumulative percentage variance for the selected two principle components were 44.04 and 22.7%, composing 66.74% feature contribution. In PCA, samples of Day 0 were separated from samples of Day 1 and samples of Day 3, demonstrating that the bacterial community obtained a significant change, with clam began to spoil since Day 1. For each sample, the deviation of Day 3 from Day 1 illustrated the continuous community variance in the process of storage. Furthermore, the relative location of samples within the same group revealed the bacterial variance in each group, illustrating that the spoiled sample from Day 1 obtained more diverse community composition compared to the closely clustered fresh clam sample at Day 0.

## Microbial Community Changes With Storage Time

To illustrate the specific bacterial change between each sample group, two-tailed Student's *t*-test was used to reveal the significantly changed genera at each storage time (**Figure 5**). Compared with samples detected at Day 0, three genera were significantly increased at Day 1 with *p*-value less than 0.05, including *Bradyrhizobium*, *Streptomyces*, and *Mycobacterium*. And 12 genera were found to get a significant decrease, in which *Pseudolabrys* obtained the biggest reduction of 3.35%. No genus was found to significantly distribute between Day 1 and Day 3.

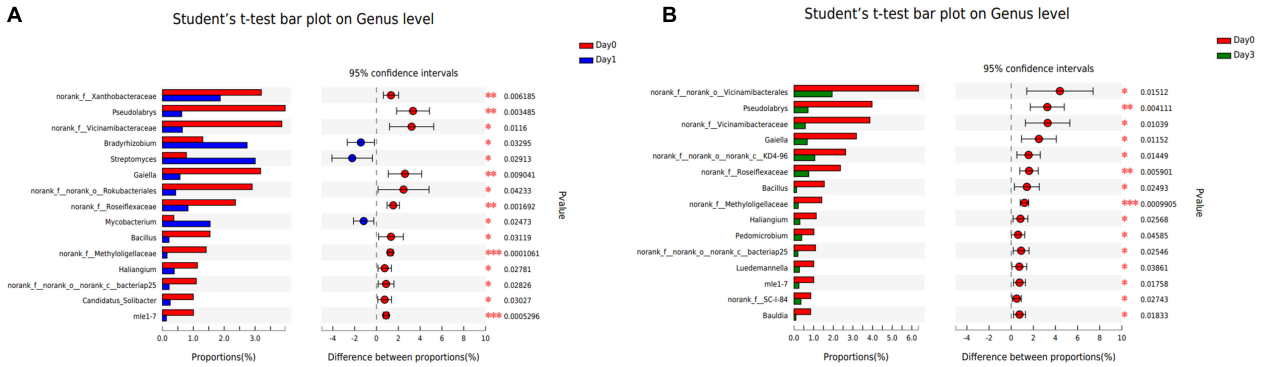


**FIGURE 5 |** Bacterial genera with significant change of relative abundance between different sample groups. **(A)** Bacterial difference between Day 0 and Day 1. **(B)** Bacterial difference between Day 0 and Day 3.

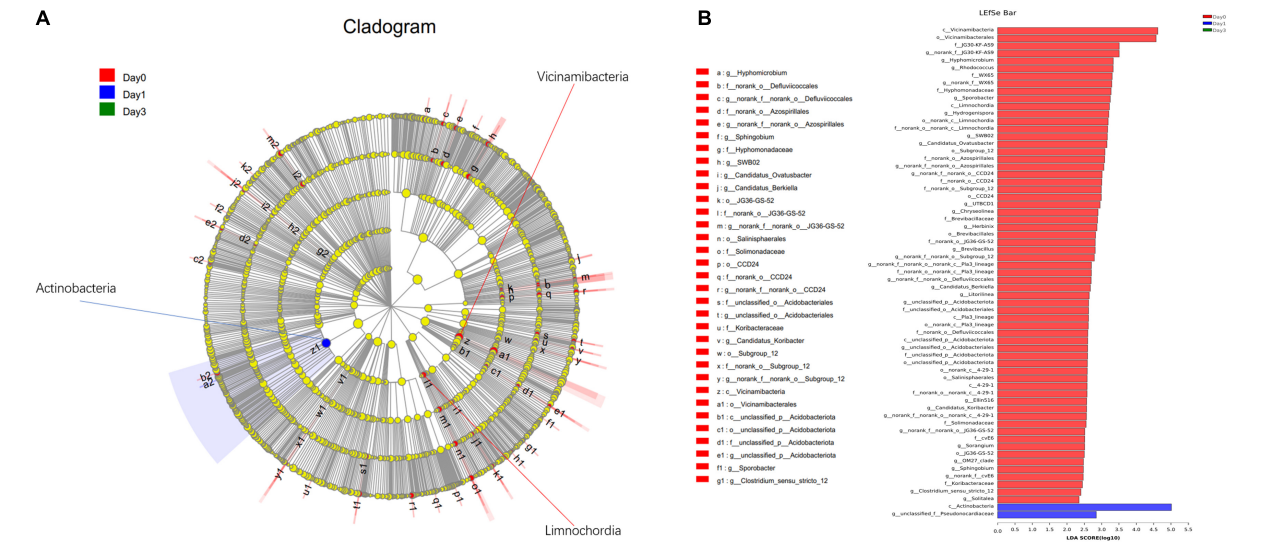


**FIGURE 6 |** Linear discriminant analysis effect size analysis of differentially distributed genera at Day 0, Day 1, and Day 3 groups. **(A)** From the center to the terminal, each layer refers to phylum, class, order, family, and genus level, respectively. Each node refers to the specific bacteria with node size proportion to the relative abundance. **(B)** Specific bacteria with LDA score more than 2 are displayed.

At Day 3, compared with Day 0, the composition proportion of 15 genera decreased with statistical significance ($p < 0.05$). The reduced proportion ranged from 0.50 to 4.40%. There was no significantly increased genus at Day 3, which is consistent with the result that the bacterial communities reached a more diverse distribution, and each genus obtained a small proportion (see section "Bacterial Composition").

In addition to the differentially distributed bacterial communities between two groups, genera for which the relative abundance changed significantly in multiple groups were also detected through LEfSe analysis. In **Figure 6**, the bacterial community with significantly different abundance is observed, which includes *Actinobacteria* class at Day 1, *Vicinamibacteria* class at Day 0, and *Limnochordia* class at Day 0. The results illustrate the significant variance of bacterial composition occurred on the storage from Day 0 to Day 1. LDA score (**Figure 6B**) showed the abundance of *Actinobacteria* and *Parviterribacter* posed a great effect at Day 1 grouping, with LDA score of 5.01 and 2.85, greater than cutoff of 2. At Day 0 classification, 63 bacteria genera, including *Vicinamibacteria* (LDA score: 4.62), *Vicinamibacterales* (LDA score: 4.57), and so on, could contribute on effective distinguish of Day 0 group.

## DISCUSSION

In this study, bacterial community in *R. philippinarum* was evaluated using high-throughput sequencing of V3–V4 regions on 16S rRNA gene of bacteria. The purpose was to observe the essentials in comprehending the bacterial dynamics in *R. philippinarum* during refrigerated storage. Based on the result, Proteobacteria, Actinobacteriota, Acidobacteriota, and Chloroflexi were the prevalent phyla. With the prolonged storage

time, the abundance of Proteobacteria increased steadily and remained at a relative high level, showing a positive correlation with storage time. On the contrary, the phyla of Acidobacteriota and Chloroflexi continued to decline. At the genus level, none of them have a majority species in the population. The abundance of *Vicinamibacterales* and *Pseudolabrys* declined rapidly from Day 1 and continued to decrease in storage. The population of *Crossfield* steadily increased to reach the most redundant genus at Day 3.

Proteobacteria are the largest group of bacteria, and all of the Proteobacteria bacterial communities belong to Gram-negative bacteria. It has been reported that Proteobacteria obtained a high abundance in fresh clam samples harvested from Nova Scotia and Quebec, Canada, and it was a typical spoilage organism for fresh seafood (Liu et al., 2020). Proteobacteria have been detected to dominate the bacterial community in fresh oyster (Madigan et al., 2014; Cao et al., 2018), fresh crisp grass carp (Pan et al., 2018), the gut of grass carp (Shi et al., 2020), frog meat (Yang et al., 2017), mussel meat (Odeyemi et al., 2019), and so on. Furthermore, the processing and storage favored the growth of Proteobacteria in clam (Liu et al., 2020), which is consistent with the observation in this study. Moreover, this phenomenon was also observed in other aquatic meat products, for example, the most dominant phyla of Proteobacteria obtained the high proportion of 77.5% in fresh oysters, with the prolong of refrigerated storage and the process of spoilage, the proportion of Proteobacteria further increased to 88.7% in spoiled oysters (Cao et al., 2018). The relative abundance of Proteobacteria identified from frog meat was 40.29% at Day 0; after storage of 12 days, Proteobacteria become prevalent phyla with abundance of 95.41% (Chen et al., 2021). The similar trend of bacterial abundance in different seafood might illustrate a common spoilage profile, which has been reported that significantly different bacterial composition in different oyster species would end up with similar bacterial profile as oyster spoiled (Madigan et al., 2014). The possible reasons which caused the change of different bacterial community might be related to selective action of different storage conditions and different bacterial activities (Ashie et al., 1996; Naum et al., 2008; Schmitt et al., 2012). Environment factors have been proven to have a crucial role in microbiota, for instance, seasonal variables and exposure to toxicants in environment alerted the composition and metabolic activity of microbiota in fresh Manila clams (Milan et al., 2018), high temperature of seawater might favor the proliferation of opportunistic bacteria in *Mytilus coruscus* (Li et al., 2018), and different sampling year of *Mytilus* also affects the alpha diversity of microbiota (Ramirez et al., 2022). Actinobacteriota was also detected with a great abundantly proportion in clam (Liu et al., 2020), grass carp (Pan et al., 2018; Shi et al., 2020), mussel meat (Odeyemi et al., 2019), and so on. Nevertheless, the abundance rank of Actinobacteriota might be varied in different species (Egerton et al., 2018; Shi et al., 2020) or seafood from different origin (Liu et al., 2020). For Acidobacteriota, the composition proportion of 19.27% at Day 0 sample was observed in this study, and the high abundance of Acidobacteriota in fresh clam was also reported previously (Liu et al., 2020). Even though Acidobacteriota was not the most redundant phylum in bacterial communities, Acidobacteriota often get together with other bacteria to account for a relatively large proportion (Odeyemi et al., 2019; Shi et al., 2020). The occurrences of Chloroflexi in shellfish are rarely reported, and recent studies indicated a decrease of Chloroflexi in seafood at the end of storage time (Odeyemi et al., 2019); in this study, the continuous decline of Chloroflexi from 12.72 to 7.77% during storage was also observed.

Between observed groups at different storage time, several genera with significant change of proportion have been detected, including *Bradyrhizobium*, *Streptomyces*, and *Mycobacterium* between Day 0 and Day 1, *Vicinamibacterales* and *Pseudolabrys* between Day 0 and Day 3, and so on. These significantly distributed genera have also been reported in other aquatic products. For example, *Bradyrhizobium* was occurred in the foot and notum epidermis of nudibranchs (Zhukova et al., 2022). In an investigation of the gut microbial community in olive flounder (*Paralichthys olivaceus*) during different growth stages, *Bradyrhizobium* was composed of the dominant genus at the grower stage of the fish (Niu et al., 2020). It was also been reported that *Bradyrhizobium* obtained the greatest number of sequences in intestinal microbiota from adult pirarucu (*Arapaima gigas*) (Pereira et al., 2017). Among the research of diet-affected changes on gut microbiome of shrimp, *Streptomyces* was one of the most abundant genera on diet-fed group (Prathiviraj et al., 2021). Several genera mentioned in this study could also been served as the probiotics in sustainable aquaculture, for example, *Streptomyces* and *Bacillus* were reported to be good candidates to protect fish and shrimp from pathogens (Tan et al., 2016), or improve feed utilization and disease resistance (Kuebutornye et al., 2019). Besides aquatic products, some genera have also occurred in environment microbiome. For instance, *Vicinamibacterales*, Xanthobacteraceae, and *Pseudolabrys* were all reported to be composed of the soil microbiome in the surface soil (Han et al., 2021); *Panax notoginseng* rhizosphere (Li M. et al., 2020) and *Andrographis paniculata* rhizosphere (Li J. et al., 2020), the abundance of these genera were correlated with the plant yields.

In the clustering and PCA of genus abundance, it is noted that the Day 0 group is separated from Day 1 and Day 3; the clustering result indicated that from Day 0 to Day 1, significant bacterial changes have occurred, followed by the further variation from Day 1 to Day 3. The result is consistent with the statistical result, in which bacterial abundance between Day 0 and other observation time is significantly varied. Meanwhile, no statistical significance can be detected between the genera at Day 1 and Day 3. In the multi-group testing of LEfSe analysis, *Actinobacteria* class had a great impact on grouping of Day 1. Similarly, this class of *Actinobacteria* has also been detected in other seafood, for example, it was reported to occur in the gut microbiota of freshwater fish (Jami et al., 2015) and composed of major bacteria in the gut microbiota of the olive flounder (*P. olivaceus*) (Niu et al., 2020). In the diversity evaluation by high-throughput sequencing data, it could be seen that the indices of Sobs, Ace, and Chao1 illustrate a great variation among the three replications from samples of Day 1. The variation within the group might give a hint on different bacterial community distribution of different tissues or positions from *R. philippinarum*. The position-specific bacterial distribution in aquatic products has been reported,

and through 16S rRNA gene sequencing, it is found that the midgut microbiome obtained higher alpha diversity indices than the foregut and hindgut microbiomes in grass carp (Wang et al., 2021) and large yellow croaker (Zhang et al., 2019). The microbiota variance in different organs and sample positions could be taken into consideration in the further study.

## CONCLUSION

This study provided insights into bacterial composition of Manila clams during refrigerated storage through high-throughput sequencing. The diversity and richness of bacterial communities were evaluated, and the relative abundance of major phyla and genera was calculated based on sequencing result. The detected bacterial change on retailed clam in the process of refrigerated storage could provide theoretical evidence of risk assessment on clam storage. Based on the result, the quality of clam could be evaluated, and different controlling strategies could be tested to prevent the bacterial change.

## DATA AVAILABILITY STATEMENT

The data presented in this study are deposited in NCBI BioProject repository, accession number: PRJNA831322. The repository can be found below: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA831322/.

## AUTHOR CONTRIBUTIONS

YY conducted the experiments. YY and JQ analyzed the data and wrote the manuscript. XW designed the experiments, modified the manuscript, and supervised the whole project. All authors contributed to the manuscript and approved the submitted version.

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## REFERENCES

Al Ashhab, A., Herzberg, M., and Gillor, O. (2014). Biofouling of reverse-osmosis membranes during tertiary wastewater desalination: microbial community composition. *Water Res.* 50, 341–349. doi: 10.1016/j.watres.2013.10.044

Amann, R. I., Ludwig, W., and Schleifer, K. H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* 59, 143–169. doi: 10.1128/mmbr.59.1.143-169.1995

Amato, K. R., Yeoman, C. J., Kent, A., Righini, N., Carbonero, F., Estrada, A., et al. (2013). Habitat degradation impacts black howler monkey (Alouatta pigra) gastrointestinal microbiomes. *ISME J.* 7, 1344–1353. doi: 10.1038/ismej.2013.16

Ashie, I. N., Smith, J. P., and Simpson, B. K. (1996). Spoilage and shelf-life extension of fresh fish and shellfish. *Crit. Rev. Food Sci. Nutr.* 36, 87–121. doi: 10.1080/10408399609527720

Boziaris, I. S., and Parlapani, F. F. (2017). "Specific Spoilage Organisms (SSOs) in Fish," in *The Microbiological Quality of Food*, eds A. Bevilacqua, M. R. Corbo, and M. Sinigaglia (Sawston: Woodhead Publishing), 61–98. doi: 10.3390/foods11030338

Cai, W., Wang, Y., Hou, Q., Zhang, Z., Tang, F., Shan, C., et al. (2021a). Rice varieties affect bacterial diversity, flavor, and metabolites of zha-chili. *Food Res. Int.* 147:110556. doi: 10.1016/j.foodres.2021.110556

Cai, W., Wang, Y., Ni, H., Liu, Z., Liu, J., Zhong, J. A., et al. (2021b). Diversity of microbiota, microbial functions, and flavor in different types of low-temperature Daqu. *Food Res. Int.* 150:110734. doi: 10.1016/j.foodres.2021.110734

Cai, W., Xue, Y. A., Tang, F., Wang, Y., Yang, S., Liu, W., et al. (2022). The Depth-Depended Fungal Diversity and Non-depth-Depended Aroma Profiles of Pit Mud for Strong-Flavor Baijiu. *Front. Microbiol.* 12:789845. doi: 10.3389/fmicb.2021.789845

Cao, R., Zhao, L., Sun, H., and Liu, Q. (2018). Characterization of microbial community in high-pressure treated oysters by high-throughput sequencing technology. *Innovat. Food Sci. Emerg. Technol.* 45, 241–248. doi: 10.1016/j.ifset.2017.11.001

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., et al. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nat. Methods* 7, 335–336. doi: 10.1038/nmeth.f.303

Caporaso, J. G., Lauber, C. L., Walters, W. A., Berg-Lyons, D., Huntley, J., Fierer, N., et al. (2012). Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J.* 6, 1621–1624. doi: 10.1038/ismej.2012.8

Chen, D., Ci, M., Dai, R., Chen, R., and Li, T. (2021). Changes in the Microbial Communities of Tiger Frog (Rana tigrina) Meat during Refrigerated Storage. *J. Food Prot.* 84, 1136–1140. doi: 10.4315/jfp-20-381

Chen, H., Liu, Z., Shi, Y., and Ding, H. H. (2016). Microbiological analysis and microbiota in oyster: a review. *ISJ* 13, 374–388. doi: 10.3389/fimmu.2021.630343

Chen, H., Wang, M., Yang, C., Wan, X., Ding, H. H., Shi, Y., et al. (2019). Bacterial spoilage profiles in the gills of Pacific oysters (Crassostrea gigas) and Eastern oysters (C. virginica) during refrigerated storage. *Food Microbiol.* 82, 209–217. doi: 10.1016/j.fm.2019.02.008

Chen, S. F., Zhou, Y. Q., Chen, Y. R., and Gu, J. (2018). fastp: an ultra-fast all-in-one FASTQ preprocessor. *Bioinformatics* 34, 884–890. doi: 10.1093/bioinformatics/bty560

Cocolin, L., Alessandria, V., Dolci, P., Gorra, R., and Rantsiou, K. (2013). Culture independent methods to assess the diversity and dynamics of microbiota during food fermentation. *Int. J. Food Microbiol.* 167, 29–43. doi: 10.1016/j.ijfoodmicro.2013.05.008

Edgar, R. C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nat. Methods* 10:996. doi: 10.1038/nmeth.2604

Egerton, S., Culloty, S., Whooley, J., Stanton, C., and Ross, R. P. (2018). The Gut Microbiota of Marine Fish. *Front. Microbiol.* 9:873. doi: 10.3389/fmicb.2018.00873

Han, S., Wang, Y., Li, Y., and Shi, K. (2021). Investigation of bacterial diversity in Cajanus cajan-planted gangue soil *via* high-throughput sequencing. *Bioengineered* 12, 6981–6995. doi: 10.1080/21655979.2021.1976043

Jami, M., Ghanbari, M., Kneifel, W., and Domig, K. J. (2015). Phylogenetic diversity and biological activity of culturable Actinobacteria isolated from freshwater fish gut microbiota. *Microbiol. Res.* 175, 6–15. doi: 10.1016/j.micres.2015.01.009

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., et al. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41:e1. doi: 10.1093/nar/gks808

Kuebutornye, F. K. A., Abarike, E. D., and Lu, Y. (2019). A review on the application of Bacillus as probiotics in aquaculture. *Fish Shellfish Immunol.* 87, 820–828. doi: 10.1016/j.fsi.2019.02.010

La Valley, K. J., Jones, S., Gomez-Chiarri, M., Dealteris, J., and Rice, M. (2009). Bacterial community profiling of the eastern oyster (crassostrea virginica): comparison of culture-dependent and culture-independent outcomes. *J. Shellfish Res.* 28, 827–835. doi: 10.2983/035.028.0412

Li, H. F., Li, Z. J., Qu, J. H., and Wang, J. S. (2017). Bacterial diversity in traditional Jiaozi and sourdough revealed by high-throughput sequencing of 16S rRNA amplicons. *LWT Food Sci. Technol.* 81, 319–325. doi: 10.1016/j.lwt.2017.04.007

Li, J., Chen, X., Li, S., Zuo, Z., Zhan, R., and He, R. (2020). Variations of rhizospheric soil microbial communities in response to continuousAndrographis paniculatacropping practices. *Bot. Stud.* 61:18. doi: 10.1186/s40529-020-00295-1

Li, M., Chen, Z., Qian, J., Wei, F., Zhang, G., Wang, Y., et al. (2020). Composition and function of rhizosphere microbiome ofPanax notoginsengwith discrepant yields. *CHIN. Med.* 15:85. doi: 10.1186/s13020-020-00364-4

Li, Y.-F., Yang, N., Liang, X., Yoshida, A., Osatomi, K., Power, D., et al. (2018). Elevated Seawater Temperatures Decrease Microbial Diversity in the Gut of Mytilus coruscus. *Front. Physiol.* 9:839. doi: 10.3389/fphys.2018.00839

Liu, X., Teixeira, J. S., Ner, S., Ma, K. V., Petronella, N., Banerjee, S., et al. (2020). Exploring the Potential of the Microbiome as a Marker of the Geographic Origin of Fresh Seafood. *Front. Microbiol.* 11:696. doi: 10.3389/fmicb.2020.00696

Madigan, T. L., Bott, N. J., Torok, V. A., Percy, N. J., Carragher, J. F., Lopes, M. A. D., et al. (2014). A microbial spoilage profile of half shell Pacific oysters (Crassostrea gigas) and Sydney rock oysters (Saccostrea glomerata). *Food Microbiol.* 38, 219–227. doi: 10.1016/j.fm.2013.09.005

Magoc, T., and Salzberg, S. L. (2011). FLASH: fast length adjustment of short reads to improve genome assemblies. *Bioinformatics* 27, 2957–2963. doi: 10.1093/bioinformatics/btr507

Milan, M., Carraro, L., Fariselli, P., Martino, M. E., Cavalieri, D., Vitali, F., et al. (2018). Microbiota and environmental stress: how pollution affects microbial communities in Manila clams. *Aqua. Toxicol.* 194, 195–207. doi: 10.1016/j.aquatox.2017.11.019

Naum, M., Brown, E. W., and Mason-Gamer, R. J. (2008). Is 16S rDNA a reliable phylogenetic marker to characterize relationships below the family level in the *Enterobacteriaceae*? *J. Mol. Evol.* 66, 630–642. doi: 10.1007/s00239-008-9115-3

Nie, H., Jiang, L., Huo, Z., Liu, L., Yang, F., and Yan, X. (2016). Transcriptomic responses to low temperature stress in the Manila clam. *Ruditapes philippinarum*. *Fish Shellfish Immunol.* 55, 358–366. doi: 10.1016/j.fsi.2016.06.008

Niu, K.-M., Lee, B.-J., Kothari, D., Lee, W.-D., Hur, S.-W., Lim, S.-G., et al. (2020). Dietary effect of low fish meal aquafeed on gut microbiota in olive flounder (Paralichthys olivaceus) at different growth stages. *Microbiologyopen* 9:e992. doi: 10.1002/mbo3.992

Odeyemi, O. A., Burke, C. M., Bolch, C. C. J., and Stanley, R. (2019). Spoilage microbial community profiling by 16S rRNA amplicon sequencing of modified atmosphere packaged live mussels stored at 4 degrees C. *Food Res. Int.* 121, 568–576. doi: 10.1016/j.foodres.2018.12.017

Pan, Z., Li, L., Shen, Z., Chen, Y., and Li, M. (2018). Characterization of the Microbiota in Air- or Vacuum-Packed Crisp Grass Carp (Ctenopharyngodon idella C. et V.) Fillets by 16S rRNA PCR-Denaturing Gradient Gel Electrophoresis and High-Throughput Sequencing. *J. Food Prot.* 81, 1022–1029. doi: 10.4315/0362-028x.Jfp-17-498

Pereira, G. D. V., da Cunha, D. G., Pedreira Mourino, J. L., Rodiles, A., Jaramillo-Torres, A., and Merrifield, D. L. (2017). Characterization of microbiota in Arapaima gigas intestine and isolation of potential probiotic bacteria. *J. Appl. Microbiol.* 123, 1298–1311. doi: 10.1111/jam.13572

Prathiviraj, R., Rajeev, R., Fernandes, H., Rathna, K., Lipton, A. N., Selvin, J., et al. (2021). A gelatinized lipopeptide diet effectively modulates immune response, disease resistance and gut microbiome in Penaeus vannamei challenged with Vibrio parahaemolyticus. *Fish Shellfish Immunol.* 112, 92–107. doi: 10.1016/j.fsi.2021.02.018

Ramirez, C., Soledad Gutierrez, M., Venegas, L., Sapag, C., Araya, C., Caruffo, M., et al. (2022). Microbiota composition and susceptibility to florfenicol and oxytetracycline of bacterial isolates from mussels (Mytilus spp.) reared on different years and distance from salmon farms. *Environ. Res.* 204:112068. doi: 10.1016/j.envres.2021.112068

Schmitt, P., Rosa, R. D., Duperthuy, M., de Lorgeril, J., Bachere, E., and Destoumieux-Garzon, D. (2012). The antimicrobial defense of the Pacific oyster, Crassostrea gigas. How diversity may compensate for scarcity in the regulation of resident/pathogenic microflora. *Front. Microbiol.* 3:160. doi: 10.3389/fmicb.2012.00160

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W. S., et al. (2011). Metagenomic biomarker discovery and explanation. *Genome Biol.* 12:R60. doi: 10.1186/gb-2011-12-6-r60

Shi, F., Zi, Y., Lu, Z., Li, F., Yang, M., Zhan, F., et al. (2020). Bacillus subtilis H2 modulates immune response, fat metabolism and bacterial flora in the gut of grass carp (Ctenopharyngodon idellus). *Fish Shellfish Immunol.* 106, 8–20. doi: 10.1016/j.fsi.2020.06.061

Stackebrandt, E., and Goebel, B. M. (1994). Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.* 44, 846–849.

Tan, L. T.-H., Chan, K.-G., Lee, L.-H., and Goh, B.-H. (2016). Streptomyces Bacteria as Potential Probiotics in Aquaculture. *Front. Microbiol.* 7:79. doi: 10.3389/fmicb.2016.00079

Tan, Y., Fang, L., Qiu, M., Huo, Z., and Yan, X. (2020). Population genetics of the Manila clam (Ruditapes philippinarum) in East Asia. *Sci. Rep.* 10:21890. doi: 10.1038/s41598-020-78923-w

Trabal, N., Mazon-Suastegui, J. M., Vazquez-Juarez, R., Asencio-Valle, F., Morales-Bojorquez, E., and Romero, J. (2012). Molecular Analysis of Bacterial Microbiota Associated with Oysters (Crassostrea gigas and Crassostrea corteziensis) in Different Growth Phases at Two Cultivation Sites. *Microb. Ecol.* 64, 555–569. doi: 10.1007/s00248-012-0039-5

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R. (2007). Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73, 5261–5267. doi: 10.1128/aem.00062-07

Wang, S.-T., Meng, X.-Z., Dai, Y.-F., Zhang, J.-H., Shen, Y., Xu, X.-Y., et al. (2021). Characterization of the intestinal digesta and mucosal microbiome of the grass carp (Ctenopharyngodon idella). *Comp. Biochem. Physiol. Part D Genomics Proteomics* 37:100789. doi: 10.1016/j.cbd.2021.100789

Yang, C., Che, Y., Qi, Y., Liang, P., and Song, C. (2017). High-Throughput Sequencing of Viable Microbial Communities in Raw Pork Subjected to a Fast Cooling Process. *J. Food Sci.* 82, 145–153. doi: 10.1111/1750-3841.13566

Zhang, C., Zheng, X., Ren, X., Li, Y., and Wang, Y. (2019). Bacterial diversity in gut of large yellow croaker Larimichthys crocea and black sea bream Sparus macrocephalus reared in an inshore net pen. *Fish. Sci.* 85, 1027–1036. doi: 10.1007/s12562-019-01349-5

Zhao, H., and Zhang, S. (2016). Identification of Jiaozhou Bay Clams (Ruditapes philippinarum) by Multi-element Fingerprinting Technique. *Food Anal. Methods* 9, 2691–2699. doi: 10.1007/s12161-016-0461-2

Zhukova, N. V., Eliseikina, M. G., Balakirev, E. S., and Ayala, F. J. (2022). Multiple bacterial partners in symbiosis with the nudibranch mollusk Rostanga alisae. *Sci. Rep.* 12:169. doi: 10.1038/s41598-021-03973-7

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# Meta-Analysis for the Global Prevalence of Foodborne Pathogens Exhibiting Antibiotic Resistance and Biofilm Formation

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Antimicrobial-resistant (AMR) foodborne bacteria causing bacterial infections pose a serious threat to human health. In addition, the ability of some of these bacteria to form biofilms increases the threat level as treatment options may become compromised. The extent of antibiotic resistance and biofilm formation among foodborne pathogens remain uncertain globally due to the lack of systematic reviews. We performed a meta-analysis on the global prevalence of foodborne pathogens exhibiting antibiotic resistance and biofilm formation using the methodology of a Cochrane review by accessing data from the China National Knowledge Infrastructure (CNKI), PubMed, and Web of Science databases between 2010 and 2020. A random effects model of dichotomous variables consisting of antibiotic class, sample source, and foodborne pathogens was completed using data from 332 studies in 36 countries. The results indicated AMR foodborne pathogens has become a worrisome global issue. The prevalence of AMR foodborne pathogens in food samples was greater than 10% and these foodborne pathogens were most resistant to β-lactamase antibiotics with *Bacillus cereus* being most resistant (94%). The prevalence of AMR foodborne pathogens in human clinical specimens was greater than 19%, and the resistance of these pathogens to the antibiotic class used in this research was high. Independently, the overall biofilm formation rate of foodborne pathogenic bacteria was 90% (95% CI, 68%–96%) and a direct linear relationship between biofilm formation ability and antibiotic resistance was not established. Future investigations should document both AMR and biofilm formation of the foodborne pathogen isolated in samples. The additional information could lead to alternative strategies to reduce the burden cause by AMR foodborne pathogens.

**Keywords: foodborne pathogens, antimicrobial resistance, biofilm, meta-analysis, global prevalence**

# INTRODUCTION

Infections caused antibiotic resistant bacteria are attributing to serious burdens for society (World Health Organization, 2021a) and where there are also threats to food safety and public health [Cania et al., 2018; FAO/OIE/WHO (Food and Agriculture Organization of the United Nations, World Organisation for Animal Health, World Health Organization), 2019]. It is estimated that 600 million people (almost 1 in 10) worldwide get sick from eating contaminated food and 420,000 die every year [World Health Organization (WHO), 2020a]. In the meanwhile, foodborne diseases cause huge economic losses in low- and middle-income countries, costing up to $110 billion annually [World Health Organization (WHO), 2020b]. Foodborne diseases are mainly caused by pathogenic microorganisms, such as *Escherichia coli*, *Staphylococcus aureus*, *Campylobacter* spp., *Salmonella* spp., and *Vibrio parahaemolyticus* [Kirk et al., 2015; World Health Organization (WHO), 2020a]. It cannot be ignored that the emergence of antibiotic resistance of foodborne pathogens has a serious impact on public health (Cania et al., 2018).

The use of antibiotics in the global food production system remains widespread and, concurrently, the development of antimicrobial resistance in humans, animals, plants, and the environment has accelerated [World Health Organization (WHO), 2021b]. There is increasing recognition that bacteria in natural ecosystems can transmit antibiotic resistance genes to humans and where the principal mode of transmission is human ingestion of food containing drug-resistant bacterial pathogens (Martínez, 2013; Hernando-Amado et al., 2019). In many clinical and food settings, foodborne pathogenic bacteria can adhere to biotic or abiotic surfaces to form biofilms, which can mitigate the action of antibiotics (Coughlan et al., 2016) and the results in decreased treatment efficacy (Guzman-Soto et al., 2021). According to the United States National Institutes of Health, biofilms mediate about 65% of human infections worldwide, and about 80% of chronic infections can be directly related to the formation of biofilms (Lebeaux et al., 2014; Melander and Melander, 2015). Moreover, a lot of research indicates that pathogens that produce biofilms are significantly more resistant to antibiotics and biocides than planktonic or free-living bacteria (Gebreyohannes et al., 2019; Karygianni et al., 2020).

Currently, foodborne pathogenic bacteria are monitored, globally, for resistance to important classes of antibiotics (Grundmann et al., 2011). And, a large number of studies of about drug resistance of foodborne pathogens and the ability to form biofilm has been published worldwide (see **Supplementary Material**). However, a systematic study of this data has yet to be reported. The aim of this meta-analysis is to systematically analyze (1) the global prevalence of antibiotic resistance and biofilm formation among foodborne pathogens and (2) the correlation between the antibiotic resistance and biofilm formation.

# MATERIALS AND METHODS

## Search Strategy

A meta-analysis based on PRISMA guidelines (Moher, 2010) and Cochrane recommendations (Cumpston et al., 2019) were employed to investigate the antibiotic resistance and biofilm production of foodborne pathogens. Information from curated databases and academic websites, including of PubMed, Web of Sciences, and China National Knowledge Infrastructure (CNKI) were evaluated for the suitability to the meta-analysis. The following keywords: "antibiotic resistance," "biofilm," "biofilm formation," "foodborne pathogenic bacteria," "foodborne disease," and "Food Poisoning" were used to search the information sources. In addition, the literature search was truncated, for articles published between 1 January 2010 and 31 December 2020, for six foodborne pathogen bacteria (*Bacillus cereus*, *Escherichia coli*, *Listeria monocytogenes*, *Salmonella* spp., *Staphylococcus aureus*, and *Vibrio parahaemolyticus*) and for ability to form biofilm. We also searched Google Scholar to ensure the comprehensiveness of the literature search, inclusive of literature from preprint repositories (bioRxiv and medRxiv).

## Selection Criteria

Literature was downloaded to EndNoteX9 and an initial screen removed duplicate information. Then eligible studies which met the following criteria was chosen: (1) pathogenic bacteria are common foodborne pathogens; (2) the pathogenic bacteria were isolates from food, patients with foodborne illness, or food practitioners with no search restrictions placed on human race, food type, sample size, and source; and (3) reported patterns of resistance of the foodborne pathogen and or the ability to form biofilm. Inclusion and exclusion of literature from the final analysis were performed, independently, by the authors, Qian Tao (TQ) and Qian Wu (WQ) by examining the titles, abstracts and full texts of the collected literature after the initial screen. This independent filtering of the literature minimized selection bias and a final selection was mediated through discussion or adjudication.

## Data Extraction and Quality Assessment

The authors, TQ and WQ, independently extracted data from the final selection by using a standardized format which included: the name of the first author, the published year, the period of sample collection, location, varieties of food, disease types, pathogenic bacteria investigated, number of isolates, method of antibiotic susceptibility testing, guideline used to interpret antimicrobial sensitivities, reported antibiotic sensitivities, number of antibiotic-resistant strains, number of multidrug-resistant strains, method of biofilm formation testing, biofilm types. If the information in a selection was unclear, an attempt was made to contact the author to verify the validity of the data. Merge tools and adapted version of the Joanna Briggs Institute (JBI) critical appraisal checklist (Moola et al., 2020) and the Newcastle-Ottawa quality-assessment scale (Wells, 2014) were also used for data assessment.

## Data Synthesis and Analysis

Stata (version 16) software was used for the statistical analysis of data from human and food studies. We used a forest plot to visualize effect sizes and 95% CI, where the estimated

antibiotic resistance rate was a pooled according to the class of antibiotics used and type of bacteria. Preliminary analysis of data revealed that antibiotic resistance and biofilm formation were heterogeneous and a random-effects models was used to account for the heterogeneity across all the studies. The $I^2$ statistic was used to quantify the heterogeneity across studies according to the following classes: a low level (less than 25%), a moderate level (25%–50%), and a high level (more than 75%; Higgins et al., 2003; IntHout et al., 2016).

Publication bias and subgroup analysis was completed by merging sample type and bacterial species. A funnel plot was used to show the extent of publication bias and a nonparametric scissor-complement analysis of publication bias was performed if bias existed. We conducted subgroup analysis from sample type, geographic location, date of sample collection (before 2010, 2011–2015, and 2016–2020) and susceptibility test.

# RESULTS

## Study Selection, Characteristics, and Quality Assessment

We identified 9,360 articles from electronic databases and 54 articles from other sources. Duplicate articles were removed, and the remaining 8,741 articles were screened for eligibility *via* the title and contents of the abstract (**Figure 1**). This screening further reduced the number of articles to 647, and a review of the content excluded another 322 articles. The exclusion criteria included five studies, where the text was not accessible. Another 33 articles were review type articles, which included meta-analyses or letters to the editor and 41 studies reported on non-foodborne pathogens. A further 44 studies where the isolates were not attributed to food or human source, and 64 studies were on pathogenic bacteria which were isolated from water or other environmental sources. Finally, 120 studies did not contain sufficient and extractable data.

In total, 325 articles were included in the meta-analysis, where 104 studies reported on antibiotic resistance in patients with foodborne illness and 244 studies reported on antibiotic resistance or biofilm formation in food. Twelve studies reported consequences for both food and patients (**Supplementary Table 1**), and 11 studies were double-counted because they reported on the result of antibiotic resistance in multiple countries or multiple foodborne pathogens. No unpublished literature met the inclusion criteria.

The final selection of studies was conducted in 36 different countries and from six continents (**Figure 2**) with the Asian ($n = 287$[82%]) and Africa regions ($n = 24$[11%]) being

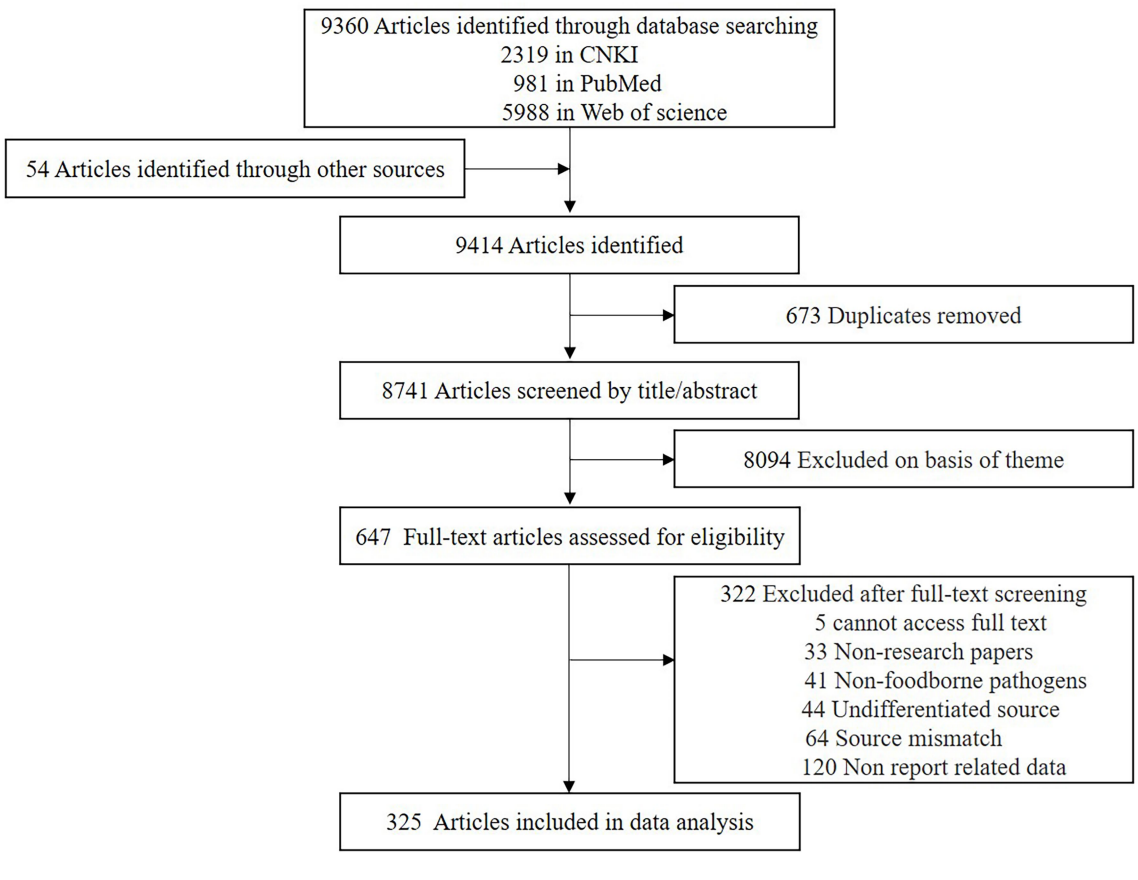


**FIGURE 1** | Study selection.


**FIGURE 2 |** Geographical distribution of reported resistance of the pathogens isolated from food or humans.

predominant. The antibiotic resistance or biofilm formation of following foodborne pathogens were evaluated: *Bacillus cereus* ($n = 9$[3%]), *Escherichia coli* ($n = 37$[11%]), *Listeria monocytogenes* ($n = 19$[5%]), *Vibrio parahaemolyticus* ($n = 129$[37%]), *Salmonella* ($n = 85$[24%]), and *Staphylococcus aureus* ($n = 58$[17%]). All the included studies used a cross-sectional design, and these studies were published in journal articles except for one which was a dissertation.

The 244 food studies included 34,746 isolates of pathogenic bacteria and these strains were isolated from a number of food groups, mainly raw meat (raw beef, mutton, chicken, duck, and pork), raw milk, aquatic products (fish, shrimp, crab, and other seafood), vegetables, and ready-to-eat foods. Only 11 studies reported on the formation of foodborne pathogenic bacteria biofilms, and the biofilm formation of these 752 isolates were measured by quantitative microtiter assay. The 104 human population studies were concentrated foodborne diarrhea patients ($n = 93$[89%]), food poisoning patients ($n = 10$[10%]), and food practitioners ($n = 1$[1%]). Further detailed of these studies study is shown in the **Table 1**.

To ensure the reliability of the meta-analysis results, we evaluated the quality of the included literature in terms of research purposes, research objects, and research methods, etc. Quality assessment results showed that overall was very complete, there was a low risk of bias (**Supplementary Table 4**). Both human studies and food studies, which purpose and results of the research are clearly described. The sample characteristics and select of study subjects may cause bias notwithstanding, there are some studies (62% food studies and 30% human studies) with poor description in this part. A full description of the measurement methods and guidelines of antimicrobial sensitivities and biofilm formation was provided in 96% ($n = 234$) of the food studies and 90% ($n = 94$) of the human studies.

## Prevalence of Antibiotic Resistant Foodborne Pathogens

The pooled prevalence of foodborne pathogens resistant to classes of tested antibiotics in food and human studies are shown in **Table 2**. The mean prevalence of antimicrobial resistant foodborne pathogens isolated in foods was ≥11% and the majority of these foodborne pathogens were highly resistant to β-lactam antibiotics. The combined or pooled prevalence of *B. cereus* resistant to β-lactams antibiotics from all the food studies was 94% (95% CI, 91%–98%), although the presence of *B. cereus* in food was only reported in six studies. The prevalence of *Escherichia coli* which was resistant to all classes of antibiotics tested ranged from 56% (95% CI, 45%–67%) to 25% (95% CI, 15%–34%). Comparatively *Salmonella* was more resistant to chloramphenicol (32, 95% CI, 21%–43%) and *V. parahaemolyticus* was mildly resistant to sulfonamides (14,

**TABLE 1** | Included studies characteristics.

| Study characteristics | Human studies (*n* = 104) | Food studies (*n* = 244) |
|---|---|---|
| **Location** | | |
| Africa | 2 (2%) | 22 (9%) |
| Asia | 99 (95%) | 188 (77%) |
| Europea | 1 (1%) | 6 (2%) |
| North America | — | 10 (4%) |
| Oceania | — | 1 (1%) |
| South America | 2 (2%) | 6 (2%) |
| **Period** | | |
| 2000–2010 | 21 (20%) | 51 (21%) |
| 2011–2015 | 48 (46%) | 108 (44%) |
| 2016–2020 | 35 (34%) | 74 (30%) |
| **Method of antimicrobial susceptibility testing** | | |
| Disk diffusion | 46 (44%) | 156 (64%) |
| MIC | 44 (42%) | 61 (25%) |
| Vitek | 10 (10%) | 10 (4%) |
| Others | — | 4 (2%) |
| Not reported | 4 (4%) | 2 (1%) |
| **Guidelines used to interpret antimicrobial sensitivities** | | |
| CLSI | 85 (82%) | 199 (82%) |
| EUCAST | 2 (2%) | 1 (1%) |
| NCCLS | 6 (6%) | 13 (5%) |
| Others | 1 (1%) | 6 (2%) |
| Not reported | 10 (10%) | 14 (6%) |
| **Bacteria studied** | | |
| *Bacillus cereus* | 1 (1%) | 8 (3%) |
| *Escherichia coli* | 11 (10%) | 26 (11%) |
| *Listeria monocytogenes* | 1 (1%) | 18 (7%) |
| *Vibrio parahaemolyticus* | 50 (48%) | 79 (32%) |
| *Salmonella* | 36 (35%) | 49 (20%) |
| *Staphylococcus aureus* | 5 (5%) | 53 (22%) |
| **Source of isolate** | | |
| Aquatic products | — | 78 (32%) |
| Meat | — | 50 (20%) |
| Milk and dairy products | — | 12 (5%) |
| RET-food | — | 13 (5%) |
| Others | — | 80 (33%) |
| Foodborne diarrhea patients | 93 (89%) | |
| Food poisoning samples | 10 (10%) | |
| Food handlers | 1 (1%) | |
| **Biofilm forming ability** | — | 11 (5%) |

**TABLE 2** | Pooled prevalence of antibiotic resistance from meta-analysis of food studies and human studies, by antibiotic category.

| | Food studies | | Human studies | |
|---|---|---|---|---|
| | Articles (*n*) | Prevalence % (95% CI) | Articles (*n*) | Prevalence % (95% CI) |
| **Gram-negative bacterium** | | | | |
| ***Escherichia coli*** | | | | |
| Aminoglycosides | 11 | 32 (19–46) | 8 | 25 (19–31) |
| β-Lactams | 19 | 56 (45–67) | 10 | 61 (51–70) |
| Chloramphenicol | 8 | 25 (15–34) | 6 | 19 (8–30) |
| Fluoroquinolones | 12 | 37 (22–52) | 8 | 38 (18–58) |
| Sulfonamides | 19 | 48 (30–65) | 9 | 39 (26–52) |
| Tetracyclines | 21 | 54 (41–57) | 9 | 49 (43–55) |
| ***Salmonella*** | | | | |
| Aminoglycosides | 31 | 39 (31–47) | 17 | 44 (27–61) |
| β-Lactams | 42 | 47 (38–55) | 33 | 56 (47–66) |
| Chloramphenicol | 18 | 32 (21–43) | 18 | 33 (24–42) |
| Fluoroquinolones | 42 | 44 (30–59) | 24 | 50 (40–60) |
| Sulfonamides | 34 | 42 (29–54) | 26 | 43 (32–54) |
| Tetracyclines | 30 | 56 (47–64) | 27 | 45 (35–54) |
| ***Vibrio parahaemolyticus*** | | | | |
| Aminoglycosides | 39 | 45 (36–53) | 14 | 22 (11–33) |
| β-Lactams | 77 | 77 (71–83) | 37 | 76 (69–82) |
| Fluoroquinolones | 7 | 13 (7–19) | 8 | 19 (−7–44) |
| Sulfonamides | 36 | 14 (10–18) | 11 | 25 (−1–52) |
| Tetracyclines | 22 | 14 (11–17) | 6 | 2 (0–4) |
| **Gram-positive bacterium** | | | | |
| ***Bacillus cereus*** | | | | |
| β-Lactams | 6 | 94 (91–98) | 1 | 81 (75–86) |
| Sulfonamides | 3 | 32 (6–58) | 1 | 66 (60–73) |
| ***Listeria monocytogenes*** | | | | |
| Aminoglycosides | 6 | 21 (8–35) | — | — |
| β-Lactams | 13 | 45 (27–63) | 1 | 54 (38–70) |
| Chloramphenicol | 8 | 30 (11–49) | - | - |
| Fluoroquinolones | 9 | 11 (6–16) | 1 | 62 (47–78) |
| Sulfonamides | 6 | 11 (3–19) | — | — |
| Tetracyclines | 13 | 22 (15–30) | 1 | 30 (15–44) |
| ***Staphylococcus aureus*** | | | | |
| Aminoglycosides | 33 | 30 (24–36) | 2 | 30 (−12–73) |
| β-Lactams | 45 | 78 (73–82) | 3 | 68 (32–102) |
| Chloramphenicol | 17 | 16 (12–19) | — | — |
| Fluoroquinolones | 27 | 23 (19–28) | 1 | 36 (8–65) |
| Sulfonamides | 19 | 31 (19–43) | 2 | 35 (5–66) |
| Tetracyclines | 43 | 41 (33–48) | 4 | 28 (4–52) |

95% CI, 10%–18%) and tetracyclines (14, 95% CI, 11%–17%). The Gram-positive pathogen, *L. monocytogenes*, was also mildly resistant to fluoroquinolones (11%; 95% CI, 6%–16%) and sulfonamides (11%; 95% CI, 3%–19%). Lastly *S. aureus* is susceptible to treatment with chloramphenicol (17 9% CI, 12%–19%) when compared to other antibiotics.

As shown in **Table 2**, the mean prevalence of antibiotic resistant foodborne pathogens isolated from human samples was ≥19% and the outlier was the mean prevalence of tetracycline resistant *V. parahaemolyticus* (2%; 95% CI, 0%–4%). In tandem to pathogens isolated from food samples, the mean prevalence of the pathogen group to β-lactams antibiotics resistance was high, with *B. cereus* at 81% (95% CI, 75%–86%), *V. parahaemolyticus* at 76% (95% CI, 69%–82%) and *L. monocytogenes* at 54% (95% CI, 38%–70%). The pattern of resistance to the classes of antibiotics tested for Gram negative and Gram-negative pathogens is similar to those isolated from the food samples with the exception of *L. monocytogenes*. The Gram-positive bacterium, *L. monocytogenes*, appears to be highly resistant to fluoroquinolones but this information is only based on 1 study.

## Subgroup Analysis by Food Types

For the food studies, studies with clear classification of food samples were included in our subgroup analysis, mainly in the following categories: aquatic products, meat, milk, and dairy products, RTE-food. **Table 3** shows the prevalence of foodborne pathogen food isolates resistant to antibiotics in different food types. The prevalence of multi-drug resistant (MDR) pathogen was ≥36% for all food types, with the highest rates in meat (52%; 95% CI, 40–60). Also in all food types, pathogens resistant to β-lactams were most common (≥57%).

**TABLE 3** | Subgroup analysis of antibiotic resistance by food types.

| | Aquatic products | | Meat | | Milk and dairy products | | RTE-food | |
|---|---|---|---|---|---|---|---|---|
| | Articles (*n*) | Prevalence % (95% CI) | Articles (*n*) | Prevalence % (95% CI) | Articles (*n*) | Prevalence % (95% CI) | Articles (*n*) | Prevalence % (95% CI) |
| MDR | 9 | 42 (26–58) | 16 | 52 (40–63) | 4 | 43 (1–84) | 8 | 36 (21–51) |
| Aminoglycosides | 38 | 43 (34–51) | 36 | 29 (31–47) | 7 | 33 (14–51) | 6 | 35 (24–46) |
| β-Lactams | 65 | 73 (66–81) | 42 | 62 (52–70) | 9 | 61 (45–77) | 10 | 57 (34–83) |
| Chloramphenicol | — | — | 19 | 36 (21–51) | 4 | 21 (17–25) | 4 | 25 (16–34) |
| Fluoroquinolones | 11 | 13 (8–19) | 38 | 39 (25–53) | 5 | 30 (14–46) | 9 | 25 (16–34) |
| Sulfonamides | 34 | 14 (11–17) | 32 | 47 (31–63) | 7 | 62 (46–78) | 7 | 43 (10–76) |
| Tetracyclines | 21 | 22 (9–35) | 36 | 62 (54–70) | 9 | 28 (2–55) | 8 | 43 (24–63) |

**TABLE 4** | Subgroup analysis of antibiotic resistance by study population.

| | Diarrhea patients | | Food poisoning samples | | Food handlers | |
|---|---|---|---|---|---|---|
| | Articles (*n*) | Prevalence % (95% CI) | Articles (*n*) | Prevalence % (95% CI) | Articles (*n*) | Prevalence % (95% CI) |
| Aminoglycosides | 34 | 32 (23–42) | 4 | 11 (0–21) | 1 | 23 (16–29) |
| β-Lactams | 78 | 65 (60–71) | 6 | 78 (62–95) | 1 | 65 (57–72) |
| Chloramphenicol | 23 | 29 (21–37) | — | — | 1 | 37 (30–45) |
| Fluoroquinolones | 41 | 42 (30–53) | 2 | 22 (6–39) | — | — |
| Sulfonamides | 46 | 39 (27–52) | 2 | 14 (2–26) | 1 | 47 (37–52) |
| Tetracyclines | 42 | 41 (33–48) | 5 | 21 (3–39) | 1 | 46 (37–52) |

**TABLE 5** | Subgroup analysis of antibiotic resistance by region, time period and susceptibility test.

| | Food studies | | Human studies | |
|---|---|---|---|---|
| | Articles *(n)* | Prevalence % (95% CI) | Articles *(n)* | Prevalence % (95% CI) |
| **Region** | | | | |
| Africa | 19 | 80 (75–86) | 2 | 92 (82–102) |
| Asia | 149 | 77 (73–81) | 93 | 82 (80–84) |
| Europe | 6 | 78 (68–89) | 1 | 48 (40–55) |
| North America | 10 | 76 (66–87) | — | — |
| Oceania | 1 | 85 (74–95) | — | — |
| South America | 6 | 97 (95–99) | 2 | 90 (80–100) |
| **Period** | | | | |
| Before 2010 | 40 | 75 (65–85) | 19 | 80 (75–84) |
| 2011–2015 | 75 | 72 (66–79) | 38 | 79 (75–83) |
| 2016–2020 | 51 | 80 (77–84) | 30 | 82 (78–86) |
| **Susceptibility test** | | | | |
| Disk diffusion | 36 | 82 (79–85) | 112 | 78 (75–81) |
| MIC | 39 | 77 (72–82) | 47 | 69 (59–79) |
| Vitek | 9 | 88 (83–93) | 9 | 90 (85–95) |

In aquatic products, the pooled prevalence of isolates resistant to fluoroquinolones and sulfonamides were both around 13%, but resistance to β-lactams was over six times higher.

## Subgroup Analysis by Study Population

Foodborne pathogens isolated from three groups in human studies also had the highest resistance to β-lactams antibiotics (**Table 4**). The mean prevalence of antibiotic resistant pathogens among foodborne diarrhea patients were ≥32%, with mean prevalence of resistant isolates to sulfonamides of 39% (95% CI, 27%–52%) and tetracyclines at 41% (95% CI, 33%–48%). In comparison to the other groups, the antibiotic resistance rate of food poisoning sample isolates were lower, except for resistance to β-Lactams which was similar. Only one study (Xu et al., 2019) delineated the antibiotic resistance *Salmonella* isolates, the predominant resistance was to β-lactams (65%), followed by that of sulfonamides (47%), tetracyclines (46%).

## Subgroup Analysis by Region, Time Period, and Susceptibility Test

**Table 5** shows the regional distribution of reported resistance profile of the pathogens isolated from food or humans and where the majority of the studies were concentrated in Asia (>75%). In South America, the pooled prevalence of antibiotic resistant pathogens was as high as 97% (food) and 90% (humans). In contrast, the prevalence of antibiotic resistant pathogens isolated from human samples was only 48% (95% CI, 40–55) in Europe. Overall, there was a decrease in the prevalence pathogens resistance of food isolate decreased from75% (95% CI, 65–85) before 2010 to 72% (95% CI, 66–79) during in 2011–2015, then increased to 80% (95% CI, 77–84) between in 2016–2020. The pathogens resistance of human isolate had changed with the time of collection, and which had the same resistance trend with food isolate. In general, we found that a trend of antimicrobial resistance of foodborne pathogens firstly decreasing and then increasing during 2000–2020. Subgroup analysis by susceptibility test group showed higher

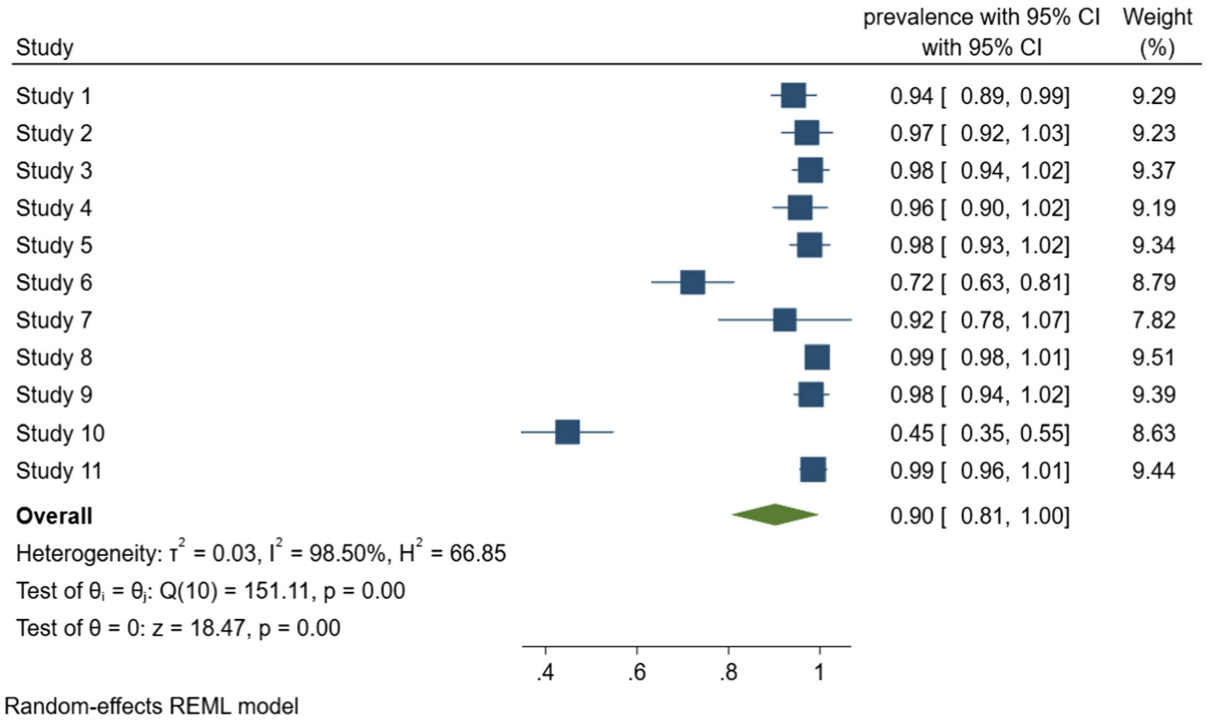


**FIGURE 3 |** Forest plot of the meta-analysis of biofilm formation rate in foodborne pathogen retrieved from food samples. Study 1: Lapierre et al. (2020). Study 2: Lopez-Leon et al. (2016). Study 3: Rodríguez-Lázaro et al. (2018). Study 4: Beshiru and Igbinosa (2018). Study 5: Beshiru et al. (2018). Study 6: Chen and Xie (2019). Study 7: Maia et al. (2020). Study 8: Ou et al. (2020). Study 9: Puah et al. (2018). Study 10: Wang et al. (2020). Study 11: Kim et al. (2018).

rates of antibiotic resistance tested with Vitek (automatic drug sensitivity analyzer) compared to used disk diffusion and minimum inhibitory concentration (MIC).

## Prevalence of Biofilm Formation in Foodborne Pathogens

According to meta-analysis of biofilm formation rate in foodborne pathogen retrieved from food samples that shown in **Figure 3**, the combined rate of biofilm formation was 90% (95% CI, 81–100). Considerable heterogeneity was detected between the studies, with $I^2$ = 98.5%, Q (10) = 151.11, $p < 0.001$, which most likely due to the inclusion of multiple species of bacteria. Among them, *Staphylococcus aureus* was the most prone to produce biofilms, all the *Staphylococcus aureus* isolates from food in the four studies can form biofilm. It is worth mentioning that Ou et al. (2020) investigated the antimicrobial resistance and biofilm formation of *staphylococcus aureus* in food, and reported 64.8% of 165 antibiotic resistant strains had strong biofilm formation ability, study describes the significant correlation between antibiotic resistance and biofilm formation.

## Publication Bias

The Begg's test for funnel plot of the resistance rate of pathogenic bacteria in food and human studies shows that both were exist visual asymmetry evidence, therefore a trim and fill procedure was executed. Funnel plot were produced for of biofilm formation rate of foodborne pathogen retrieved from food samples too, which no evidence of publication bias. Funnel plot shown in Appendix, **Supplementary Material**.

# DISCUSSION

## Prevalence of Antibiotic Resistance of Foodborne Pathogens

This meta-analysis showed that foodborne pathogens present high levels of antibiotic resistance, both in food samples and clinical specimens. Worldwide, rates of resistance to β-lactams were the highest, irrespective of types of foodborne pathogens. Analogously, a meta-analysis by Jia et al. (Kai et al., 2020) retrieved resistance data of *Staphylococcus aureus* isolates from retail foods, between 2007 and 2017 from70, The main finding was that resistance by *S. aureus* found in retail foods to seven types of antibiotics ranged from 8% to 87% with the most serious resistance being to beta-lactam antibiotics.

Overall, human isolates of *Salmonella* and *Listeria monocytogenes* are significantly more resistant to various antibiotics than food isolates. According to the Microbiological Risk Assessment 2020 report, Salmonellosis is one of the most common zoonotic diseases; the number of listeriosis had increased compared to before [EFSA (European Food Safety Authority), 2020], which might be related to the increased antibiotic resistance of pathogenic bacteria. Our research found

a resistance rate increase of 41%–52% in the proportion of isolates that were *Listeria monocytogenes* resistant to fluoroquinolones in human studies compared with food studies. By contrast, for the other pathogens, there is no absolute difference in antibiotic resistance between humans and food isolates. For example, for *Escherichia coli*, food isolates are more resistant to β-lactams and fluoroquinolones than human isolates, which had an opposite of resistance to the other four types of antibiotics.

In the subgroup analysis by food types, foodborne pathogenic bacteria in meat were significantly more resistant to antibiotics than other food isolates, which might be related to the amount of antibiotics used. In farming, antibiotic use in food animal production accounts for two-thirds of the overall antibiotic usage (Done et al., 2015). Meat contamination is mainly caused by *Escherichia coli*, *Salmonella*, and *Staphylococcus aureus* (Adesokan et al., 2020), our reported pooled multi-drug resistance rate of foodborne pathogens in meat was 52%, which was similar to the reported antimicrobial resistance in zoonotic and indicator bacteria from food by EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control) (2021). Meanwhile, we find that the resistance of pathogenic bacteria in meat to antibiotics were, in increasing order of resistance, aminoglycosides, chloramphenicol, fluoroquinolones, sulfonamides, β-lactams, and tetracyclines. To 62% resistance to tetracyclines and β-lactams was observed.

In the subgroup analysis by study population, we found that pathogenic bacteria isolated from food handlers and foodborne diarrhea patients have similar drug resistance. The antibiotic resistance of pathogenic bacteria in food handlers was significantly stronger than that in food poisoning samples, except for resistance to β-lactams. Because of direct or indirect contact with food, food handlers are likely to transmit drug-resistant foodborne pathogens to consumers through food. Franck et al. (2014) found that in the 191 foodborne disease outbreaks in Denmark between 2005 and 2011, food handlers played an indispensable role. There have also been 43 cases of infections caused by *Salmonella* carried by food handlers in the United States (Kimura et al., 2005).

In general, the antibiotic resistance rates of foodborne pathogens from six continents were at high levels, regardless of the source or food samples isolated from clinical specimens. A notable finding was the difference in the summary analysis value of antibiotic resistance rate of foodborne pathogens between regions, which was significantly higher in Africa and South America compared to others. While most countries in South America and Africa are low-income and middle-income countries, antibiotic consumption was greater than in high-income countries (Klein et al., 2020). Heavy use of antibiotics will make the enhanced bacterial resistance (Oliveira et al., 2021), in the European Union and the United States, according to reports, agriculture accounts for more than 75% of the annual antibiotic use; between 2011 and 2014, the use of antibiotics in 24 European Union countries fell by 12% [OECD (Organization for Economic Cooperation and Development), 2016]. By time period, in our subgroup analysis, antibiotic resistance of foodborne pathogens also declined during 2011–2015. In general, we detected that the resistance rate foodborne pathogens in food samples and human specimens had increased.

In the studies included, most of the studies applied the antibiotic susceptibility testing methods (disk diffusion and MIC) recommended by the Clinical and Laboratory Standards Institute (CLSI Clinical and Laboratory Standards Institute, 2019). Our meta-analysis shows that foodborne pathogens measured with Vitek had a higher resistance rate, which might be related to the higher sensitivity of the automatic drug sensitivity analyzer (Nakasone et al., 2007). In fact, sensitivity testing methods will not have a substantially impact on bacterial resistance. The resistance of foodborne pathogens bacteria depends on several factors, such as type of bacteria, the type of food and disease, the source of the sample, and other related factors.

## Prevalence of Biofilm Formation of Foodborne Pathogens

We described the biofilm formation rate of foodborne pathogen, and most foodborne pathogens have the ability to form biofilms in the food studies included. Due to lack of relevant research, we did not find evidence of biofilm formation of foodborne pathogens isolated from humans.

The mechanisms by which biofilms promote resistance of bacteria to antimicrobials is very complicated. A series of molecular mechanisms occur can conduce to the stability of the biofilm microbial community (Hall and Mah, 2017). These occur in the main in the following three ways. First, interaction between biofilm matrix components and antibiotics; there are factors in the biofilm that can reduce the penetration rate of antibiotics (Boudjemaa et al., 2016; Singh et al., 2016). Second, bacteria in biofilms evade external stimuli by reducing their growth rate (Walters et al., 2003; Borriello et al., 2004). Third, the role of specific genetic determinants of antibiotic resistance in biofilms, such as efflux pumps (Van Acker and Coenye, 2016), quorum sensing (Chua et al., 2016), colony variants (Proctor et al., 2006), et al. The results of our meta-analysis show that 90% of the 752 foodborne pathogens can form biofilms, this provides the possibility of enhancing their resistance. However, due to the limitations of existing research, we cannot provide evidence of a direct linear relationship between biofilm formation ability and antibiotic resistance.

## Strengths and Limitations

To our knowledge, this study is the first comprehensive meta-analysis of global prevalence of antibiotic resistance and biofilm formation in foodborne pathogens. We were able to include 11 studies (**Supplementary Table 3**) with assess the biofilm formation of foodborne pathogens in food, and the relationship between the ability of biofilm formation and antibiotic resistance was discussed. One of our main advantages is that strict compliance with PRISMA guidelines (Moher, 2010), we have adopted strict standards and limited to research that analyzed the resistance of foodborne pathogen in food and humans, the reservoirs of pathogenic bacteria,

and the main vehicle of antibiotic resistance transmitted through the food chain.

Our meta-analysis has some limitations. First, there was significant heterogeneity among the included studies in the analysis. As we undertook a bundled meta-analysis (Tang et al., 2017), it is reasonable that no relevant reduction of heterogeneity was detected when we conducted subgroup analysis from sample type, region, date of sample collection and susceptibility test. Furthermore, another potential source of heterogeneity may be related to antibiotic consumption patterns in various regions. Second, because of we limited by study quality, there was potential data reporting bias in this meta-analysis. Studies which met the inclusion criteria and which reported the prevalence of antibiotic resistance of foodborne pathogens isolated from food handlers was scarce and is, as a consequence, poorly represented.

## CONCLUSION

In conclusions, this study provides a comprehensive overview of global antibiotic resistance of foodborne pathogen in food and humans, which shows worrying levels of resistance in some parts of the world, where one possible explanation is large and irregular use of antibiotics. Following preliminary pooling of data relating to biofilm formation of pathogenic bacteria, our analysis shows that foodborne pathogens have a high tendency to form biofilms. It is less clear that a direct linear relationship exists between the ability to form biofilms and antibiotic resistance. It is recommended that future research should thoroughly explore the relationship between the ability of biofilm formation and the antibiotic resistance of foodborne pathogens and provide a new theoretical basis for the discovery of the mechanism of foodborne pathogens' antibiotic resistance. Therefore, frequent monitoring of types, antibiotic resistance and biofilm characteristics of foodborne pathogens in clinical and environmental is urgently needed to raise our awareness of antibiotic resistance and its spread, and prompt the development of effective strategies to improve food safety and prevent foodborne illness infection.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, and further inquiries can be directed to the corresponding authors.

## AUTHOR CONTRIBUTIONS

QT, QW, and ZZ interpreted the literature sources and drafted the manuscript. QT and QW conceived and planned the data collection and analysis. JL, CT, and ZH involved in data verification. ZZ, PM, and YP critically reviewed and revised the manuscript. ZZ and YZ conceptualized the idea and edited the manuscript. All authors contributed to the article and approved the submitted version.

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## REFERENCES

Adesokan, H. K., Funso-Adu, K., and Okunlade, O. A. (2020). Foodborne pathogens on meat stored in major central cold rooms in Ibadan and their susceptibility to antimicrobial agents. *Folia Vet.* 64, 1–10. doi: 10.2478/fv-2020-0011

Beshiru, A., and Igbinosa, E. O. (2018). Characterization of extracellular virulence properties and biofilm-formation capacity of Vibrio species recovered from ready-to-eat (RTE) shrimps. *Microb. Pathogenesis* 119, 93–102. doi: 10.1016/j.micpath.2018.04.015

Beshiru, A., Igbinosa, I. H., and Igbinosa, E. O. (2018). Biofilm formation and potential virulence factors of Salmonella strains isolated from ready-to-eat shrimps. *PloS One* 13:e0204345. doi: 10.1371/journal.pone.0204345

Borriello, G., Werner, E., Roe, F., Kim, A. M., Ehrlich, G. D., and Stewart, P. S. (2004). Oxygen limitation contributes to antibiotic tolerance of *Pseudomonas aeruginosa* in biofilms. *Antimicrob. Agents Chemother.* 48, 2659–2664. doi: 10.1128/AAC.48.7.2659-2664.2004

Boudjemaa, R., Briandet, R., Revest, M., Jacqueline, C., Caillon, J., Fontaine-Aupart, M. P., et al. (2016). New insight into daptomycin bioavailability and localization in *S. aureus* biofilms by dynamic fluorescence imaging. *Antimicrob. Agents Chemother.* 60, 4983–4990. doi: 10.1128/AAC.00735-16

Cania, M., Manageiro, V., Abriouel, H., Moran-Gilad, J., and Franz, C. M. A. P. (2018). Antibiotic resistance in foodborne bacteria. *Trends Food Sci Technol.* 84, 41–44. doi: 10.1016/j.tifs.2018.08.001

Chen, Q., and Xie, S. (2019). Genotypes, enterotoxin gene profiles, and antimicrobial resistance of *Staphylococcus aureus* associated with foodborne outbreaks in Hangzhou, China. *Toxins* 11:307. doi: 10.3390/toxins11060307

Chua, S. L., Yam, J., Hao, P. L., Adav, S. S., Salido, M. M., Liu, Y., et al. (2016). Selective labelling and eradication of antibiotic-tolerant bacterial populations in *Pseudomonas aeruginosa* biofilms. *Nat. Commun.* 7:10750. doi: 10.1038/ncomms10750

CLSI Clinical and Laboratory Standards Institute (2019). Performance Standards for Antimicrobial Susceptibility Testing; Fifteenth Informational Supplement. CLSI Document M100es20. Wayne, PA: CLSI.

Coughlan, L. M., Cotter, P. D., Hill, C., and Alvarez-Ordonez, A. (2016). New weapons to fight old enemies: novel strategies for the (bio)control of bacterial biofilms in the food industry. *Front. Microbiol.* 7:1641. doi: 10.3389/fmicb.2016.01641

Cumpston, M., Li, T. J., Page, M. J., Chandler, J., Welch, V. A., Higgins, J. P. T., et al. (2019). Updated guidance for trusted systematic reviews: a new edition of the Cochrane Handbook for Systematic Reviews of Interventions. *Cochrane Database Syst Rev.*:ED000142, 10. doi: 10.1002/14651858.ED000142

Done, H. Y., Venkatesan, A. K., and Halden, R. U. (2015). Does the recent growth of aquaculture create antibiotic resistance threats different from those associated with land animal production in agriculture? *AAPS J.* 17, 513–524. doi: 10.1208/s12248-015-9722-z

EFSA (European Food Safety Authority), (2020). Annual report of the Scientific Network on Microbiological Risk Assessment 2020. EFSA supporting publication 2020: EN-1991. 10 pp. doi: 10.2903/sp.efsa.2020. EN-1991

EFSA (European Food Safety Authority) and ECDC (European Centre for Disease Prevention and Control) (2021). The European Union Summary Report on Antimicrobial Resistance in zoonotic and indicator bacteria from humans, animals and food in 2018/2019. *EFSA J.* 19:e06490. doi: 10.2903/j.efsa.2021.6490

FAO/OIE/WHO (Food and Agriculture Organization of the United Nations, World Organisation for Animal Health, World Health Organization) (2019). Tripartite Collaboration Project (FAO, OIE, PAHO/WHO): working together to fight antimicrobial resistance. Available at: http://www.fao.org/antimicrobial-resistance/projects/ongoing/project-7/zh/ (Accessed 3 September 2021).

Franck, K. T., Lisby, M., Fonager, J., Schultz, A. C., Bottiger, B., Villif, A., et al. (2014). Sources of calicivirus contamination in foodborne Outbreaks in Denmark, 2005–2011—the role of the asymptomatic food handler. *J. Infect. Dis.* 211, 563–570. doi: 10.1093/infdis/jiu479

Gebreyohannes, G., Nyerere, A., Bii, C., and Sbhatu, D. B. (2019). Challenges of intervention, treatment, and antibiotic resistance of biofilm-forming microorganisms. *Heliyon* 5:e02192. doi: 10.1016/j.heliyon.2019.e02192

Grundmann, H., Klugman, K. P., Walsh, T., Ramon-Pardo, P., Sigauque, B., Khan, W., et al. (2011). A framework for global surveillance of antibiotic resistance. *Drug Resist. Updat.* 14, 79–87. doi: 10.1016/j.drup.2011.02.007

Guzman-Soto, I., McTiernan, C., Gonzalez-Gomez, M., Ross, A., Gupta, K., Suuronen, E. J., et al. (2021). Mimicking biofilm formation and development: recent progress in *in vitro* and *in vivo* biofilm models. *Iscience* 24:102443. doi: 10.1016/j.isci.2021.102443

Hall, C. W., and Mah, T. F. (2017). Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. *FEMS Microbiol Rev.* 41, 276–301. doi: 10.1093/femsre/fux010

Hernando-Amado, S., Coque, T. M., Baquero, F., and Martinez, J. L. (2019). Defining and combating antibiotic resistance from one health and global-health perspectives. *Nat. Microbiol.* 4, 1432–1442. doi: 10.1038/s41564-019-0503-9

Higgins, J. P., Thompson, S. G., Deeks, J. J., and Altman, D. G. (2003). Measuring inconsistency in meta-analyses. *BMJ* 327, 557–560. doi: 10.1136/bmj.327.7414.557

IntHout, J., Ioannidis, J. P., Rovers, M. M., and Goeman, J. J. (2016). Plea for routinely presenting prediction intervals in meta-analysis. *BMJ* 6:e010247. doi: 10.1136/bmjopen-2015-010247

Kai, J., Tai, S. F., Xiang, W., Liu, Y. T., Sun, W. X., Wang, Y. R., et al. (2020). Antibiotic resistance patterns of *Staphylococcus aureus* isolates from retail foods in mainland China: a meta-analysis. *Foodborne Pathog. Dis.* 17, 296–307. doi: 10.1089/fpd.2019.2686

Karygianni, L., Ren, Z., Koo, H., and Thurnheer, T. (2020). Biofilm Matrixome: extracellular components in structured microbial communities. *Trends Microbiol.* 28, 668–681. doi: 10.1016/j.tim.2020.03.016

Kim, H. J., Oh, T., and Baek, S. Y. (2018). Multidrug resistance, biofilm formation, and virulence of *Escherichia coli* isolates from commercial meat and vegetable products. *Foodborne Pathog. Dis.* 15, 782–789. doi: 10.1089/fpd.2018.2448

Kimura, A. C., Palumbo, M. S., Meyers, H., Abbott, S., Rodriguez, R., and Werner, S. B. (2005). A multi-state outbreak of Salmonella serotype Thompson infection from commercially distributed bread contaminated by an ill food handler. *Epidemiol. Infect.* 133, 823–828. doi: 10.1017/S0950268805004127

Kirk, M. D., Pires, S. M., Black, R. E., Caipo, M., Crump, J. A., Devleesschauwer, B., et al. (2015). World Health Organization estimates of the global and regional disease burden of 22 foodborne bacterial, protozoal, and viral diseases, 2010: a data synthesis. *PLoS Med.* 12:e1001921. doi: 10.1371/journal.pmed.1001921

Klein, E. Y., Milkowska-Shibata, M., Tseng, K. K., Sharland, M., Gandra, S., Pulcini, C., et al. (2020). Assessment of WHO antibiotic consumption and access targets in 76 countries, 2000–15: an analysis of pharmaceutical sales data. *Lancet Infect. Dis.* 21, 107–115. doi: 10.1016/S1473-3099(20)30332-7

Lapierre, L., Cornejo, J., Zavala, S., Galarce, N., Sánchez, F., Benavides, M. B., et al. (2020). Phenotypic and Genotypic Characterization of Virulence Factors and Susceptibility to Antibiotics in Salmonella Infantis Strains Isolated from Chicken Meat: First Findings in Chile. *Animals* 10:1049. doi: 10.3390/ani10061049

Lebeaux, D., Ghigo, J. M., and Beloin, C. (2014). Biofilm-related infections: bridging the gap between clinical management and fundamental aspects of recalcitrance toward antibiotics. *Microbiol. Mol. Biol. Rev.* 78, 510–543. doi: 10.1128/MMBR.00013-14

Lopez-Leon, P., Luna-Gonzalez, A., Escamilla-Montes, R., Flores-Miranda, M. D., Fierro-Coronado, J. A., Alvarez-Ruiz, P., et al. (2016). Isolation and characterization of infectious Vibrio parahaemolyticus, the causative agent of AHPND, from the whiteleg shrimp (*Litopenaeus vannamei*). *Lat. Am. J. Aquat. Res.* 44, 470–479. doi: 10.3856/vol44-issue3-fulltext-5

Maia, D. S. V., Haubert, L., Kroning, I. S., Soares, K. D., Oliveira, T. L., and da Silva, W. P. (2020). Biofilm formation by *Staphylococcus aureus* isolated from food poisoning outbreaks and effect of Butia odorata Barb. Rodr. Extract on planktonic and biofilm cells. *LWT* 117:108685. doi: 10.1016/j.lwt.2019.108685

Martínez, J. L. (2013). Bacterial pathogens: from natural ecosystems to human hosts. *Environ. Microbiol.* 15, 325–333. doi: 10.1111/j.1462-2920.2012.02837.x

Melander, R. J., and Melander, C. (2015). "*Innovative Strategies for Combating Biofilm-Based Infections*." in *Biofilm-based Healthcare-associated Infections. Vol. II*, pp. 69–71. (Cham: Springer Int Publishing).

Moher, D. (2010). Corrigendum to: preferred reporting items for systematic reviews and meta-analyses: The PRISMA statement. *Int. J. Surg.* 8, 336–341. doi: 10.1016/j.ijsu.2010.07.299

Moola, S., Munn, Z., Tufanaru, C., Aromataris, E., and Mu, P. F. (2020). "Chapter 7: systematic reviews of etiology and risk" in *JBI Manual for Evidence Synthesis*. eds. E. Aromataris and Z. Munn (JBI)

Nakasone, I., Kinjo, T., Yamane, N., Kisanuki, K., and Shiohira, C. M. (2007). Laboratory-based evaluation of the colorimetric VITEK-2 compact system for species identification and of the advanced expert system for detection of antimicrobial resistances: VITEK-2 compact system identification and antimicrobial susceptibility testing. *Diagn. Microbiol. Infect. Dis.* 58, 191–198. doi: 10.1016/j.diagmicrobio.2006.12.008

OECD (Organization for Economic Cooperation and Development) (2016). OECD Antimicrobial resistance-policy insights. Available at: http://www.oecd.org/health/health-systems/AMR-Policy-Insights-November2016.pdf (Accessed September 3, 2021).

Oliveira, T., Nascimento, B. O. D., Santos, I. D. S., and Paixo, J. A. D. (2021). The use of Antibiotics and the Contribution to the Increase in Bacterial Resistance: A Perspective from a Research in a Health unit in Salvador. *Int. J. Adv. Eng. Sci.* 8, 336–346. doi: 10.22161/ijaers.82.42

Ou, C. J., Shang, D. Q., Yang, J. X., Chen, B., Chang, J., Jin, F. N., et al. (2020). Prevalence of multidrug-resistant Staphylococcus aureus isolates with strong biofilm formation ability among animal-based food in Shanghai. *Food Control.* 112:107106. doi: 10.1016/j.foodcont.2020.107106

Proctor, R., Eiff, C. V., Kahl, B. C., Becker, K., McNamara, P., Herrmann, M., et al. (2006). Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections. *Nat. Rev. Microbiol.* 4, 295–305. doi: 10.1038/nrmicro1384

Puah, S. M., Tan, J. A. M. A., Chew, C. H., and Chua, K. H. (2018). Diverse profiles of biofilm and adhesion genes in *Staphylococcus aureus* food strains isolated from sushi and sashimi. *J. Food Sci.* 83, 346–351. doi: 10.1111/1750-3841.14300

Rodríguez-Lázaro, D., Alonso-Calleja, C., Oniciuc, E. A., Capita, R., Gallego, D., Gonzalez-Machado, C., et al. (2018). Characterization of biofilms formed by foodborne methicillin-resistant *Staphylococcus aureus*. *Front. Microbiol.* 9:3004. doi: 10.3389/fmicb.2018.03004

Singh, R., Sahore, S., Kaur, P., Rani, A., and Ray, P. (2016). Penetration barrier contributes to bacterial biofilm-associated resistance against only select antibiotics, and exhibits genus-, strain- and antibiotic specific differences. *Pathog. Dis.* 74:ftw056. doi: 10.1093/femspd/ftw056

Tang, K. L., Caffrey, N. P., Nóbrega, D. B., Cork, S. C., Ronksley, P. E., Barkema, H. W., et al. (2017). Restricting the use of antibiotics in food-producing animals and its associations with antibiotic resistance in food-producing animals and human beings: a systematic review and meta-analysis. *Lancet Planet Health* 1, e316–e327. doi: 10.1016/S2542-5196(17)30141-9

Van Acker, H., and Coenye, T. (2016). The role of efflux and physiological adaptation in biofilm tolerance and resistance. *J. Biol. Chem.* 291, 12565–12572. doi: 10.1074/jbc.R115.707257

Walters, M. C., Roe, F., Bugnicourt, A., Franklin, M. J., and Stewart, P. S. (2003). Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. *Antimicrobial Agents Chemother.* 47, 317–323. doi: 10.1128/AAC.47.1.317-323.2003

Wang, W., Wang, L., Su, J. Y., and Xu, Z. B. (2020). Antibiotic susceptibility, biofilm-forming ability, and incidence of class 1 integron of *Salmonella* spp., *Escherichia coli*, and *Staphylococcus aureus* isolated from various foods in a school canteen in China. *Foodborne Pathog. Dis.* 17, 269–275. doi: 10.1089/fpd.2019.2694

Wells, G. A. (2014). "The Newcastle-Ottawa Scale (NOS) for assessing the quality of nonrandomized studies in meta-analyses." in *Symposium on Systematic Reviews: Beyond the Basics*.

World Health Organization (2021a). Global Antimicrobial Resistance and Use Surveillance System (GLASS). Available at: https://www.who.int/publications/i/item/9789240027336 (Accessed 3 September, 2021).

World Health Organization (WHO) (2020a). Food-borne disease. Available at: https://www.who.int/health-topics/foodbornediseases#tab=tab_1 (Accessed 3 September, 2021).

World Health Organization (WHO) (2020b). Food safety. Key facts. Available at: https://www.who.int/news-room/fact-sheets/detail/food-safety (Accessed 3 September, 2021).

World Health Organization (WHO) (2021b). World leaders and experts call for significant reduction in the use of antimicrobial drugs in global food systems. Departmental news. Available at: https://www.who.int/news/item/24-08-2021-world-leaders-and-experts-call-for-significant-reduction-in-the-use-of-antimicrobial-drugs-in-global-food-systems (Accessed 3 September, 2021).

Xu, H. Y., Zhang, W., Guo, C., Xiong, H. P., Chen, X., Jiao, X. N., et al. (2019). Prevalence, serotypes, and antimicrobial resistance profiles among salmonella isolated from food catering workers in Nantong, China. *Foodborne Pathog. Dis.* 16, 346–351. doi: 10.1089/fpd.2018.2584

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# New Insights Into the Persistent Effects of Acute Exposure to $AFB_1$ on Rat Liver

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Aflatoxin $B_1$ ($AFB_1$) has mutagenesis, carcinogenesis and teratogenesis effects and mainly found in food crops and their processed foods. $AFB_1$ exposure can cause acute or chronic liver poisoning, but there were few studies on the persistent effects of acute $AFB_1$ exposure on the liver. In this study, rat liver injury models were established 2 and 7 days after single exposure to high and low doses of $AFB_1$. The persistent effects of $AFB_1$ single acute exposure (ASAE) on rat liver were analyzed from the phenotypic and genetic levels. The results showed that compared with the control group, liver function indexes, MDA content in liver and the number of apoptotic hepatocytes in model groups increased to the highest on the 2nd day after ASAE ($p < 0.001$). However, the changes of liver coefficient were most significant on the 7th day after ASAE ($p < 0.01$). The results of liver pathology showed that the liver injury was not alleviated and the activities of antioxidant enzymes GSH-Px and SOD were the lowest on the 7th day ($p < 0.001$). RNA-Seq results indicated that there were 236, 33, 679, and 78 significantly differentially expressed genes (DEGs) in the model groups (LA-2d, LA-7d, HA-2d, HA-7d) compared with the control group. Among them, the *Gtse1* gene related to the proliferation, differentiation and metastasis of liver cancer cells, the *Lama5* and *Fabp4* gene related to the inflammatory response were significantly DEGs in the four model groups, and the differential expression of the immune system-related Bcl6 gene increased with the prolonged observation time after ASAE. In conclusion, ASAE can cause persistent liver damage in rats. The persistently affected genes *Lama5*, *Gtse1*, *Fabp4*, and *Bcl6* possess the potential to be therapeutic targets for liver disease induced by $AFB_1$.

**Keywords: aflatoxin $B_1$, RNA-seq, hepatotoxicity, target gene, oxidative stress**

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## INTRODUCTION

Aflatoxins (AFs) is a general term for a class of highly toxic secondary metabolites produced by *Aspergillus parasiticus* and *Aspergillus flavus*. Crops such as grain and oilseed are easily contaminated by AFs during planting, harvesting, storage and processing. In addition, its physical and chemical properties are stable. Therefore, there is a great risk of harming animal and human health through the food chain (Rushing and Selim, 2019). At present, more than 20 kinds of AF

**TABLE 1 |** Different animal models were established to explore the effects of $AFB_1$ on liver.

| Species | Dose | Modeling time (d) | Breeding time after modeling (h) | Effect (liver injury) | References |
|---|---|---|---|---|---|
| Rat | 2 mg/kg | 1 | 48 | Acute | Deng et al., 2020 |
| Rat | 1 mg/kg | 1 | 96 | Acute | Monmeesil et al., 2019 |
| Mice | 3 mg/kg | 2 | 12 | Acute | Ishida et al., 2020 |
| Mice | 100 μg/day | 14 | 12 | Chronic | Long et al., 2016 |
| Mice | 0.75 mg/kg | 30 | 24 | Chronic | Xu et al., 2019 |
| Rat | 20 μg/day | 42 | 24 | Chronic | Nayak and Sashidhar, 2010 |
| Rat | 25 μg/day | 90 | 24 | Chronic | El-Agamy, 2010 |

have been found, among which $AFB_1$ is the most toxic and most common, and the target organ of its toxic effect is mainly the liver (Hussein and Brasel, 2001). A warm and humid environment is easy to cause a large amount of $AFB_1$ pollution (Abrar et al., 2013), so acute or chronic poisoning is often caused by dietary exposure to $AFB_1$. **Table 1** lists the effects of different doses of $AFB_1$ and modeling time on the liver in some studies. It can be seen from the table that $AFB_1$ can cause acute or chronic poisoning in the liver. But viewed from the model, there are few studies on the lasting effects of $AFB_1$ exposure on the liver.

$AFB_1$ is metabolized by the P450 enzyme system in the liver into the ultimate carcinogen aflatoxin $B_1$-8, 9-epoxide (AFBO), which has strong oxidation ability and can induce the body to produce a large amount of reactive oxygen species (ROS) (Guengerich et al., 1998). AFBO can covalently bind to DNA guanine N7 to form adduct $AFB_1$-N7-guanine (Lin et al., 2014), and oxidize guanine to produce DNA damage marker 8-OH-deoxyguanine (Bailey et al., 1996; Wang and Groopman, 1999). In addition, excessive ROS will break the dynamic balance of the redox system and cause oxidative stress reaction. It also attacks cells, causing cells damage and inducing apoptosis. Therefore, this study explored the liver injury in model rats by analyzing the changes of phenotypic indicators related to oxidative stress response.

RNA-seq is a widely used parallel sequencing method. Through this technology, the complete transcript of the test sample and its expression level can be found (Wang et al., 2009). From the obtained data, we can not only know individual genes but also understand the functional pathways of gene enrichment through the analysis of functional database. RNA-seq data has been used to analyze the underlying mechanism of carotid atherosclerosis (CAS). Differential and functional enrichment analysis of sequencing data from CAS patients and control group showed that inflammation and immune response were the potential pathogenesis of CAS, and significant DEGs *CCR5*, *NPY,* and *NPY5R* were potential therapeutic targets of CAS (Li et al., 2021). The method is also often used to study cancer therapeutic targets. Bioinformatics analysis of DEGs in ovarian cancer and normal tissue showed that they were mainly concentrated in metabolic, cell cycle regulation and antibiotic biosynthesis pathways. Meanwhile, 10 central genes including *CCNB2*, *TYMS* and *KIF11* were analyzed (Yang et al., 2020). Sorafenib is the only FDA approved oral multi-target tyrosine kinase inhibitor that promotes apoptosis, reduces angiogenesis and inhibits tumor cell proliferation in the treatment of liver cancer. However, drug resistance and side effects of varying degrees were found in the process of drug use (Trojan and Zeuzem, 2013; Rota Caremoli and Labianca, 2014). Therefore, more potential genes for targeted treatment of liver diseases can be searched through gene sequencing technology.

This paper explores the sustained effects of $AFB_1$ on the rat liver by establishing a model of ASAE. Based on this model, the RNA-Seq data were analyzed to find potential target genes for the treatment of liver diseases caused by $AFB_1$, providing a new research basis for the development of targeted therapy for liver diseases.

**TABLE 2 |** Primer information.

| Gene | Primer sequence (5′to′) | Number of bases |
|---|---|---|
| β-*Actin* | Forward: AGCGTGGCTACAGCTTCACC | 20 |
| | Reverse: AAGTCTAGGGCAACATAGCACAGC | 24 |
| *Lama5* | Forward: TTCACAGGAGAGCGGTGTTC | 20 |
| | Reverse: CGTTGCAGTCACAGTTCACG | 20 |
| *Ccnd1* | Forward: TCAAGTGTGACCCGGACTG | 19 |
| | Reverse: GACCAGCTTCTTCCTCCACTT | 21 |
| *Cdkn1a* | Forward: TGTGATATGTACCAGCCACAGG | 22 |
| | Reverse: GCGAAGTCAAAGTTCCACCG | 20 |
| *Gpx2* | Forward: ATCAGTTCGGACATCAGGAGA | 21 |
| | Reverse: TCACCATTCACCTCGCACTT | 20 |

# MATERIALS AND METHODS

## Chemicals

Aflatoxin $B_1$ (≥ 99%) and Dimethyl sulfoxide (DMSO) were purchased from Shanghai Acmec Biochemical Co., Ltd., and Sangon Biotech Co., Ltd. (Shanghai, China), respectively. Assay kits for the measurements of malondialdehyde (MDA), superoxide dismutase (SOD) Glutathione peroxidase (GSH-Px) and total protein (TP) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Assay kits for the total RNA extraction, PowerUp SYBR Green Master Mix (Thermo Fisher Scientific, America) and HiScript III-RT SuperMix for qPCR (Vazyme, China).

## Animals

A total of 36 male Wistar rats were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. Body weight (BW): 180-200 g. All rats were housed in an environmentally controlled room at

22-24°C with a relative humidity of 50-55% under a cycle of 12 h each light/dark. Food and water were available *ad libitum*. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Shanghai University of Traditional Chinese Medicine and approved by the Animal Ethics Committee of Animal Center of China and Shanghai University of Traditional Chinese Medicine (Ethics number: PZSHUTCM210115014).

## Experimental Design

After one week of adaptive feeding, 36 rats were randomly divided into 3 groups (12 in each group): blank group (CK): normal breeding; $AFB_1$ high-dose (HA) group: $AFB_1$ was intraperitoneal injection with a dose of 2 mg/kg BW, only once; $AFB_1$ low-dose (LA) group: $AFB_1$ was intraperitoneal injection at a dose of 1 mg/kg BW, only once ($AFB_1$ soluble in 4% DMSO). The experimental doses of $AFB_1$ were determined according to references and results of pre-experiment (Monmeesil et al., 2019; Deng et al., 2020).

The above dose groups were determined based on references and preliminary experiments. The concentration of DMSO did not affect the normal growth of rats (Chen et al., 2020). Blood samples were collected from ocular venous plexus at the 1st, 4th and 7th day after ASAE and serum were prepared. On the 2nd day after ASAE, 6 rats in each group were randomly selected to weigh their final body weight. The rats were anesthetized with 10% chloral hydrate (350 mg/kg). After anesthesia, the rats were dissected, the blood of abdominal aorta was taken, the liver was separated, washed with normal saline, sucked dry and weighed. Liver tissues of an appropriate size were taken from the same part of the liver of each rat and soaked in 4% paraformaldehyde solution, which was fully fixed for histopathological study, and the rest tissues were frozen in liquid nitrogen and stored in a cryogenic refrigerator at −80°C for use. The remaining 6

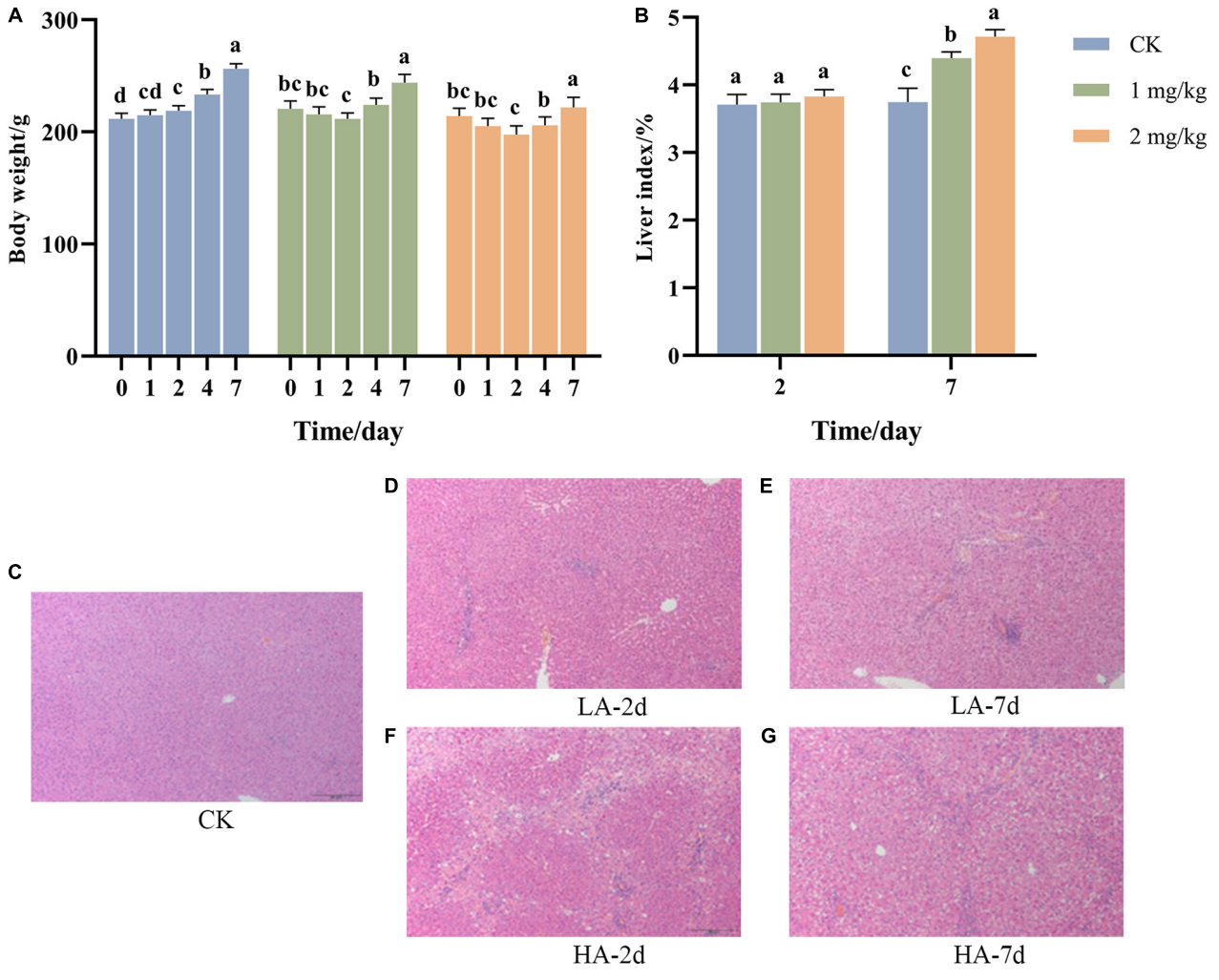



**FIGURE 1 |** Changes of **(A)** body weight, **(B)** liver index, and **(C–G)** liver pathological morphology with time after ASAE in rats (HE, × 100). 0: weight before ASAE; 1 and 4: weight on 1st and 4thr days after ASAE; 2 and 7: weight and index on 2nd and 7th days after ASAE. LA-2d and HA-2d: rats were sacrificed on the 2nd day after ASAE. LA-7d and HA-7d: rats were sacrificed on the 7th day after ASAE. The data are expressed as mean ± standard deviation. Different letters representing significant difference ($p < 0.05$) (the same below).

rats in each group were treated with the same method on the 7th day after ASAE.

## Hepatic Pathological Examination

After being fixed in 4% paraformaldehyde for 24 h, the liver was removed and dehydrated in graded alcohol series, transparent in xylene. Tissues were embedded in paraffin, and paraffin blocks were then cut to 4 μm thickness using a microtome (RM2016, Shanghai Leica Instrument Co., Ltd., Germany). These sections were deparaffinized, rehydrated and stained using hematoxylin-eosin (HE) and then analyzed by optical microscopy (Nikon Eclipse E100, Japan) to evaluate histopathological changes.

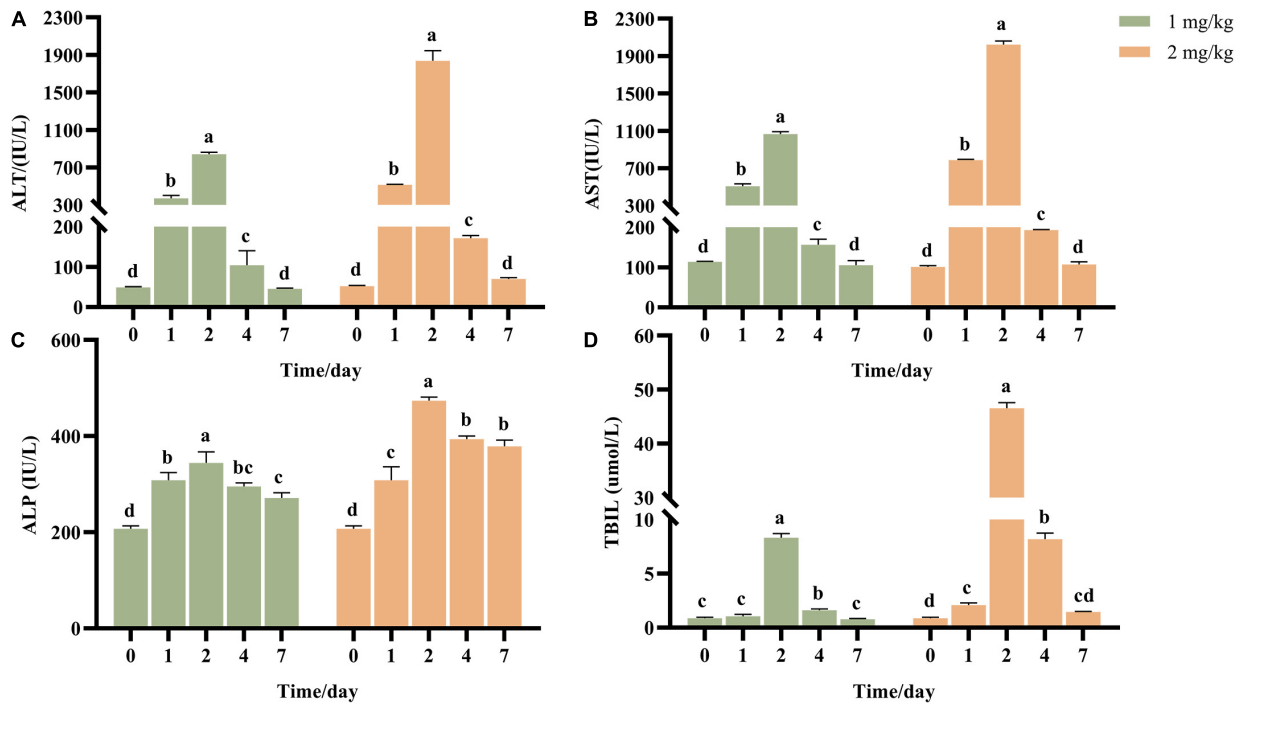



**FIGURE 2 |** Effect of $AFB_1$ on serum liver function indicators in rats. **(A)** Serum alanine aminotransferase (ALT), **(B)** aspartate aminotransferase (AST), **(C)** alkaline phosphatase (ALP) activities, and **(D)** total bilirubin (TBIL) were measured. 0: serum samples before ASAE; 1, 2, 4 and 7: serum samples on 1st, 2nd, 4th and 7th days after ASAE.

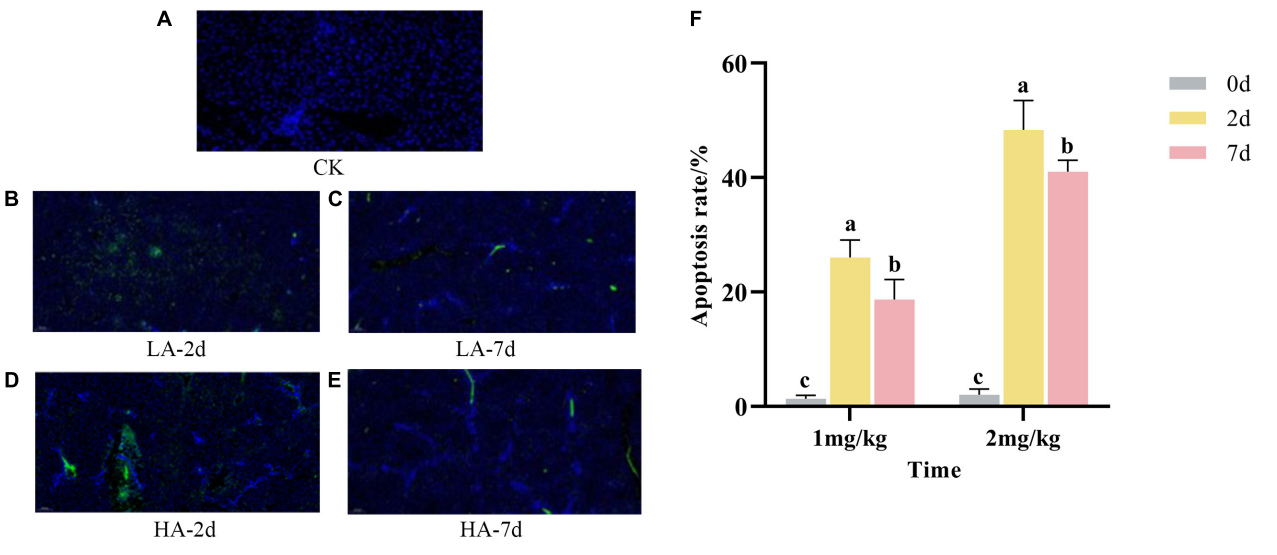



**FIGURE 3 |** Effect of $AFB_1$ on apoptosis in rat hepatocytes. **(A–E)**: representative images of TUNEL staining (× 200), **(F)**: TUNEL assay for percentage of hepatocyte apoptosis.

Observe the morphologies of the liver and spleen tissues were observed at 100 ×.

## Determination of Biochemical Indices in Serum and Liver

The serum was separated and measured the levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP), aspartate aminotransferase (AST) and total bilirubin (TBIL) by automated chemistry analyzer (ADVIA 2120i, Hitachi, Ltd., Japan). The activities of SOD and GSH-Px, as well as the level of MDA were examined using commercial assay kits.

## Determination of Hepatocyte Apoptosis Rate

The apoptosis rate of rat hepatocytes in the model groups were detected by TUNEL method. Paraffin sections were taken in sequence for deparaffinize and rehydrate, antigen retrieval, permeabilization, equilibration at room temperature, TUNEL reaction, BSA blocking, addition of double antibodies, DAPI counterstain in nucleus and then sealed for microscopic examination. Stained nuclei were blue under UV excitation and positive apoptotic cells were green. The number of apoptotic hepatocytes in the total field of view was counted as well as the total number of hepatocytes. Calculation formula: Hepatocyte apoptosis rate = (Number of apoptotic hepatocytes/Total number of hepatocytes) × 100%.

## Illumina RNA Sequencing

Total RNA in the rats' liver was extracted using the MagMAX$^{TM}$ mir Vana$^{TM}$ Total RNA Isolation Kit following the specification (Thermo Scientific$^{TM}$ KingFisher$^{TM}$ Flex$^{TM}$, Finland). Total RNA of each sample was quantified and qualified by Agilent 2100/2200 Bioanalyzer (Agilent Technologies, Palo Alto, CA, United States), NanoDrop (Thermo Fisher Scientific Inc.). 1 μg total RNA was used for following library preparation. Illumina RNA sequencing experiments used three parallel samples. The sequencing library construction and Illumina sequencing were conducted at GENEWIZ.

## Real-Time Quantitative PCR Analysis

To verify the reliability of the expression profiles observed in the RNA-Seq data, four genes were selected for real-time quantitative PCR (Q-PCR) analysis with the same experimental samples as in the RNA-Seq experiments. Total RNA was extracted using a King Fisher Flex automated nucleic acid extractor and accompanying kit, and reverse transcribed after RNA electrophoresis to determine RNA quality. β-*Actin* was used as an internal reference and primers designed by Primer 5 are shown in **Table 2**, and relative expression was calculated according to the $2^{-\Delta\Delta ct}$ relative quantification formula.

## Statistical Analysis

Results were analyzed using GraphPad Prism 8.0 (GraphPad Software, La Jolla, California), SPSS 25 (IBM, NY, United States) and Origin 8.0 software (Origin Inc., Northampton, MA, United States). Analysis of variance (ANOVA) was performed followed by Tukey's test with a confidence interval of 95% ($p \leq 0.05$).

# RESULTS

## Body Weight and Liver Index Changes in Rats

During the experiment, the BW of the rats was weighed before ASAE and on the 1st, 2nd, 4th and 7th after ASAE (**Figure 1A**). The BW of rats in the CK group showed an upward trend in the whole experimental cycle, while that in the model groups decreased and then increased.

The organ index is one of the main indicators of the biological properties of animals (Zou et al., 2010). **Figure 1B** shows that there was no significant difference in liver index between rats treated on the 2nd day after ASAE and the CK group. Compared on the 7th day after ASAE with the CK group, the liver coefficient of the model groups was significantly higher ($p < 0.05$), and the HA group was significantly different from the CK group ($p < 0.001$).

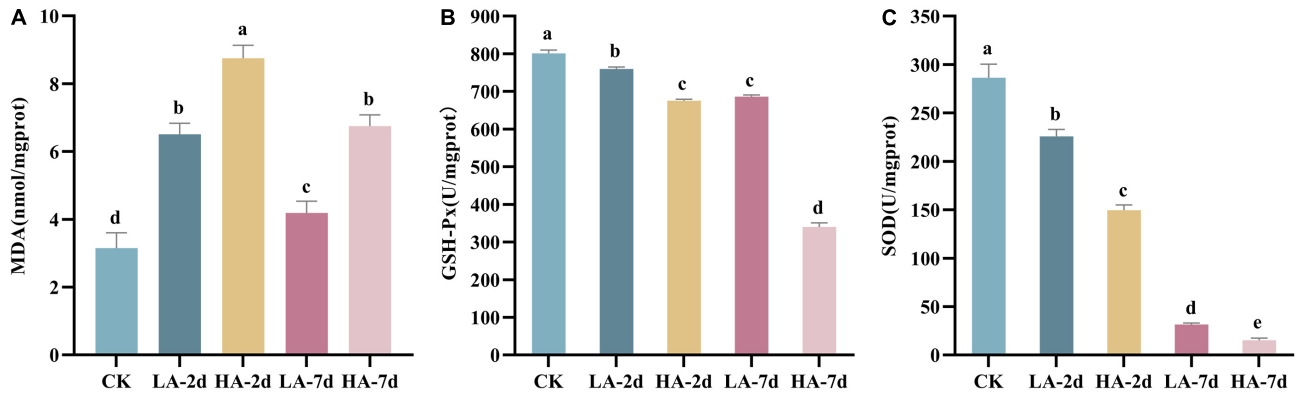
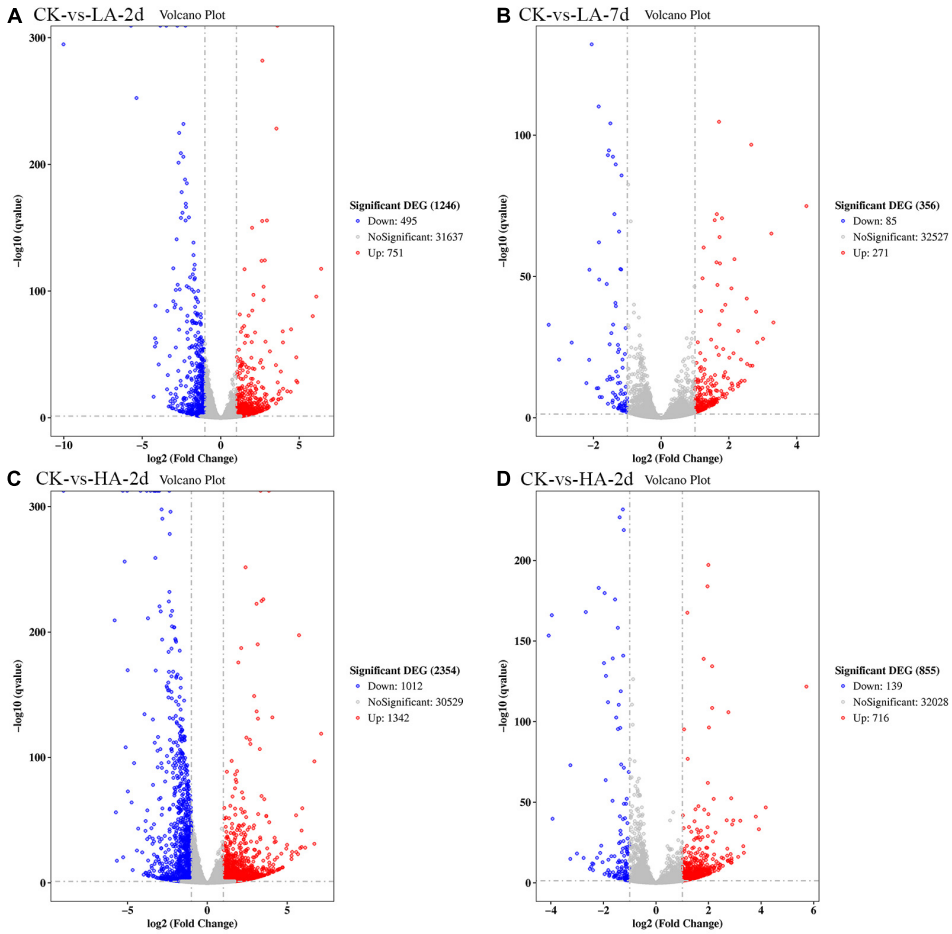



**FIGURE 4 |** Effect of $AFB_1$ on hepatic tissue lipid peroxidation level and antioxidant enzyme activities in rats. **(A)** Hepatic malondialdehyde (MDA) content, **(B)** hepatic glutathione peroxidase (GSH-Px) and **(C)** hepatic superoxide dismutase (SOD) activity.



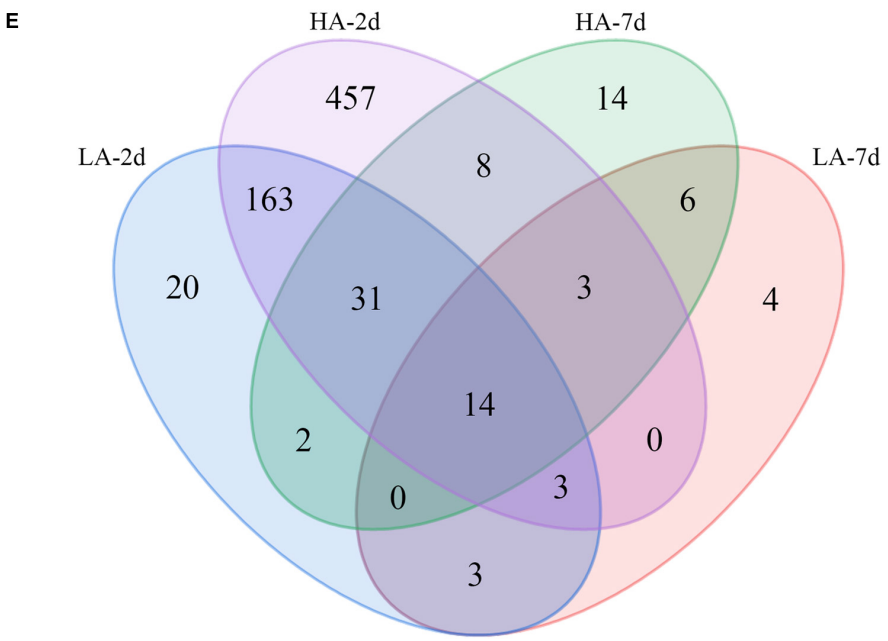




**FIGURE 5 |** The Volcano plot and Venn diagram of DGEs. **(A–D)** Red spots indicate significantly up-regulated genes; blue spots indicate significantly down-regulated genes; gray spots are genes of no differential expression. The horizontal line at false discovery rate (FDR) = 0.05; vertical line at | log2FC| = 1. **(E)** There were special DEGs in the different analyses of each group (Venn diagram, FDR ≤ 0.01 and | log2FC| ≥ 2).

## Histopathological Changes of Liver Tissue in Rats

Representative hepatic histopathological examination results of each group were shown in **Figures 1C–G**. The rats in the CK group had a normal liver lobular architecture and cell structure. The nucleolus was clear, homogenous cytoplasm (**Figure 1C**). Livers illustrated different degrees of pathological changes in the model groups. In LA-2d group, the liver exhibited moderate spotty necrosis and degeneration (**Figure 1D**). In LA-7d group, moderate spotty necrosis, vacuolar degeneration, and mild inflammatory cell infiltration were observed in the portal area (**Figure 1E**). Hepatocyte lytic necrosis, called hepatocyte bridging necrosis, was widely found in the liver pathological sections of rats in HA-2d group. This phenomenon was common in liver poisoning or severe chronic hepatitis (**Figure 1F**). In HA-7d group, in addition to severe bridging necrosis of hepatocytes, there were also vacuolar degeneration of hepatocytes and fibrous tissue hyperplasia (**Figure 1G**).

## Effect of $AFB_1$ on Liver Function

Transaminase ALT and AST are common indicators to determine whether the liver is damaged. ALT mainly exists in the plasma of hepatocytes, and the elevation of ALT in serum reflects the injury of hepatocytes. AST mainly exists in mitochondria of hepatocytes, and elevated AST indicates more serious liver injury. ALP and TBIL are the detection indexes reflecting hepatic bile metabolism. After ASAE, ALT, AST, ALP and TBIL in the model groups increased first and then decreased (**Figure 2**). It reached the peak on the 2nd day after ASAE, which was significantly different from that in the CK group ($p < 0.001$). Except ALP, other indexes returned to normal level on the 7th day after ASAE.

## Analysis of Apoptosis in Rat Hepatocytes

Apoptosis refers to the process by which cells are affected by abnormal factors and controlled by genes to end their life autonomously. As shown in **Figures 3A-E**, the nuclear membranes of normal hepatocytes were intact and stained blue; the nuclear membranes of apoptotic hepatocytes were clustered or fragmented and stained green. As can be seen from **Figure 3F**, compared with the CK group, the apoptosis rate in the model groups was significantly increased in a dose-dependent manner. However, the apoptosis rate of hepatocytes on the 7th after ASAE was slightly lower than that on the 2nd after ASAE, and there was still significant difference ($p < 0.05$).

## Hepatic Antioxidative Status and Lipid Peroxidation

The content of MDA can reflect the degree of lipid peroxidation and indirectly reflect the degree of cell damage. In this study, the MDA content in model groups were significantly higher than that in CK group ($p < 0.05$) (**Figure 4A**), indicating that $AFB_1$ caused hepatocyte injury. It was also found that MDA content in the liver of the rats after ASAE not only showed a dose-dependent manner, but also first increasing and then decreasing trend with prolonged observation. However, it was still significantly higher than the CK group and did not return to the normal levels on the 7th day ($p < 0.05$).

Studies have shown that $AFB_1$ can lead to the imbalance of the body's oxidative system homeostasis and cause oxidative stress response. The antioxidant enzymes SOD and GSH-Px are the body's first line of defense against free radicals. In this study, compared with the CK group, the activities of SOD and GSH-Px in the model groups decreased significantly ($p < 0.05$) (**Figures 4B,C**). And with the prolonged observation time after ASAE, the activities of SOD and GSH-Px became lower and lower ($p < 0.001$).

## Differential Expression Genes and Functional Analysis

In this study, the DEGs between the model groups and the CK group were obtained by RNA-Seq technology, and a volcano map was drawn (**Figures 5A–D**). The volcano plot showed that LA-2d, LA-7d, HA-2d and HA-7d groups had 1246, 356, 2354, and 855 DEGs, respectively (FDR $\leq 0.05$ and $|\log_2 FC| \geq 1$). From this result, it can be found that the number of DEGs was related to the dose of ASAE and the observation time after ASAE: the higher the dose, the more DEGs; the number of DEGs decreased with the prolonged time of continuous impact. **Table 3** shows the classification and number of entries of KEGG pathways enriched in each group of DEGs. Looking at the total number of KEGG pathways in each model group, there were fewer on the 7th day after ASAE than on the 2nd day. The number of entries for each classification in each group shows that the main difference was in the metabolic category. This is related to the fact that the liver is the largest metabolic organ of the body as well as being extremely self-repairing.

In order to explore the genes that were continuously affected and their functions, the screening criteria were strengthened, and the Venn diagram as shown was drawn (FDR $\leq 0.01$ and $|\log_2 FC| \geq 2$) (**Figure 5E**). Compared with CK group under the above filter conditions, the LA-2d, LA-7d, HA-2d and HA-7d groups had 236, 33, 679, and 78 differential genes, respectively. There were 211 common genes in LA-2d and HA-2d, 23 common genes in LA-7d and HA-7d, 20 common genes in LA-2d and LA-7d, and 56 common genes in HA-2d and HA-7d. In order to analyze the accuracy of the results, the first 20 independent DEGs were selected from each group, and all the ones less than

**TABLE 3 |** Number of pathways for each KEGG classification in the model group.

| Level | LA-2d | LA-7d | HA-2d | HA-7d |
|---|---|---|---|---|
| Cellular Processes | 22 | 20 | 22 | 21 |
| Environmental Information Processing | 33 | 32 | 34 | 33 |
| Genetic Information Processing | 13 | 13 | 16 | 14 |
| Human Diseases | 84 | 75 | 84 | 82 |
| Metabolism | 83 | 48 | 91 | 67 |
| Organismal Systems | 84 | 74 | 85 | 84 |
| **Total** | 320 | 262 | 333 | 301 |

**TABLE 4 |** Effects of AESE dose on DEGs enriched pathways and expression levels.

| Gene ID (ENSRNOG-) | Gene Symbol | Log2FC | | Pathway |
|---|---|---|---|---|
| | | LA-2d | HA-2d | |
| **Cellular Processes** | | | | |
| 00000003897 | *Col1a1* | 2.041 | 2.096 | ko04510 |
| 00000020918 | *Ccnd1* | 3.553 | 3.511 | ko04510 |
| 00000043451 | *Spp1* | 3.669 | 5.572 | ko04510 |
| 00000053691 | *Lama5* | 4.817 | 5.954 | ko04510 |
| **Environmental Information Processing** | | | | |
| 00000051171 | *G6pc* | −2.272 | −3.933 | ko04068, ko04152 |
| 00000020918 | *Ccnd1* | 3.553 | 3.511 | ko04068, ko04152 |
| 00000021814 | *Tnfrsf25* | 2.572 | 3.138 | ko04060 |
| 00000000521 | *Cdkn1a* | 2.646 | 3.340 | ko04068 |
| 00000003546 | *Tnfrsf12a* | 2.193 | 2.872 | ko04060 |
| 00000007002 | *Lif* | 2.572 | 4.111 | ko04060 |
| 00000013018 | *Eda2r* | 2.202 | 3.829 | ko04060 |
| 00000013090 | *Gadd45g* | | −3.231 | ko04068 |
| 00000013552 | *Scd* | −5.715 | −5.805 | ko04152 |
| **Metabolism** | | | | |
| 00000020420 | *Pklr* | −2.539 | −3.275 | ko01100 |
| 00000020775 | *Cyp2b2* | −2.553 | −3.338 | ko01100, ko00830, ko00590, ko00140 |
| 00000001379 | *Cyp3a62* | 2.022 | 2.739 | ko01100, ko00830, ko00140, ko00591 |
| 00000001388 | *Sds* | −2.858 | −5.113 | ko00260, ko01100 |
| 00000001466 | *LOC100361492* | 4.823 | 5.824 | ko01100, ko00830, ko00590, ko00140, ko00591 |
| 00000002597 | *Hsd17b6* | −2.134 | −2.066 | ko01100, ko00830, ko00140 |
| 00000003244 | *Ltc4s* | −2.421 | −2.386 | ko01100, ko00590 |
| 00000005358 | *Pmm1* | 2.194 | 2.932 | ko01100 |
| 00000009080 | *Atp6v1d* | 2.604 | 3.158 | ko01100 |
| 00000009734 | *Akr1b8* | 2.027 | 3.264 | ko01100, ko00561 |
| 00000009754 | *Nampt* | −2.115 | −2.145 | ko01100 |
| 00000009875 | *Akr1b7* | 2.249 | 3.880 | ko01100, ko00561 |
| 00000010633 | *Acsl1* | −2.374 | −3.016 | ko01100, ko01212, ko00071 |
| 00000011200 | *Bhmt* | −2.378 | −2.263 | ko00260, ko01100 |
| 00000011250 | *Inmt* | −4.178 | −2.046 | ko00380 |
| 00000011381 | *Acsbg1* | −2.141 | −3.408 | ko01100, ko01212, ko00071 |
| 00000011420 | *Mtmr7* | −2.012 | −2.820 | ko01100 |
| 00000013241 | *Cyp2c24* | 3.631 | 4.291 | ko01100, ko00830, ko00590, ko00140, ko00591 |
| 00000013552 | *Scd* | −5.715 | −5.805 | ko01212, ko01040 |
| 00000015076 | *Cyp26b1* | −2.858 | −2.474 | ko01100, ko00830 |
| 00000016791 | *Chka* | −2.533 | −2.863 | ko01100 |
| 00000016826 | *Pla2g2d* | 2.266 | 3.716 | ko01100, ko00590, ko00591 |
| 00000016924 | *Acly* | −2.206 | −3.237 | ko01100 |
| 00000017311 | *Me3* | −2.881 | −3.343 | ko01100 |
| 00000017619 | *Aldh1a1* | 2.654 | 2.398 | ko01100, ko00830 |
| 00000017878 | *Aldh1a7* | 3.526 | 4.368 | ko01100, ko00830 |
| 00000022268 | *Pnpla3* | −2.701 | −2.661 | ko01100, ko00561 |
| 00000025691 | *Pla2g7* | 2.749 | 3.884 | ko01100 |
| 00000026764 | *Pla2g4c* | | −3.987 | ko01100, ko00590, ko00591 |
| 00000045743 | *Etnppl* | −2.880 | −2.627 | ko01100 |
| 00000052810 | *Cyp2c11* | −3.469 | −4.585 | ko01100, ko00830, ko00590, ko00140, ko00591 |
| 00000053448 | *AC123346.1* | −2.741 | −2.936 | ko00410, ko00260, ko01100 |
| 00000053691 | *Lama5* | 4.817 | 5.954 | ko01100 |
| 00000055221 | *Acot1* | | 3.033 | ko01100, ko01040 |
| 00000055672 | *Gpx2* | 2.490 | 3.264 | ko00590 |
| 00000057470 | *Pla2g12a* | 2.566 | 2.566 | ko01100, ko00590, ko00591 |

*(Continued)*

**TABLE 4 |** (Continued)

| Gene ID (ENSRNOG-) | Gene Symbol | Log2FC | | Pathway |
|---|---|---|---|---|
| | | LA-2d | HA-2d | |
| 00000051171 | *G6pc* | −2.272 | −3.933 | ko01100, ko00500 |
| **Organismal Systems** | | | | |
| 00000005250 | *Abcg5* | | −3.045 | ko04979, ko04975 |
| 00000005420 | *Abcg8* | −2.088 | −2.148 | ko04979, ko04976, ko04975 |
| 00000009686 | *Aqp7* | 3.037 | 3.037 | ko03320, ko04923 |
| 00000010633 | *Acsl1* | −2.374 | −3.016 | ko03320 |
| 00000010805 | *Fabp4* | 3.685 | 5.909 | ko03320, ko04923 |
| 00000011381 | *Acsbg1* | −2.141 | −3.408 | ko03320 |
| 00000013552 | *Scd* | −5.715 | −5.805 | ko03320 |
| 00000016826 | *Pla2g2d* | 2.266 | 3.716 | ko04975 |
| 00000024947 | *Fabp2* | 2.752 | 3.916 | ko03320, ko04975 |
| 00000051171 | *G6pc* | −2.272 | −3.933 | ko04973 |
| 00000057470 | *Pla2g12a* | 2.566 | 2.566 | ko04975 |
| | | **LA-7d** | **HA-7d** | |
| **Cellular Processes** | | | | |
| 00000016148 | *Gtse1* | 2.691 | 3.323 | ko04115 |
| **Environmental Information Processing** | | | | |
| 00000001843 | *Bcl6* | 2.524 | 2.167 | ko04068 |
| 00000013552 | *Scd* | | −2.671 | ko04152 |
| **Organismal Systems** | | | | |
| 00000010805 | *Fabp4* | 2.321 | 3.915 | ko03320 |
| 00000013552 | *Scd* | | −2.671 | ko03320 |

20 were selected for KEGG enrichment analysis. According to KEGG enrichment analysis, these genes are mainly enriched in four types of biological metabolic pathways: Cellular Processes, Environmental Information Processing, Metabolism and Organismal Systems (**Tables 4**, **5**).

By analyzing the DEGs in **Tables 4**, **5**, it was found that DEGs of LA-2d and HA-2d were mainly enriched in pathways related to carbohydrate, lipid and amino acid metabolism, while DEGs of LA-7d and HA-7d were mainly enriched in four pathways, which were related to cell cycle regulation, inflammatory response and oxidation reaction. *Lama5*, *Gtse1* and *Fabp4* gene in DEGs were all highly expressed in the model groups. *Bcl6* gene was highly expressed on the 7th day after ASAE, and the expression level was higher than that on the 2nd day after ASAE. *Lama5* gene enrichment in focal adhesion (ko04510) and metabolic pathways (ko01100). Focal adhesion pathway plays an important role in cell motility, cell proliferation, and cell differentiation (Ye et al., 2020). The dysregulation of focal adhesion is considered to be an essential step in tumor invasion, it can promote tumor invasiveness and metastasis (Shen et al., 2018). Metabolic pathway plays an important role in the metabolism of sugar, fat and protein in the liver. Metabolic dysfunction not only affects the normal growth of the body, but also causes various diseases. *Gtse1* gene enrichment in p53 signaling pathway (ko04115). The protein encoded by the *p53* gene is a transcription factor that controls the cell cycle. The occurrence of oxidative stress and DNA damage in the body can cause *p53* mutation, resulting in cell cycle dysregulation (Lacroix et al., 2020; Bailey et al., 2021). *Fabp4* gene enrichment in regulation of lipolysis in adipocytes (ko04923) and PPAR signaling pathways (ko03320). Regulation of lipolysis in adipocytes pathway plays an important role in the dynamic equilibrium of lipid droplet formation and decomposition (Yang and Mottillo, 2020). Dysregulation of this pathway leads to lipid accumulation in the liver causes hepatocyte damage and portal inflammation (An et al., 2021). PPAR signaling pathway plays an essential role in the maintenance of metabolic homeostasis, it can adjust the balance between anabolism and oxidation of adipose tissue to prevent peroxidation (Corrales et al., 2018). *Bcl6* gene enrichment in FoxO signaling pathway (ko04068). The pathway can inhibit cell metabolism, growth, differentiation, oxidative stress, and aging, it is suggested to play a pivotal functional role as a tumor suppressor in cancers (Farhan et al., 2017; Lee and Dong, 2017).

## Quantitative Validation

Quantitative validation of the predicted differential genes based on transcriptome sequencing data was carried out for *Lama5*, *Ccnd1*, *Cdkn1a* and *Gpx2*, which were randomly selected. RNA integrity is shown in the plots from electrophoresis experiments using a fully automated nucleic acid electrophoresis analyzer as in **Figure 6**. The validation results are shown in **Table 6**. The transcriptome sequenced genes with Q-PCR showed the same trend in different model groups.

**TABLE 5 |** Effects of AESE duration on DEGs enrichment pathway and expression.

| Gene ID (ENSRNOG-) | Gene symbol | Log2FC | | Pathway |
|---|---|---|---|---|
| | | LA-2d | LA-7d | |
| Cellular processes | | | | |
| 00000016148 | *Gtse1* | 3.755 | 2.691 | ko04115 |
| 00000020918 | *Ccnd1* | 3.553 | | ko04115 |
| Environmental information processing | | | | |
| 00000013552 | *Scd* | −5.715 | | ko04152 |
| 00000020918 | *Ccnd1* | 3.553 | | ko04068, ko04152 |
| 00000051171 | *G6pc* | −2.272 | | ko04068, ko04152 |
| Organismal systems | | | | |
| 00000007152 | *Bhlhe40* | −2.860 | −2.116 | ko04710 |
| 00000007545 | *Angptl4* | −2.032 | | ko03320 |
| 00000010633 | *Acsl1* | −2.374 | | ko03320 |
| 00000010805 | *Fabp4* | 3.685 | 2.321 | ko03320, ko04923 |
| 00000013552 | *Scd* | −5.715 | | ko03320, ko04212 |
| 00000038047 | *Mt1* | −2.472 | 3.249 | ko04212 |
| 00000043098 | *Mt2A* | −2.189 | 2.657 | ko04212 |
| | | HA-2d | HA-7d | |
| Environmental information processing | | | | |
| 00000000521 | *Cdkn1a* | 3.340 | 2.015 | ko04068 |
| 00000013552 | *Scd* | −5.805 | −2.671 | ko04068 |
| 00000051171 | *G6pc* | −3.933 | | ko04068 |
| Metabolism | | | | |
| 00000017878 | *Aldh1a7* | 4.368 | 2.877 | ko00830 |
| 00000019058 | *Gstm3l* | 4.218 | 2.875 | ko00983 |
| 00000026764 | *Pla2g4c* | −3.987 | | ko00590, ko00591 |
| 00000055672 | *Gpx2* | 3.264 | 2.715 | ko00590 |
| 00000001466 | *LOC100361492* | 5.824 | 3.129 | ko00140, ko00590, ko00591, ko00830 |
| 00000005341 | *Upp2* | | 2.226 | ko00983 |
| 00000013241 | *Cyp2c24* | 4.291 | 2.711 | ko00140, ko00590, ko00591, ko00830 |
| Organismal systems | | | | |
| 00000005794 | *Slc10a1* | −4.743 | | ko04976 |
| 00000010805 | *Fabp4* | 5.909 | 3.915 | ko03320 |
| 00000013552 | *Scd* | −5.805 | −2.671 | ko03320 |
| 00000024947 | *Fabp2* | 3.916 | | ko03320 |

## DISCUSSION

In this study, a rat model of acute injury was established by a single intraperitoneal injection of 1 and 2 mg/kg $AFB_1$, and then the rats were fed normally for 2 and 7 days, respectively. The aim was to study the persistent effects of ASAE on rat liver. We found that other indexes except antioxidant enzyme and liver coefficient showed a trend of recovery with the continuous influence time of the $AFB_1$ on liver. Among them, ALT, AST and TBIL returned to normal, ALP, hepatocyte apoptosis rate and MDA content still had significant differences compared with CK group ($p < 0.05$). Antioxidant enzyme activity continued to decrease with the prolonged exposure time of $AFB_1$. The liver coefficient is rising. Meanwhile, the model was used to explore the persistent effects of acute $AFB_1$ exposure on liver genes, and we found that the number of DEGs decreased significantly over time.

The data in this article shows that although there is no significant difference in the weight of rats after ASAE compared with that before ASAE, it still presents a downward trend. It illustrates that $AFB_1$ leads to the reduction of food intake in rats, and the decline of the body's metabolic capacity, so that the nutrients cannot be fully absorbed (Jindal et al., 1994). This is consistent with the results of previous research (Rastogi et al., 2001; Knipstein et al., 2015). Compared with the CK group, SOD and GSH-Px activities decreased, MDA content increased, and cell apoptosis rate increased on the 2nd day after ASAE, indicating that $AFB_1$ can cause oxidative stress reaction and liver cell apoptosis, resulting in severe liver damage. This result is similar to other published papers on acute liver injury caused by $AFB_1$ (Wang et al., 1991; Kumagai et al., 1998; Benkerroum, 2020; Ruggeberg et al., 2020). On the 4th day after ASAE, ALT and AST values decreased, but they were still significantly higher than the CK group ($p < 0.05$), which was consistent with previous

**TABLE 6 |** Summary of Q-PCR results and RAN-Seq results for randomly selected genes.

| | Fold change | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | CK | LA-2d | | LA-7d | | HA-2d | | HA-7d | |
| Relative gene expression level | | Q-PCR | RNA-Seq | Q-PCR | RNA-Seq | Q-PCR | RNA-Seq | Q-PCR | RNA-Seq |
| *Lama5* | 1 | 22.91 | 28.19 | 13.78 | 9.93 | 38.45 | 61.99 | 19.19 | 18.08 |
| *Ccnd1* | 1 | 7.08 | 11.74 | 1.25 | 0 | 4.88 | 11.4 | 2.17 | 3.07 |
| *Cdkn1a* | 1 | 396.9 | 6.26 | 154.39 | 3.3 | 446.12 | 10.13 | 189.54 | 4.04 |
| *Gpx2* | 1 | 0.51 | 5.62 | 0.39 | 2.55 | 0.35 | 9.61 | 0.18 | 6.57 |

studies (Monmeesil et al., 2019). At the same time, we found that ALT, AST, and TBIL returned to normal on day 7th after ASAE, but other phenotypic indexes still showed significant differences compared with the CK group. However, compared with the 2nd day after ASAE, the phenotypic indexes except antioxidant enzymes and liver coefficient showed significant differences, but showed a recovery trend. These results showed that $AFB_1$ still caused rats liver damage to a certain extent after feeding for 7 days after ASAE. The recovery of liver function indexes ALT, AST and TBIL may be related to the strong self-repair ability of liver (Ko et al., 2020), and the change trend of apoptosis rate and MDA content also indicates that the compensatory function of liver plays a role (Hall et al., 2021). The ALP value is related to whether the intrahepatic and extrahepatic bile ducts are blocked. The ALP value on the 7th day after ASAE was still significantly higher than that of the CK group, this is related to the fact that cholestasis caused by bile duct blockage does not self-adjust and recover in a short time (Boyer, 2013; Taylor et al., 2020). The changes of pathological section and liver coefficient further indicated that liver morphological damage was difficult to recover spontaneously in a short time. The antioxidant enzymes GSH-Px and SOD activities continued to decrease with the prolongation of $AFB_1$ influence time. On the 2nd day after ASAE, the change trend of antioxidant enzyme activity was similar to that reported in the previous paper (Deng et al., 2020). The changes of antioxidant enzymes on the 7th day after ASAE may be related to the fact that the liver damage of rats is not alleviated and the intake of antioxidant nutrients is insufficient due to decreased appetite (Michiels et al., 1994; Peskin, 1997; Liochev and Fridovich, 2010). However, the reasons for the changes in lipid peroxide content and antioxidant enzyme activity in this model need to be further explored through related experiments.

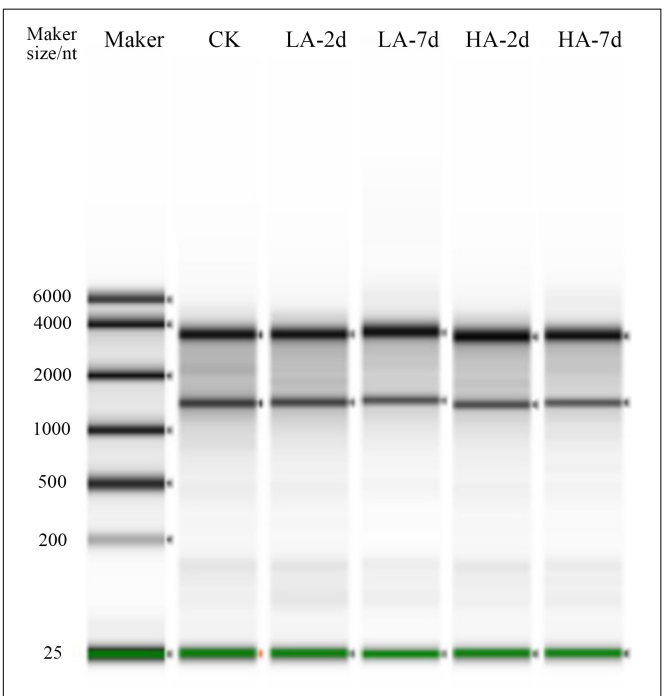



**FIGURE 6 |** Electrophoresis diagram of the Total RNA Automatic Nucleic Acid Electrophoresis Analyzer.

We obtained the gene data of each experimental group by RNA-Seq technique and found that the number of DEGs was affected by the dose and duration of $AFB_1$ exposure, among which *Lama5*, *Gtse1*, *Fabp4* and *Bcl6* genes were continuously affected and associated with liver injury. Their mechanism of action is shown in **Figure 7**. *Lama5* gene plays a role in promoting angiogenesis during tumor development and is a key promoter of liver metastatic growth (Yousif et al., 2013; Gordon-Weeks et al., 2019). Tumor-infiltrating myeloid cells can drive tumor cells to express *Lama5* through *NF-κB* gene transduction, thereby promoting angiogenesis (Bonnans et al., 2014). The Lama5 protein chain is an important element of the extracellular matrix (ECM). Thus, the *Lama5* gene links two markers of cancer progression (Inflammation and Angiogenesis) to extracellular matrix (ECM) protein deposition. Related studies had shown that down-regulation of *Lama5* can reduce the formation of vascular branches, and even tend to normalize (Carmeliet and Jain, 2011; Di Russo et al., 2017). *Fabp4* has also been shown to be involved in inflammatory responses. Cytokine levels, including TNF-α and IL-1β, were significantly increased in macrophages with high *Fabp4* expression (Makowski et al., 2001). And *Fabp4* plays an important role in transporting fatty acids between fat cells and cancer cells (Besnard et al., 2002). These effects ultimately may lead to fibrosis of tissues (Nieman et al., 2011). The proto-oncogene *Bcl6* is an important transcriptional regulator of the immune system, and its upregulation is a marker of B cell accumulation through the germinal center (GC) transport process. In tumors, its dysregulated expression may

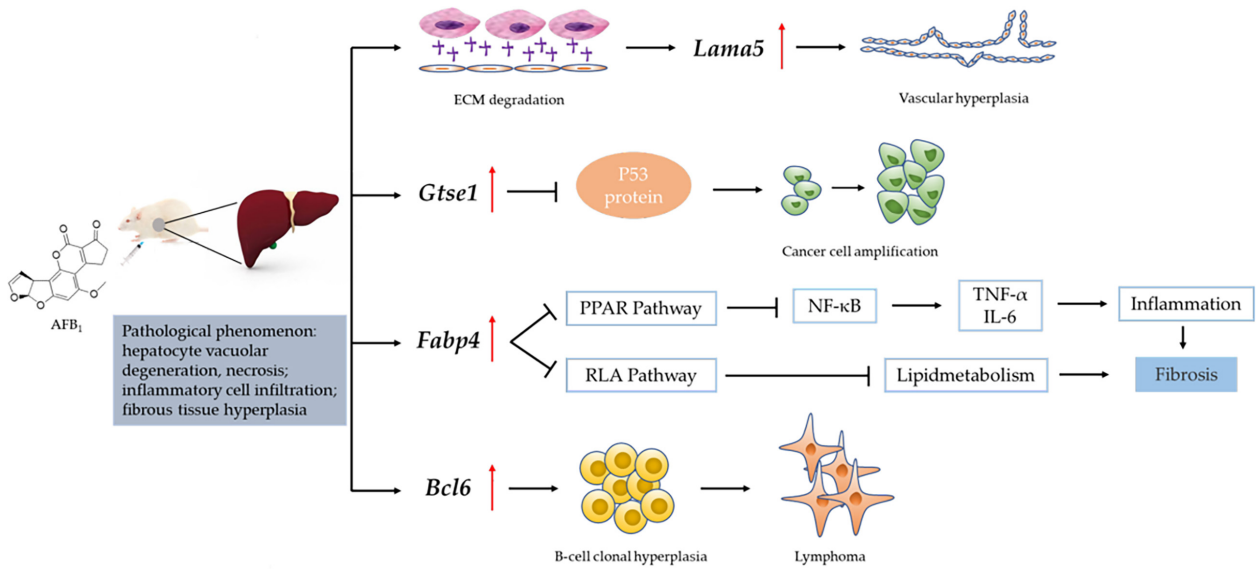



**FIGURE 7 |** Mechanism of liver injury induced by *Lama5*, *Gtse1*, *Fabp4*, and *Bcl6* genes. RAF: Regulation of lipolysis in adipocytes; ECM: Extracellular matrix; "┼": vascular endothelial growth factor (VEGF); "—/↑": promote/up-regulate; "—|": inhibition.

promote lymphoma by increasing B cell resistance to apoptosis and inhibiting B cell differentiation in the GC (Murakami et al., 1999; Niu, 2002). Low-grade B-cell lymphomas can occur in the liver and manifest as a characteristic massive infiltration of lymphocytes within the confluent zone. *Lama5*, *Fabp4* and *Bcl6* gene related to inflammation and immune response were all up-regulated, and the expression of *Bcl6* gene was higher at 7 days than at 2 days after ASAE. Their changing trend explained that the pathological phenomena of liver was not relieved the 7th day after ASAE to some extent. And there were still different degrees of liver cell necrosis, inflammatory cell infiltration and fibrous tissue hyperplasia. *Gtse1* has been found to be overexpressed in a variety of cancers (Tian et al., 2011). Previous studies have shown that it inhibits apoptosis induced by the tumor suppressor protein p53 (Liu et al., 2010). Cell level experiments showed that *Gtse1* was down-regulated, the protein content of p53 in cells increased, and the dephosphorylation of protein kinase B and cyclin B1 decreased, thus inhibiting the proliferation of HCC cells and promoting apoptosis (Guo et al., 2016). Our data showed that, the expression level of *Gtse1* on the 7th day after ASAE was lower than that on the 2nd day after ASAE, which may explain why the apoptosis rate of liver cells decreased on the 7th day after ASAE. However, the specific regulatory mechanism needs further study.

## CONCLUSION

In summary, ASAE can cause persistent liver damage. The experiment period of this study was 7 days, and the damage of liver during the extended experiment period could be further studied. The persistently affected *Lama5*, *Gtse1*, *Fabp4* and *Bcl6* gene can be potential target genes for the treatment of $AFB_1$ induced liver diseases, but their effectiveness needs to be further explored in experiments.

## DATA AVAILABILITY STATEMENT

The data presented in the study are deposited in the NCBI repository, accession number BioProject ID PRJNA823496.

## ETHICS STATEMENT

The animal study was reviewed and approved by Shanghai University of Traditional Chinese Medicine.

## AUTHOR CONTRIBUTIONS

JY: conceptualization, methodology, formal analysis, investigation, resources, data curation, writing—original draft preparation, and visualization. LC: methodology, formal analysis, and resources. LZ: investigation and resources. ZZ: validation, writing—review and editing and funding acquisition. YZ: supervision, project administration, and funding acquisition. YW: conceptualization, validation, and project administration. JO: conceptualization, validation, writing—review and editing, supervision, and project administration. All authors contributed to the article and approved the submitted version.

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## REFERENCES


Abrar, M., Anjum, F. M., Butt, M. S., Pasha, I., Randhawa, M. A., Saeed, F., et al. (2013). Aflatoxins: biosynthesis, occurrence, toxicity, and remedies. *Crit. Rev. Food Sci. Nutr.* 53, 862–874. doi: 10.1080/10408398.2011.563154

An, Y. A., Chen, S., Deng, Y., Wang, Z. V., Funcke, J. B., Shah, M., et al. (2021). The mitochondrial dicarboxylate carrier prevents hepatic lipotoxicity by inhibiting white adipocyte lipolysis. *J. Hepatol.* 75, 387–399. doi: 10.1016/j.jhep.2021.03.006

Bailey, E. A., Iyer, R. S., Stone, M. P., Harris, T. M., and Essigmann, J. M. (1996). Mutational properties of the primary aflatoxin $b_1$-DNA adduct. *Proc. Natl. Acad. Sci. U. S. A.* 93, 1535–1539. doi: 10.1073/pnas.93.4.1535

Bailey, K. L., Cartwright, S. B., Patel, N. S., Remmers, N., Lazenby, A. J., Hollingsworth, M. A., et al. (2021). Porcine pancreatic ductal epithelial cells transformed with kras(g12d) and sv40t are tumorigenic. *Sci. Rep.* 11:13436. doi: 10.1038/s41598-021-92852-2

Benkerroum, N. (2020). Chronic and acute toxicities of aflatoxins: mechanisms of action. *Int. J. Environ. Res. Public Health* 17:423. doi: 10.3390/ijerph17020423

Besnard, P., Niot, I., Poirier, H., Clément, L., and Bernard, A. (2002). New insights into the fatty acid-binding protein (fabp) family in the small intestine. *Mol. Cell Biochem.* 239, 139–147. doi: 10.1023/A:1020505512364

Bonnans, C., Chou, J., and Werb, Z. (2014). Remodelling the extracellular matrix in development and disease. *Nat. Rev. Mol. Cell Biol.* 15, 786–801. doi: 10.1038/nrm3904

Boyer, J. L. (2013). Bile formation and secretion. *Compr. Physiol.* 3, 1035–1078. doi: 10.1002/cphy.c120027

Carmeliet, P., and Jain, R. K. (2011). Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. *Nat. Rev. Drug Discov.* 10, 417–427. doi: 10.1038/nrd3455

Chen, Z., Xiao, J., Liu, H., Yao, K., Hou, X., Cao, Y., et al. (2020). Astaxanthin attenuates oxidative stress and immune impairment in d-galactose-induced aging in rats by activating the nrf2/keap1 pathway and suppressing the nf-kappab pathway. *Food Funct.* 11, 8099–8111. doi: 10.1039/d0fo01663b

Corrales, P., Vidal-Puig, A., and Medina-Gómez, G. (2018). Ppars and metabolic disorders associated with challenged adipose tissue plasticity. *Int. J. Mol. Sci.* 19:2124. doi: 10.3390/ijms19072124

Deng, Z. J., Zhao, J. F., Huang, F., Sun, G. L., Gao, W., Lu, L., et al. (2020). Protective effect of procyanidin b2 on acute liver injury induced by aflatoxin $b_1$ in rats. *Biomed. Environ. Sci.* 33, 238–247. doi: 10.3967/bes2020.033

Di Russo, J., Luik, A. L., Yousif, L., Budny, S., Oberleithner, H., Hofschröer, V., et al. (2017). Endothelial basement membrane laminin 511 is essential for shear stress response. *Embo. J.* 36, 183–201. doi: 10.15252/embj.201694756

El-Agamy, D. S. (2010). Comparative effects of curcumin and resveratrol on aflatoxin b-1-induced liver injury in rats. *Arch. Toxicol.* 84, 389–396. doi: 10.1007/s00204-010-0511-2

Farhan, M., Wang, H., Gaur, U., Little, P. J., Xu, J., and Zheng, W. (2017). Foxo signaling pathways as therapeutic targets in cancer. *Int. J. Biol. Sci.* 13, 815–827. doi: 10.7150/ijbs.20052

Gordon-Weeks, A., Lim, S. Y., Yuzhalin, A., Lucotti, S., Vermeer, J. A. F., Jones, K., et al. (2019). Tumour-derived laminin α5 (lama5) promotes colorectal liver metastasis growth, branching angiogenesis and notch pathway inhibition. *Cancers* 11:630. doi: 10.3390/cancers11050630

Guengerich, F. P., Johnson, W. W., Shimada, T., Ueng, Y. F., Yamazaki, H., and Langouët, S. (1998). Activation and detoxication of aflatoxin $b_1$. *Mutat. Res.* 402, 121–128. doi: 10.1016/s0027-5107(97)00289-3

Guo, L., Zhang, S., Zhang, B., Chen, W., Li, X., Zhang, W., et al. (2016). Silencing gtse-1 expression inhibits proliferation and invasion of hepatocellular carcinoma cells. *Cell Biol. Toxicol.* 32, 263–274. doi: 10.1007/s10565-016-9327-z

Hall, Z., Chiarugi, D., Charidemou, E., Leslie, J., Scott, E., Pellegrinet, L., et al. (2021). Lipid remodeling in hepatocyte proliferation and hepatocellular carcinoma. *Hepatology* 73, 1028–1044. doi: 10.1002/hep.31391

Hussein, H. S., and Brasel, J. M. (2001). Toxicity, metabolism, and impact of mycotoxins on humans and animals. *Toxicology* 167, 101–134. doi: 10.1016/s0300-483x(01)00471-1

Ishida, Y., Yamasaki, C., Iwanari, H., Yamashita, H., Ogawa, Y., Yanagi, A., et al. (2020). Detection of acute toxicity of aflatoxin b1 to human hepatocytes in vitro and in vivo using chimeric mice with humanized livers. *PLoS One* 15:e0239540. doi: 10.1371/journal.pone.0239540

Jindal, N., Mahipal, S. K., and Mahajan, N. K. (1994). Toxicity of aflatoxin b1 in broiler chicks and its reduction by activated charcoal. *Res. Vet. Sci.* 56, 37–40. doi: 10.1016/0034-5288(94)90193-7

Knipstein, B., Huang, J., Barr, E., Sossenheimer, P., Dietzen, D., Egner, P. A., et al. (2015). Dietary aflatoxin-induced stunting in a novel rat model: evidence for toxin-induced liver injury and hepatic growth hormone resistance. *Pediatr. Res.* 78, 120–127. doi: 10.1038/pr.2015.84

Ko, S., Russell, J. O., Molina, L. M., and Monga, S. P. (2020). Liver progenitors and adult cell plasticity in hepatic injury and repair: knowns and unknowns. *Annu. Rev. Pathol.* 15, 23–50. doi: 10.1146/annurev-pathmechdis-012419-032824

Kumagai, S., Sugita-Konishi, Y., Hara-Kudo, Y., Ito, Y., Noguchi, Y., Yamamoto, Y., et al. (1998). The fate and acute toxicity of aflatoxin b1 in the mastomys and rat. *Toxicon* 36, 179–188. doi: 10.1016/s0041-0101(97)00071-8

Lacroix, M., Riscal, R., Arena, G., Linares, L. K., and Le Cam, L. (2020). Metabolic functions of the tumor suppressor p53: implications in normal physiology, metabolic disorders, and cancer. *Mol. Metab.* 33, 2–22. doi: 10.1016/j.molmet.2019.10.002

Lee, S., and Dong, H. H. (2017). Foxo integration of insulin signaling with glucose and lipid metabolism. *J. Endocrinol.* 233, R67–R79. doi: 10.1530/joe-17-0002

Li, Z., Hao, J., Chen, K., Jiang, Q., Wang, P., Xing, X., et al. (2021). Identification of key pathways and genes in carotid atherosclerosis through bioinformatics analysis of rna-seq data. *Aging* 13, 12733–12747. doi: 10.18632/aging.202943

Lin, Y. C., Li, L., Makarova, A. V., Burgers, P. M., Stone, M. P., and Lloyd, R. S. (2014). Error-prone replication bypass of the primary aflatoxin b1 DNA adduct, afb1-n7-gua. *J. Biol. Chem.* 289, 18497–18506. doi: 10.1074/jbc.M114.561563

Liochev, S. I., and Fridovich, I. (2010). Mechanism of the peroxidase activity of cu, zn superoxide dismutase. *Free Radic. Biol. Med.* 48, 1565–1569. doi: 10.1016/j.freeradbiomed.2010.02.036

Liu, X. S., Li, H., Song, B., and Liu, X. (2010). Polo-like kinase 1 phosphorylation of g2 and s-phase-expressed 1 protein is essential for p53 inactivation during g2 checkpoint recovery. *EMBO Rep.* 11, 626–632. doi: 10.1038/embor.2010.90

Long, M., Zhang, Y., Li, P., Yang, S.-H., Zhang, W.-K., Han, J.-X., et al. (2016). Intervention of grape seed proanthocyanidin extract on the subchronic immune injury in mice induced by aflatoxin b1. *Int. J. Mol. Sci.* 17:516. doi: 10.3390/ijms17040516

Makowski, L., Boord, J. B., Maeda, K., Babaev, V. R., Uysal, K. T., Morgan, M. A., et al. (2001). Lack of macrophage fatty-acid-binding protein ap2 protects mice deficient in apolipoprotein e against atherosclerosis. *Nat. Med.* 7, 699–705. doi: 10.1038/89076

Michiels, C., Raes, M., Toussaint, O., and Remacle, J. (1994). Importance of se-glutathione peroxidase, catalase, and cu/zn-sod for cell survival against oxidative stress. *Free Radic. Biol. Med.* 17, 235–248. doi: 10.1016/0891-5849(94)90079-5

Monmeesil, P., Fungfuang, W., Tulayakul, P., and Pongchairerk, U. (2019). The effects of astaxanthin on liver histopathology and expression of superoxide dismutase in rat aflatoxicosis. *J. Vet. Med. Sci.* 81, 1162–1172. doi: 10.1292/jvms.18-0690

Murakami, J., Shimizu, Y., Kashii, Y., Kato, T., Minemura, M., Okada, K., et al. (1999). Functional b-cell response in intrahepatic lymphoid follicles in chronic hepatitis c. *Hepatology* 30, 143–150. doi: 10.1002/hep.510300107

Nayak, S., and Sashidhar, R. B. (2010). Metabolic intervention of aflatoxin b-1 toxicity by curcumin. *J. Ethnopharmacol.* 127, 641–644. doi: 10.1016/j.jep.2009.12.010

Nieman, K. M., Kenny, H. A., Penicka, C. V., Ladanyi, A., Buell-Gutbrod, R., Zillhardt, M. R., et al. (2011). Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nat. Med.* 17, 1498–1503. doi: 10.1038/nm.2492

Niu, H. (2002). The proto-oncogene bcl-6 in normal and malignant b cell development. *Hematol. Oncol.* 20, 155–166. doi: 10.1002/hon.689

Peskin, A. V. (1997). Cu,zn-superoxide dismutase gene dosage and cell resistance to oxidative stress: a review. *Biosci. Rep.* 17, 85–89. doi: 10.1023/a:1027343519591

Rastogi, R., Srivastava, A. K., and Rastogi, A. K. (2001). Biochemical changes induced in liver and serum of aflatoxin $b_1$-treated male wistar rats: preventive effect of picroliv. *Pharmacol. Toxicol.* 88, 53–58. doi: 10.1034/j.1600-0773.2001.088002053.x

Rota Caremoli, E., and Labianca, R. (2014). Tivantinib: critical review with a focus on hepatocellular carcinoma. *Expert Opin. Investig. Drugs* 23, 1563–1574. doi: 10.1517/13543784.2014.949339

Ruggeberg, K. G., O'Sullivan, P., Kovacs, T. J., Dawson, K., Capponi, V. J., Chan, P. P., et al. (2020). Hemoadsorption improves survival of rats exposed to an acutely lethal dose of aflatoxin b(1). *Sci. Rep.* 10:799. doi: 10.1038/s41598-020-57727-y

Rushing, B. R., and Selim, M. I. (2019). Aflatoxin b1: a review on metabolism, toxicity, occurrence in food, occupational exposure, and detoxification methods. *Food Chem. Toxicol.* 124, 81–100. doi: 10.1016/j.fct.2018.11.047

Shen, J., Cao, B., Wang, Y., Ma, C., Zeng, Z., Liu, L., et al. (2018). Hippo component yap promotes focal adhesion and tumour aggressiveness via transcriptionally activating thbs1/fak signalling in breast cancer. *J. Exp. Clin. Cancer Res.* 37:175. doi: 10.1186/s13046-018-0850-z

Taylor, S. A., Yeap, X. Y., Wang, J. J., Gromer, K. D., Kriegermeier, A., Green, R. M., et al. (2020). A novel murine model of reversible bile duct obstruction demonstrates rapid improvement of cholestatic liver injury. *Physiol. Rep.* 8:e14446. doi: 10.14814/phy2.14446

Tian, T., Zhang, E., Fei, F., Li, X., Guo, X., Liu, B., et al. (2011). Up-regulation of gtse1 lacks a relationship with clinical data in lung cancer. *Asian Pac. J. Cancer Prev.* 12, 2039–2043. doi: 10.1007/978-3-642-16602-0_13

Trojan, J., and Zeuzem, S. (2013). Tivantinib in hepatocellular carcinoma. *Expert Opin. Investig. Drugs* 22, 141–147. doi: 10.1517/13543784.2013.741586

Wang, C. J., Shiow, S. J., and Lin, J. K. (1991). Effects of crocetin on the hepatotoxicity and hepatic DNA binding of aflatoxin b1 in rats. *Carcinogenesis* 12, 459–462. doi: 10.1093/carcin/12.3.459

Wang, J. S., and Groopman, J. D. (1999). DNA damage by mycotoxins. *Mutat. Res.* 424, 167–181. doi: 10.1016/s0027-5107(99)00017-2

Wang, Z., Gerstein, M., and Snyder, M. (2009). Rna-seq: a revolutionary tool for transcriptomics. *Nat. Rev. Genet.* 10, 57–63. doi: 10.1038/nrg2484

Xu, F., Wang, P., Yao, Q., Shao, B., Yu, H., Yu, K., et al. (2019). Lycopene alleviates afb(1)-induced immunosuppression by inhibiting oxidative stress and apoptosis in the spleen of mice. *Food Funct.* 10, 3868–3879. doi: 10.1039/c8fo02300j

Yang, A., and Mottillo, E. P. (2020). Adipocyte lipolysis: from molecular mechanisms of regulation to disease and therapeutics. *Biochem. J.* 477, 985–1008. doi: 10.1042/bcj20190468

Yang, D., He, Y., Wu, B., Deng, Y., Wang, N., Li, M., et al. (2020). Integrated bioinformatics analysis for the screening of hub genes and therapeutic drugs in ovarian cancer. *J. Ovarian. Res.* 13:10. doi: 10.1186/s13048-020-0613-2

Ye, K., Ouyang, X., Wang, Z., Yao, L., and Zhang, G. (2020). Sema3f promotes liver hepatocellular carcinoma metastasis by activating focal adhesion pathway. *DNA Cell Biol.* 39, 474–483. doi: 10.1089/dna.2019.4904

Yousif, L. F., Di Russo, J., and Sorokin, L. (2013). Laminin isoforms in endothelial and perivascular basement membranes. *Cell Adh. Migr.* 7, 101–110. doi: 10.4161/cam.22680

Zou, Y. H., Liu, X., Khlentzos, A. M., Asadian, P., Li, P., Thorling, C. A., et al. (2010). Liver fibrosis impairs hepatic pharmacokinetics of liver transplant drugs in the rat model. *Drug Metab. Pharmacokinet.* 25, 442–449. doi: 10.2133/dmpk.dmpk-10-rg-031

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# Genomic Investigation of *Proteus mirabilis* Isolates Recovered From Pig Farms in Zhejiang Province, China

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*Proteus mirabilis* is a common opportunistic zoonotic pathogen, and its ongoing acquisition of antimicrobial resistance genes poses challenges to clinical treatments. Human-sourced whole genomic sequencing of human *P. mirabilis* isolates has been reported, but pig-sourced isolates have not been thoroughly investigated even though these animals can serve as reservoirs for human infections. In the current study, we report a molecular epidemiological investigation to unravel the antimicrobial and virulence gene risk factors for *P. mirabilis* contamination in 9 pig farms in 3 different cities in Zhejiang Province, China. We collected 541 swab samples from healthy pigs and 30 were confirmed as *P. mirabilis*. All 30 isolates were resistant to tetracyclines, macrolides, sulfonamides, β-lactams and chloramphenicol, and all were multiple drug-resistant and 27 were strong biofilm formers. Phylogenetic analyses indicated these 30 isolates clustered together in 2 major groups. Whole genome sequencing demonstrated that the isolates possessed 91 different antimicrobial resistance genes belonging to 30 antimicrobial classes including *rmtB, sul1, qnrS1, AAC(6′) – Ib – cr, blaCTX – M – 65* and *blaOXA – 1.* All isolates contained mobile genetic elements including integrative conjugative elements (ICEs) and integrative and mobilizable elements (IMEs). Minimum inhibitory concentration (MIC) testing indicated direct correlates between cognate genes and antimicrobial resistance. We also identified 95 virulence factors, almost all isolates contained 20 fimbrial and flagellar operons, and this represents the greatest number of these operon types found in a single species among all sequenced bacterial genomes. These genes regulate biofilm formation and represent a confounding variable for treating *P. mirabilis* infections. Our *P. mirabilis* isolates were present in healthy animals, and multiple drug resistance in these isolates may serve as a reservoir for other intestinal and environmental Enterobacteriaceae members. This prompts us to more strictly regulate veterinary antibiotic use.

**Keywords: antimicrobial resistance, biofilm information, whole genome sequencing, virulence factor, *Proteus mirabilis***

## INTRODUCTION

*Proteus mirabilis* is an Enterobacteriaceae member and a significant opportunistic and food-borne pathogen and second only to enteropathogenic *Escherichia coli* (EPEC) in prevalence for urinary tract infection. *P. mirabilis* can exist in a variety of environments including the intestinal tracts of humans and wild and domestic animals and can cause infections in immunocompromised hosts resulting in diarrhea, urinary tract infection (UTI) and keratitis (Mobley and Belas, 1995; Drzewiecka, 2016; Gong et al., 2019). Importantly, antimicrobial resistance of *P. mirabilis* has become a serious impediment to clinical treatment and resistance to colistin, nitrofurans, tigecycline, tetracycline, and β-lactams have been documented (Stock, 2003).

China is the largest global antibiotic producer and consumer and more than half are used to treat food animals (Gilbert, 2012). Livestock farms are environments with high bacterial loads and high antimicrobial selective pressures; a combination favoring development of resistant bacteria (Klein et al., 2018; Qian et al., 2018). There is abundant direct and indirect evidence that the antibiotic use on farms correlates with the rise and spread of associated antibiotic resistance genes (ARG) in human pathogens and even the direct transfer of antibiotic-resistant bacteria from animals to humans (Marshall and Levy, 2011). Pigs are major meat source worldwide, especially in China and ARGs are increasingly detected in bacterial isolates from pig feces implicating pig husbandry in ARG development and spread (Larson, 2015; Shi et al., 2021). In particular, mobile genetic elements (MGE) including insertion sequences (IS), resistance plasmids, gene castle, transposons, integrative conjugative elements (ICE), and integrative and mobilizable elements (IME) allow ARG movement within or between DNA molecules. These MGE are now frequently detected in *P. mirabilis* isolates (Shelenkov et al., 2020; Wang et al., 2021).

*P. mirabilis* is also a biofilm former allowing its engraftment on living or abiotic surfaces and complicates disease treatment and enhances viability through antimicrobial and disinfectant resistance. Treatment of UTIs due to indwelling catheters can be complicated through biofilm formation that prolong bacterial presence and can lead to bladder and kidney infections that may progress to bacteremia or sepsis (Høiby et al., 2011). *P. mirabilis* contains numerous virulence factors related to adhesion, colonization, biofilm formation and pathogenicity. These include the MR/P and PMF fimbriae and the uroepithelial cell adhesin (UCA) and all can combine to enable urinary cell attachment and biofilm formation.

The emergence of multidrug-resistant (MDR) bacterial pathogens is now considered a public health risk and sources of these pathogens have been found in humans, livestock, wild animals and food. Importantly, molecular typing studies have identified animal strains that were transmitted to humans (Reich et al., 2013; Olonitola et al., 2015; Lei et al., 2016). Previously reported 7.07% *P. mirabilis* from broiler chicken samples (Li et al., 2022) and pig operations in China are currently experiencing large outbreaks of diarrheal disease in piglets (Li, 2021). As a pathogen that can cause diarrhea, *P. mirabilis* has not attracted enough attention. Therefore, the objectives were to investigated the prevalence, antimicrobial resistance and virulence genes for *P. mirabilis* to characterize risk factors of *P. mirabilis* contamination in Zhejiang Province, China. Whole genome sequencing and bioinformatics analyses are cost-effective methods for the investigation and characterization of zoonotic pathogens (Biswas et al., 2020; Liu et al., 2020, 2021). We conducted a study on 9 farms in 3 different cities in Zhejiang Province, China, which were rarely reported, to obtain *P. mirabilis* isolates from pig swab sources. The recovered strains were subjected to whole genome sequencing followed by *in silico* analysis to identify antimicrobial resistance and virulence genes. We also determined antibiotic MICs to correlate with ARG presence.

## MATERIALS AND METHODS

### Sample Collection and Characterization of *Proteus mirabilis*

A total of 541 pig swab specimens were obtained from 9 farms located in 3 different cities in Zhejiang Province, China from May to December 2021. The strains isolated from Jinhua, Hangzhou, and Quzhou were designated PM 1 ($n = 101$), PM 2 ($n = 240$), and PM 3 ($n = 200$), respectively (**Table 1**). In brief, swabs were placed in sterile centrifuge tubes containing 3 ml Selenite Cystine Broth (SC; Hopebiol, Qingdao, China) and incubated at 37°C for 12 h. Loopfuls were then streaked on Salmonella-Shigella agar plates (SS; Becton Dickinson, Franklin Lakes, NJ, United States) and incubated as per above (Li et al., 2022). Suspected *P. mirabilis* colonies were streak-purified a second time. Presumptive *P. mirabilis* colonies displayed black centers with transparent edges on SS agar.

### Identification and Phylogenetic Analyses of *Proteus mirabilis*

Suspected *P. mirabilis* were selected for genomic DNA extraction using a TIANamp bacteria DNA kit (Tiangen Biotech, Beijing, China) according to the instructions of the manufacturer and

**TABLE 1** | Sampling design and prevalence of *Proteus mirabilis* from different sources.

| Sources | | No. of samples | Group | No. of positive | Percentage of isolates |
|---|---|---|---|---|---|
| City | Farm | | | | |
| Jinhua | 1 | 31 | PM1 | 2 (6.45%) | 8.91% |
| | 2 | 33 | | 4 (12.12%) | |
| | 3 | 38 | | 3 (7.89%) | |
| Hangzhou | 4 | 78 | PM2 | 6 (7.69%) | 4.58% |
| | 5 | 80 | | 3 (3.75%) | |
| | 6 | 82 | | 2 (2.44%) | |
| Quzhou | 7 | 63 | PM3 | 5 (7.94%) | 5.00% |
| | 8 | 74 | | 3 (4.05%) | |
| | 9 | 63 | | 2 (3.17%) | |
| Total | | 541 | | 30 | 5.55% |

DNA was stored at −20°C. *P. mirabilis* identities were confirmed using PCR detection of the *atpD* gene (5′-AGAGTTTGATC CTGGCTCAG-3′5′-ACGGGCGGTGTGTRC-3′) as previously described (Bi et al., 2013). In addition, the 16S rDNA genes (5′-AGAGTTTGATCCTGGCTCAG-3′5′-ACGGGCGGTGTG TRC-3) from these isolates were sequenced. Pure cultures of identified strains were cryopreserved at −80°C in 30% glycerol.

## Biofilm Formation Ability

Biofilm formation was assayed using the crystal violet (CV) staining method as previously described (Stepanović et al., 2007; Han et al., 2015; Lajhar et al., 2018). Briefly, bacterial suspension turbidities were adjusted to 0.5 McFarland standard (~$10^8$ CFU/ml) and 20 μl was then added to prepared microtiter plates containing 180 μl Luria Bertani (LB) broth per well; negative control wells contained only LB. The plates were then covered and incubated for 24 h at 37°C under static conditions. The liquid was then discarded and the wells were washed 3× with phosphate-buffered saline (PBS) and 200 μl 0.1% CV (Sigma, Aldrich, United States) was then added per well and the plates were allowed to stand for 30 min at room temperature. The CV was decanted and the bacteria were then heat-fixed by exposure to a stream of hot air at 60°C for 60 min. The dye bound to the cells was then resolubilized using three rinses with 200 μl of 95% ethanol per well. The optical density (OD) of combined washes was measured at 595 nm using a microtiter plate reader. The average $OD_{595}$ values were calculated for triplicates and the tests were repeated three times.

## Genomic Sequencing and Bioinformatic Analysis

Illumina pair-end sequencing of each strain utilized 1 μg genomic DNA for library construction. The qualified library was used for Illumina NovaSeq 6,000 sequencing (150 bp * 2) at Shanghai Biozeron. The raw paired end reads were trimmed and quality controlled by Trimmomatic version 0.36. ABySS[1] was used for genome assembly with multiple-Kmer parameters for optimization. GapCloser[2] was subsequently applied to fill remaining local inner gaps and correct single base polymorphisms for the final assembly results. *Ab initio* prediction methods were used to obtain gene models for *P. mirabilis* strains and were identified using GeneMark and then used for BLASTp searches against the non-redundant (NR) NCBI database. Potential virulence factors were assessed by searches against the Virulence Factors of Pathogenic Bacteria Database (VFDB) and BLAST results were considered significant at $E < 1e^{-5}$ and is a generally accepted consensus cut-off (Chen et al., 2005). ARGs of the 30 *P. mirabilis* strains we sequenced were also examined using the Comprehensive Antibiotic Resistance Database (CARD), and ICEs were identified using ICEfinder (Liu et al., 2019). The public data of reference genome of *P. mirabilis* (HI4320) was obtained from NCBI[3]; gene annotation information including functional annotations and gene family information were also downloaded. The datasets generated for this study can be found in the NCBI Bioproject with the accession number no. PRJNA841796.

## Phenotypic Antimicrobial Resistance Testing

Broth dilution method was used for MIC test for a panel of 15 antimicrobial agents belonging to 9 classes as described previously (Kobylka, Kuth, Müller, Geertsma, & Pos, 2020). MIC results were interpreted according to the recommendations of the Clinical Laboratory Standard Institute guidelines (CLSI, 2017; **Table 2**). The antimicrobial agents were grouped in the following classes: tetracyclines (tetracycline, TET, 8–256 μg/ml; minocycline, MIN, 4–256 μg/ml), fluoroquinolones (ofloxacin, OFX, 2–64 μg/ml; nalidixic acid, NAL, 2–64 μg/ml; ciprofloxacin, CIP, 2–64 μg/ml), macrolides (azithromycin, AZ, 16–512 μg/ml; erythromycin, EM, 16–512 μg/ml), β-lactamase inhibitors (amoxicillin/clavulanic acid, AMC, 4/2–128/64 μg/ml), penicillins (ampicillin, AMP, 16–512 μg/ml), carbapenems (meropenem, MEM, 1–64 μg/ml), cephalosporins (ceftiofur, CEF, 16–512 μg/ml), aminoglycosides (amikacin, AMK, 2–64 μg/ml; gentamicin, GEN, 2–128 μg/ml), phenicols (chloramphenicol, CHL, 4–128 μg/ml; rifampicin, RIF, 1–64 μg/ml).

*Proteus mirabilis* CMCC 49005 was used for quality control.

## Data Analysis

GraphPad Prism 8 software (San Diego, CA, United States) was used for figure generation. ANOVA was performed using least squares techniques with IBM SPSS Statistics 20 software (SPSS, Chicago, IL, United States). A significant difference was established at $p < 0.05$. MIC results of intermediate susceptibility were merged with resistance and each test result (resistant or susceptible) was compared with the detection (presence or absence) of the corresponding ARG *in silico*.

# RESULTS AND DISCUSSION

*Proteus mirabilis* has been frequently incriminated in food-borne and urinary tract infections in humans. We first explored the prevalence and characteristics of *P. mirabilis* in pig farm isolates from Zhejiang province, China.

## *Proteus mirabilis* Prevalence

We isolated 30 *P. mirabilis* strains from 541 samples (5.55%) using *atpD* PCR detection, 16S rDNA and whole genome sequencing (**Table 1**). The PM1 group (Jinhua) was the most contaminated (8.91%), and this level was close to that previously reported (7.07%) from broiler chicken samples (Li et al., 2022) but lower than from human sources (Mirzaei et al., 2019; Tabatabaei et al., 2021). Phylogenetic analysis identified 2 major clusters including Group 1 that contained reference strain

[1]http://www.bcgsc.ca/platform/bioinfo/software/abyss
[2]https://sourceforge.net/projects/soapdenovo2/files/GapCloser/
[3]https://www.ncbi.nlm.nih.gov/assembly/GCF_000069965.1/

**TABLE 2** | Antimicrobial susceptibility tests for *P. mirabilis* strains used in this study ($n = 30$).

| Antimicrobial drug | | Abbreviation | Breakpoint (μg/ml) | | | Results (%) | | |
|---|---|---|---|---|---|---|---|---|
| Species | Name | | *S* | *I* | *R* | *S* | *I* | *R* |
| **Tetracyclines** | | | | | | | | |
| Tetracycline | | TET | ≤4 | 8 | ≥16 | 0% (0/30) | 0% (0/30) | 100.00% (30/30) |
| Minocycline | | MIN | ≤4 | 8 | ≥16 | 0% (0/30) | 0% (0/30) | 100.00% (30/30) |
| **Fluoroquinolones** | | | | | | | | |
| Ofloxacin | | OFX | ≤2 | 4 | ≥8 | 30.00% (9/30) | 26.67% (8/30) | 43.33% (13/30) |
| Naphthaleneic acid | | NAL | ≤16 | – | ≥32 | 26.67% (8/30) | 0% (0/30) | 73.33% (22/30) |
| Ciprofloxacin | | CIP | ≤0.25 | 0.5 | ≥1 | 23.33% (7/30) | 0% (0/30) | 76.67% (23/30) |
| **Macrolide** | | | | | | | | |
| Azithromycin | | AZ | ≤2 | 4 | ≥8 | 0% (0/30) | 0% (0/30) | 100.00% (30/30) |
| Erythromycin | | EM | ≤0.5 | 1 | ≥8 | 0% (0/30) | 0% (0/30) | 100.00% (30/30) |
| **β-Lactam combinations** | | | | | | | | |
| Ampicillin | | AMP | ≤8 | – | ≥16 | 10.00% (3/30) | 0% (0/30) | 90.00% (27/30) |
| Amoxicillin/clavulanic acid | | AMC | ≤8/4 | – | ≥16/8 | 10.00% (3/30) | 0% (0/30) | 90.00% (27/30) |
| Ceftiofur | | CEF | ≤2 | 4 | ≥8 | 16.67% (5/30) | 6.67% (2/30) | 76.67% (23/30) |
| Meropenem | | MEM | ≤1 | 2 | ≥4 | 40.00% (12/30) | 60.00% (18/30) | 0% (0/30) |
| **Aminoglycosides** | | | | | | | | |
| Amikacin | | AMK | ≤16 | 32 | ≥64 | 70.00% (21/30) | 10.00% (3/30) | 20.00% (6/30) |
| Gentamicin | | GEN | ≤4 | 8 | ≥16 | 30.00% (9/30) | 13.33% (4/30) | 56.67% (17/30) |
| **Phenicols** | | | | | | | | |
| Chloramphenicol | | CHL | ≤8 | 16 | ≥32 | 0% (0/30) | 0% (0/30) | 100.00% (30/30) |
| **Rifamycin** | | | | | | | | |
| Rifamycin | | RIF | ≤1 | 2 | ≥4 | 26.67% (8/30) | 33.33% (10/30) | 40.00% (12/30) |

LR738973_1 from fecal samples of weaned piglets in Brazil and the smaller Group 2 that included model strain HI4320, MK758055.1 from human feces, AM231709.1 from fish bowels and OL629222.1 from raccoon feces in China (**Figure 1A**). The isolates were also clustered closely in the phylogenetic analysis of core SNPs especially between PM1 and PM3 (**Figure 1B**), and PCA analysis indicated PM 1 and PM 3 were closely linked (**Figure 1C**), which might because Jinhua and Quzhou are closer together.

## Biofilm Information Ability

The 30 *P. mirabilis* isolates we identified were also biofilm formers (**Figure 2**). For example, isolates PM1-3, PM1-4 and PM1-6 displayed a moderate biofilm formation capacity ($0.2314 \leq OD_{595} \leq 0.4628$) while 27 isolates were strong biofilm formers ($OD_{595} \geq 0.4628$). All PM2 and PM3 isolates were in this biofilm formation capacity category and significantly different from isolates from PM1 ($p < 0.05$). Bacterial biofilms are resistant to antibiotics, disinfectants and phagocytosis as well as other components of innate and adaptive inflammatory systems (Høiby et al., 2011). Compared with planktonic cells, biofilm cells are less sensitive to antimicrobial agents and limit antibiotic entry to low levels (Tseng et al., 2013). In this study, 90% (27/30) of our isolates were strong biofilm formers and these could be ranked as PM3 > PM2 > PM1. Interestingly, this ranking was consistent with the MIC results (see below) and intensity of biofilm formation has been previously linked to antibiotic resistance (Mirzaei et al., 2019) and MDR strains are often biofilm formers, similar to our findings (Sanchez et al., 2013).

## Phenotypic Antimicrobial Resistance

MIC testing of our 30 isolates indicated different degrees of resistance to the 15 antimicrobials we analyzed. *P. mirabilis* is naturally resistant to tetracycline, tigecycline, and polymyxin, but sensitive to carbapenems and aminoglycosides although amikacin and meropenem resistance has been increasing and is linked with their abuse (Kang et al., 2021). Our results were consistent with these observations and most isolates were sensitive to the aminoglycoside amikacin (21/30) and the carbapenem meropenem (12/30). These levels were lower than those found in two recent hospital studies in Iran that documented 71% and 82% and 82% and 82%, respectively (Mirzaei et al., 2019; Tabatabaei et al., 2021), and higher than the 10.5% sensitive to meropenem in a Chinese hospital (Hu et al., 2012; Han et al., 2015). All our strains were resistant to the tetracyclines (tetracycline, minocycline), macrolides (azithromycin, erythromycin) and chloramphenicol (chloramphenicol) classes and to a lesser extent to amoxicillin/clavulanic acid (27/30), ampicillin (7/30), ciprofloxacin (23/30), and rifampicin (22/30; **Table 2**). Rifampicin is a broad-spectrum antibiotic used primarily in clinical treatment of tuberculosis (Chen et al., 2017). Chloramphenicol was forbidden for veterinary use in China in 2002

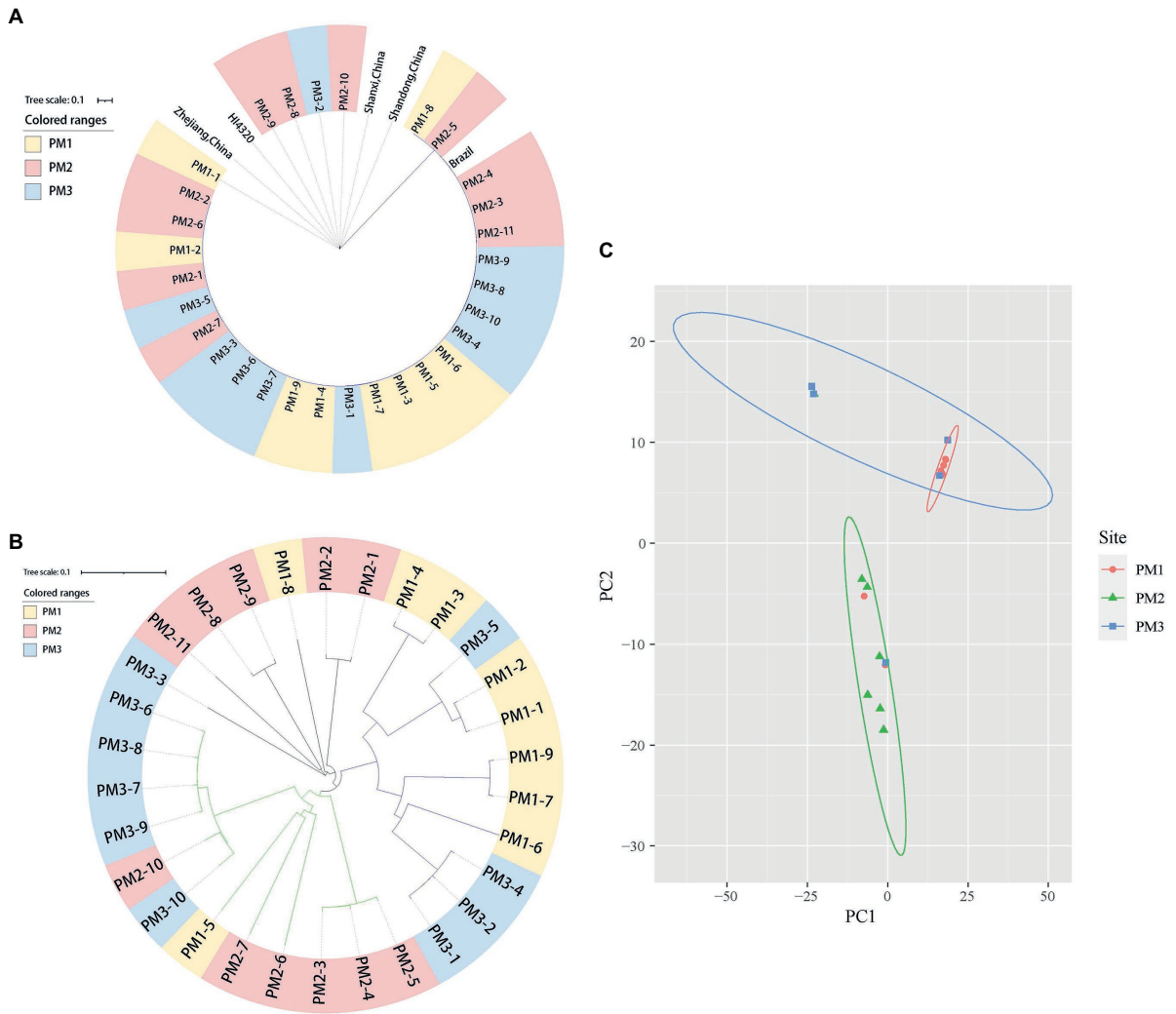


**FIGURE 1 |** Phylogenetic tree of 31 *P. mirabilis* isolates in this study. **(A)** 16S rDNA tree. **(B)** SNP tree. **(C)** PCA analysis.

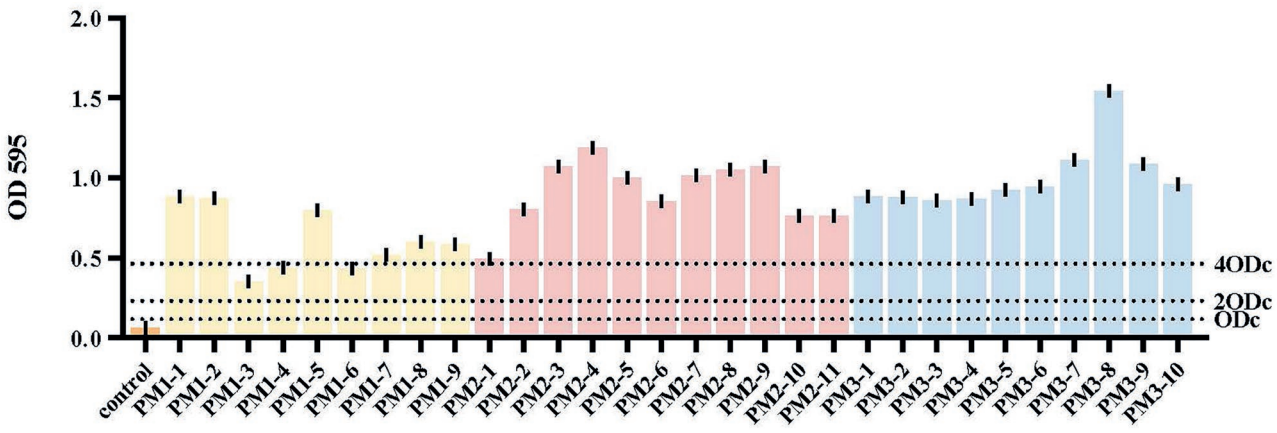


**FIGURE 2 |** Biofilm formation. Yellow, PM1; pink, PM2; blue, PM3. The cut-off value (ODc) was defined as 3 SD above the mean $OD_{595}$ of the negative control: ODc = average $OD_{595}$ of negative control + (3 × SD of negative control). $OD_{595} \leq ODc$ = absence of biofilm; $ODc \leq OD_{595} \leq 2 \times ODc$ = weak biofilm producer; $2 \times ODc \leq OD_{595} \leq 4 \times ODc$ = moderate biofilm producer; $4 \times ODc \leq OD_{595}$ = strong biofilm producer.

(The Ministry of Agriculture of the People's Republic of China, 2002) so it is intriguing why we found such high levels of resistance to these drugs.

Our PM3 group displayed the highest prevalence for antibiotic resistance to amoxicillin/clavulanic acid, ampicillin, gentamycin and amikacin (**Figure 4A**). Moreover, all our isolates were resistant to at least 3 antimicrobial classes and were considered MDR (**Figure 3**). These data were similar to previous findings of resistance in animals in China (Li et al., 2022). These high levels of drug resistance and prevalence of MDR *P. mirabilis* on these farms pose a grave public health threat and would render clinical treatment protocols problematic.

## ARG Predictions

Genomic analysis identified the presence of 91 ARGs in our isolates that encoded resistance to 30 antimicrobial classes. The most prevalent (total 43 genes) resistance mechanisms were antibiotic efflux pumps (42.86%), antibiotic inactivation (27.47%), antibiotic target alteration (15.38%), antibiotic target protection (6.59%) and antibiotic target replacement (7.69%). Interestingly, 43 (47.25%) ARGs were present in all isolates. These ARGs included members of the ATP-binding cassette (ABC), major facilitator superfamily (MFS), *pmr* phosphoethanolamine transferase, resistance-nodulation-cell division (RND), glycopeptide resistance gene cluster, *Acinetobacter* mutant *lpx* gene conferring resistance to colistin and ABC-F ATP-binding cassette ribosomal protection protein MDR gene family members *bcr-1*, *tet(J)*, *tetA(48)*, *msbA*, *mdtB*, and *mexA* (**Supplementary Table S1**).

The remaining 48 ARGs were variably present among the isolates. These were associated with different antibiotic classes: *blaCTX-M-65*, *blaDHA-17*, *blaNmcR*, *blaOXA-1*, *blaPER-4*, and *blaPER-4* for β-lactams; *sul1*, *sul2*, *sul3* for sulfonamides; *rmtB* and AAC, ANT, APH groups for aminoglycosides, *dfr*A groups for trimethoprim, *lnu* for lincosamide, *ereA2*, *mphE*, *emrB* for macrolides; *qnrD1*, *qnrS1* for quinolones; *fos* for fosfomycin; *fact*, *arr-3* for rifamycin and *cml*, *flo*R, *cat* for phenicols. In particular, >50% of these isolates contained *hmrM*, *emrB*, *ImrC*, *floR*, *sul1*, *fact*, *arr-3*, *blaOXA-1*, *catB3* and *AAC(6′)-Ib-cr* that encode resistance to fluoroquinolones, macrolides, phenicols, sulfonamides, rifamycin, aminoglycosides, and β-lactams, respectively. We also detected the *qacH* gene in 5 isolates; this gene encodes resistance to disinfecting agents and antiseptics (**Figure 5C**).

The extended-spectrum β-lactamases (ESBL) were reported for the first time in 1983 (Knothe et al., 1983) and *P. mirabilis* is highly represented as an ESBL host. These representatives include the cephalosporinase (AmpC) and TEM and CTX-M type carbapenemases that can degrade penicillin, piperacillin, and the cephalosporins (Bontron et al., 2019). We found the presence of blaCTX-M in our *P. mirabilis* isolates that

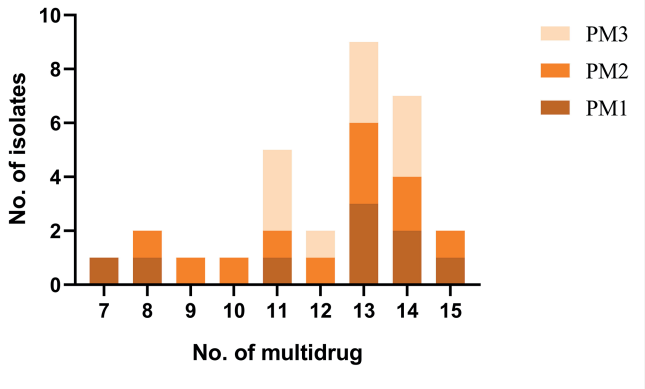


**FIGURE 3 |** MDR tests of *P. mirabilis* isolates.

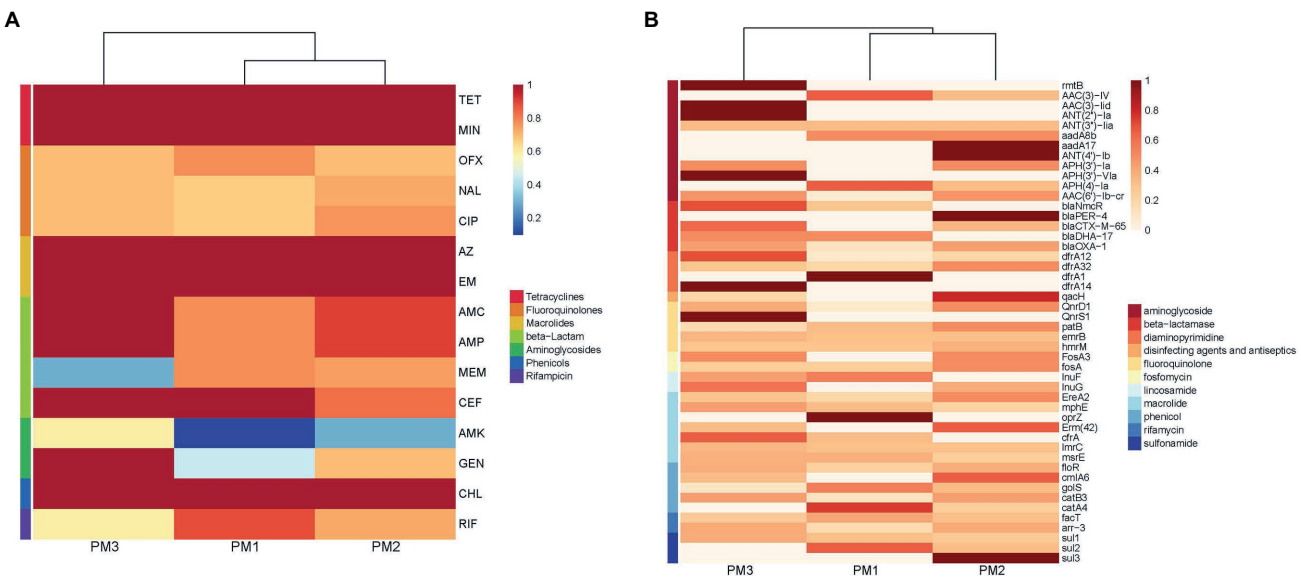


**FIGURE 4 |** Heatmap of antimicrobial resistance of *P. mirabilis* isolates according to **(A)** phenotype and **(B)** genotype.

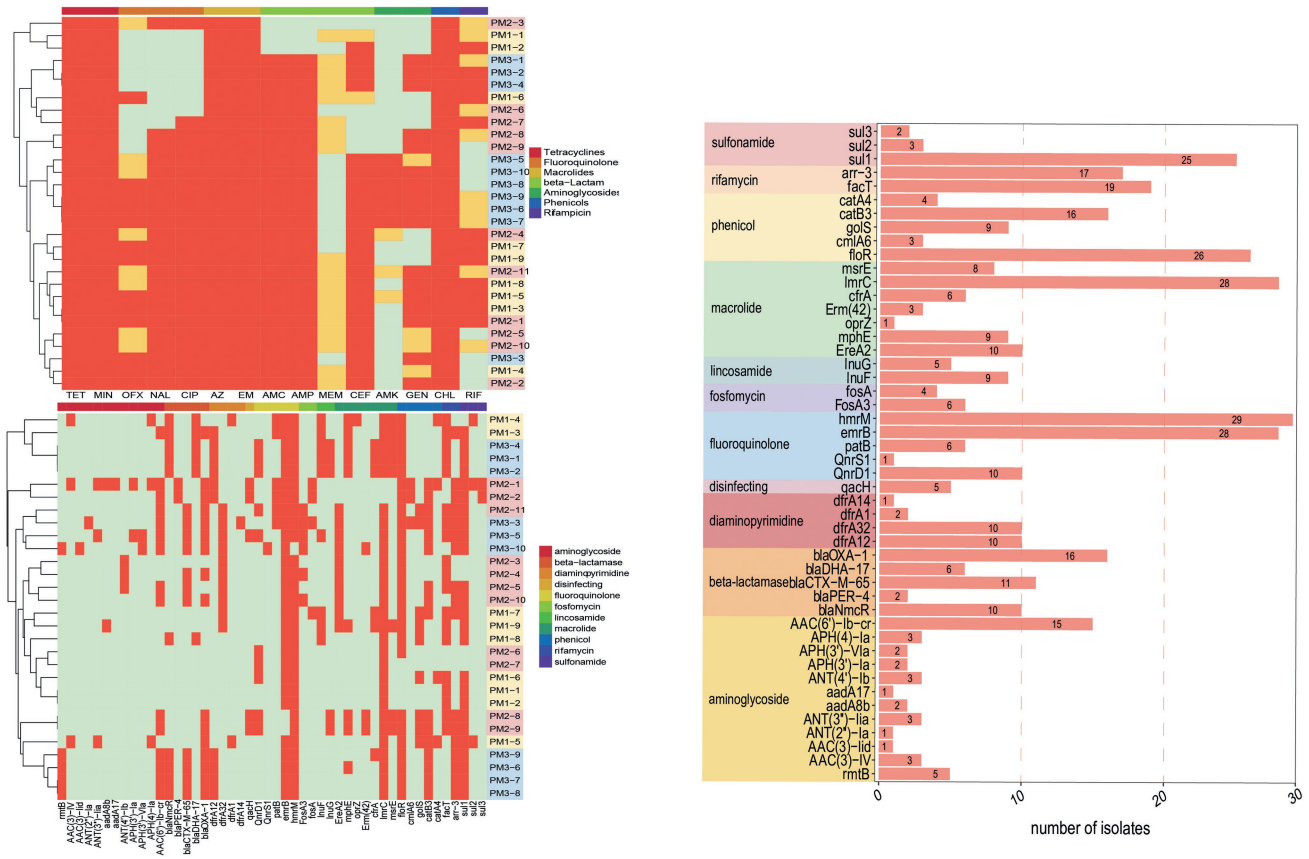


**FIGURE 5** | ARGs present in *P. mirabilis* genomes of study isolates. **(A)** Phenotype; PM 1–5, PM 2–12 were resistant to all the tested drugs. **(B)** Genotype, excepting ARGs that all strains contained. **(C)** Fluoroquinolone, macrolide, phenicol, and sulfonamide prevalence. Red, positive; green, negative.

enables resistance to ceftiofur while piperacillin and cephalosporin resistance was linked to the presence of *blaOXA-1* gene (Algammal et al., 2021). In addition, both *blaOXA-1* and *blaCTX-M* genes synergistically enable *P. mirabilis* to resist β-lactams combination treatments (Schultz et al., 2015). In the current study, our isolates displayed a high prevalence of *blaOXA-1* (53.33%), *blaCTX-M-65* (36.67%), *blaNmcR* (33.33%), *blaDHA-17* (20%), and *blaPER-4* (13.33%) that are β-lactams resistance gene (**Figure 5C**). Remarkably, 10 isolates contained both *blaOXA-1* and *blaCTX-M-65* and 7 in PM3 that also displayed the highest phenotypic resistance to the β-lactams ceftiofur, ampicillin, and amoxicillin/clavulanic acid (**Supplementary Material**; **Figure 3A**). The primary aminoglycoside ARG carried by all isolates in our study was *AAC (6′)-lb-cr* (50%) and 5 *rmtB* 16S rRNA methyltransferases (*rmt*) were detected only in PM3. Resistance to amikacin was also prevalent in this group (**Figure 4A**). The *rmtB* gene is the most frequently detected ARG in Enterobacteriaceae from humans and animals and mediates a very high-level resistance to aminoglycosides including amikacin and is usually associated with MDR bacteria (Fang et al., 2019) and was consistent with our results. We also detected a large number of tetracycline, macrolide, peptide and fluoroquinolone resistance genes and PM3 displayed resistance to tetracycline, macrolide and phenicols (**Figures 4A,B**). Furthermore, we evaluated the relationship between phenotypic drug resistance and the presence or absence of corresponding ARGs. All the isolates were resistant to tetracycline, minocycline, azithromycin, erythromycin, and chloramphenicol ARG, and 76.67% resistant to ciprofloxacin, ceftiofur, and gentamicin, and 60% to meropenem and possessed the cognate ARGs. However, genotypic and phenotypic resistance are sometimes not directly linked although the drug-resistant phenotypes and genotypes of most bacteria were consistent (**Table 3**). Eight strains did not contain any β-lactamase resistance and only one (PM2-3) was sensitive to all the β-lactams, 6 were partially sensitive and PM1-9 displayed resistance similar to the other 22 isolates. For rifampicin, fluoroquinolone, and aminoglycoside resistance, we could not identify any significant correlation between genotype and phenotype and resistance may be mediated by efflux pump mechanisms (**Supplementary Table S2**).

Our results indicated that the distribution of drug-resistant phenotypes was correlated with genotypes for β-lactams and aminoglycosides although these differed by degree. These may

**TABLE 3** | Phenotypic and genotypic analyses of antimicrobial resistance of *P. mirabilis* isolates.

| Antimicrobial drugs | | Coherent results | | Incoherent results | | |
|---|---|---|---|---|---|---|
| Species | Name | Both susceptible | Both resistant | Phenotype resistance and genotype susceptible | Phenotype susceptible and genotype resistance | Percentage of coherence |
| **Tetracyclines** | | | | | | |
| Tetracycline | | 0 | 30 | 0 | 0 | 100.00% (30/30) |
| Minocycline | | 0 | 30 | 0 | 0 | 100.00% (30/30) |
| **Fluoroquinolones** | | | | | | |
| Ofloxacin | | 0 | 21 | 0 | 9 | 70.00% (21/30) |
| Naphthaleneic acid | | 0 | 22 | 0 | 8 | 73.33% (22/30) |
| Ciprofloxacin | | 0 | 23 | 0 | 7 | 76.67% (23/30) |
| **Macrolide** | | | | | | |
| Azithromycin | | 0 | 30 | 0 | 0 | 100.00% (30/30) |
| Erythromycin | | 0 | 30 | 0 | 0 | 100.00% (30/30) |
| **β-Lactam combination** | | | | | | |
| Ampicillin | | 3 | 17 | 9 | 0 | 70.00% (21/30) |
| Amoxicillin/clavulanic acid | | 3 | 17 | 9 | 0 | 70.00% (21/30) |
| Ceftiofur | | 3 | 20 | 5 | 2 | 76.67% (23/30) |
| Meropenem | | 4 | 14 | 4 | 8 | 60.00% (18/30) |
| **Aminoglycosides** | | | | | | |
| Amikacin | | 10 | 9 | 0 | 11 | 63.33% (19/30) |
| Gentamicin | | 6 | 17 | 4 | 3 | 76.67% (23/30) |
| **Phenicols** | | | | | | |
| Chloramphenicol | | 0 | 30 | 0 | 0 | 100.00% (30/30) |
| **Rifamycin** | | | | | | |
| Rifamycin | | 0 | 19 | 3 | 8 | 63.33% (19/30) |

**TABLE 4** | MGE prediction for the 30 isolates examined in this study.

| Group | Isolates | MGE numbers | | Number positive | |
|---|---|---|---|---|---|
| | | ICE | IME | ICE | IME |
| PM1 ($n = 9$) | PM1-7 | 1 | 0 | 33.33% (3/9) | 0.00 (0/9) |
| | PM1-8 | 2 | 0 | | |
| | PM1-9 | 1 | 0 | | |
| PM2 ($n = 11$) | PM2-3 | 0 | 1 | 18.18% (2/11) | 36.36% (4/11) |
| | PM2-5 | 0 | 1 | | |
| | PM2-6 | 1 | 2 | | |
| | PM2-10 | 1 | 1 | | |
| PM3 ($n = 10$) | PM3-1 | 2 | 0 | 80.00% (8/10) | 20.00% (2/10) |
| | PM3-2 | 1 | 0 | | |
| | PM3-3 | 1 | 0 | | |
| | PM3-4 | 1 | 0 | | |
| | PM3-5 | 1 | 1 | | |
| | PM3-6 | 1 | 0 | | |
| | PM3-8 | 0 | 1 | | |
| | PM3-9 | 1 | 0 | | |
| | PM3-10 | 1 | 0 | | |
| Total | 53.33% (16/30) | 15 | 7 | 43.33% (13/30) | 20.00% (6/30) |

due to the complex drug resistance mechanisms in agreement with a study indicating that the phenotypic test and not possession of a specific ARG is the gold standard for assessment of bacterial drug resistance (Wu et al., 2021).

## MGE Prediction

The presence of MGEs in *P. mirabilis* are a great public health threat due to the capacity for autonomous ARG transfer (Partridge et al., 2018). ICEs are integrated DNA regions in the chromosome that are mobilizable by conjugation *via* type IV secretion systems that mediate cell to cell DNA transfer (Delavat et al., 2017). Tyrosine and serine recombinases can mediate *att* site-specific recombination between circular ICE and chromosomal targets (Johnson and Grossman, 2015). IMEs can be mobilized using ICE machinery or conjugative plasmids when ICEs erode and accumulate within the host chromosome leading to inactivation of their independent mobility (Partridge et al., 2018).

In our study, we identified 15 ICEs and 7 IMEs and 53.33% of our isolates contained ICEs or IMEs with 13 of 30 containing ICEs and 6 of 30 containing IMEs (**Table 4**). These results were higher than previously reported (23.53%) in *P. mirabilis* from the tree shrew (Gu et al., 2020). Most MGEs were detected in PM3 (90%) and 2 PM1 strains possessed the same ICE type (**Table 5**). This indicated that transconjugation was frequent for this group. Moreover, strains with ICEs from the same group carried similar resistance genes especially in PM1 and PM3. All of the PM1 strains with MGEs (PM1-7, PM1-8, PM1-9) were clustered. Strains with MGEs in PM3 gathered into 3 small groups and carried the same resistance gene (PM3-8, PM3-9

**TABLE 5** | Details of 15 predicted ICEs and 7 IMEs.

| Isolate | Location (nt) | GC content (%) | Length (bp) | Direct repeats | Type |
|---|---|---|---|---|---|
| PM1-7 | 1809473..1902135 | 44.85 | 92,663 | | Putative ICE with T4SS |
| PM1-8 | 2873874..2966537 | 44.85 | 92,664 | † | Putative ICE with T4SS |
| | 3727325..4093850 | 41.37 | 366,526 | attL: 3727325..3727339 (agaggtcattgtgca) | Putative ICE with T4SS |
| | | | | attR: 4093836..4093850 (agaggtcattgtgca) | |
| PM1-9 | 1810225..1902887 | 44.85 | 92,663 | ≠ | Putative ICE with T4SS |
| PM2-6 | 1741700..1935382 | 41.39 | 193,683 | attL: 1741700..1741714 (ttttgaatgacataa) | Putative ICE with T4SS |
| | | | | attR: 1935368..1935382 (ttttgaatgacataa) | |
| PM2-10 | 3589109..3824596 | 40.54 | 235,488 | attL: 3589109..3589124 (taattgccattatatt) | Putative ICE with T4SS |
| | | | | attR: 3824581..3824596 (taattgccattatatt) | |
| PM3-1 | 3795192..3926392 | 44.63 | 131,201 | attL: 3795192..3795206 (agatacattttgttt) | Putative ICE with T4SS |
| | | | | attR: 3926378..3926392 (agatacattttgttt) | |
| | 1308600..1414471 | 43.22 | 105,872 | attL: 1308600..1308614 (tattgccgctttaat) | Putative ICE with T4SS |
| | | | | attR: 1414457..1414471 (tattgccgctttaat) | |
| PM3-2 | 1678276..1746776 | 43.40 | 68,501 | attL: 1678276..1678290 (taaaagcaacagcat) | Putative ICE with T4SS |
| | | | | attR: 1746762..1746776 (taaaagcaacagcat) | |
| PM3-3 | 3696382..3796909 | 41.98 | 100,528 | attL: 3696382..3696396 (taatgctatttttttt) | Putative ICE with T4SS |
| | | | | attR: 3796895..3796909 (taatgctatttttttt) | |
| PM3-4 | 1678055..1746555 | 43.40 | 68,501 | attL: 1678055..1678069 (taaaagcaacagcat) | Putative ICE with T4SS |
| | | | | attR: 1746541..1746555 (taaaagcaacagcat) | |
| PM3-5 | 3524314..3652232 | 42.17 | 127,919 | attL: 3524314..3524328 (ataaaatacttttta) | Putative ICE with T4SS |
| | | | | attR: 3652218..3652232 (ataaaatacttttta) | |
| PM3-6 | 3603558..3866601 | 40.46 | 263,044 | attL: 3603558..3603573 (tctgtgcagtaaaaaa) | Putative ICE with T4SS |
| | | | | attR: 3866586..3866601 (tctgtgcagtaaaaaa) | |
| PM3-9 | 3601557..3864543 | 40.45 | 262,987 | attL: 3601557..3601572 (tctgtgcagtaaaaaa) | Putative ICE with T4SS |
| | | | | attR: 3864528..3864543 (tctgtgcagtaaaaaa) | |
| PM3-10 | 3716853..3757384 | 44.69 | 40,532 | – | Putative ICE with T4SS |
| PM2-3 | 3790440..3819943 | 44.38 | 29,504 | attL: 3790440..3790454 (caaaaccataaaacc) | Putative IME |
| | | | | attR: 3819929..3819943 (caaaaccataaaacc) | |
| PM2-5 | 3363919..3384240 | 40.87 | 20,322 | attL: 3363919..3363933 (ccaaaaaatgcatta) | Putative IME |
| | | | | attR: 3384226..3384240 (ccaaaaaatgcatta) | |
| PM2-6 | 3316029..3385857 | 36.68 | 69,829 | attL: 3316029..3316044 (aaaatattagtgagta) | Putative IME |
| | | | | attR: 3385842..3385857 (aaaatattagtgagta) | |
| | 3853151..3907640 | 45.54 | 54,490 | †† | Putative IME |
| PM2-10 | 3847018..3872472 | 42.51 | 25,455 | attL: 3847018..3847032 (cgctgatgcagtaac) | Putative IME |
| | | | | attR: 3872458..3872472 (cgctgatgcagtaac) | |
| PM3-5 | 1510679..1556480 | 40.12 | 45,802 | attL: 1510679..1510694 (cagcaatggatattta) | Putative IME |
| | | | | attR: 1556465..1556480 (cagcaatggatattta) | |
| PM3-8 | 3864568..3889007 | 42.45 | 24,440 | attL: 3864568..3864582 (cgctgatgcagtaac) | Putative IME |
| | | | | attR: 3888993..3889007 (cgctgatgcagtaac) | |

† *attL: 2873874..2873925 (atggtgcccggactcggaatcgaaccaaggacacggggattttcaatcccct); attR: 2966486..2966537 (atggtgcccggactcggaatcgaaccaaggacacggggattttcaatcccct).*
≠ *attL: 1810225..1810276 (atggtgcccggactcggaatcgaaccaaggacacggggattttcaatcccct); attR: 1902836..1902887 (atggtgcccggactcggaatcgaaccaaggacacggggattttcaatcccct).*
†† *attL: 3853151..3853228 (ggattgttcacccactaatagggaacgtgagctgggtttagaccgtcgtgagacaggttagttttaccctactgatga); attR: 3907563..3907640 (ggattgttcacccaccaataggaacgtgagctgggtttagaccgtcgtgagacaggttagttttaccctactgatga).*

and PM3-1, PM3-2, PM3-4; **Figure 5** and **Table 5**). Such results indicate the serious drug resistance of PM3 group may be associated with MGEs. The high MDR prevalence could be attributed to a combination of these factors. However, we did not perform a validation analysis of these detected MGE functions and further analyses are needed to understand the effect of these elements on drug resistance.

## Virulence Gene Prediction

Most of the endemic strains in our study were MDR and the number of virulence genes identified would contribute to the establishment of persistent and drug-tolerant infections. In our 30 isolates, we identified 95 of 2,741 possible virulence factors composed of 10 categories: transporter, fimbriae, flagella, hemin, metal, enzyme, protein, regulator, type VI secretion system, and adhesin. Enzyme represented 22 virulence factors related to factors such as DP-heptose synthase, UDP-glucose 6-dehydrogenase, and decarboxylase. The flagella and fimbrial groups were also abundant and 20 different genes were represented. The least abundant were the type VI secretion system and adhesin (**Figure 6**).

All isolates contained 63/95 virulence factors associated with biofilm formation, adhesin, carbon storage, hemin, metal and motility and 32/95 were variably present among

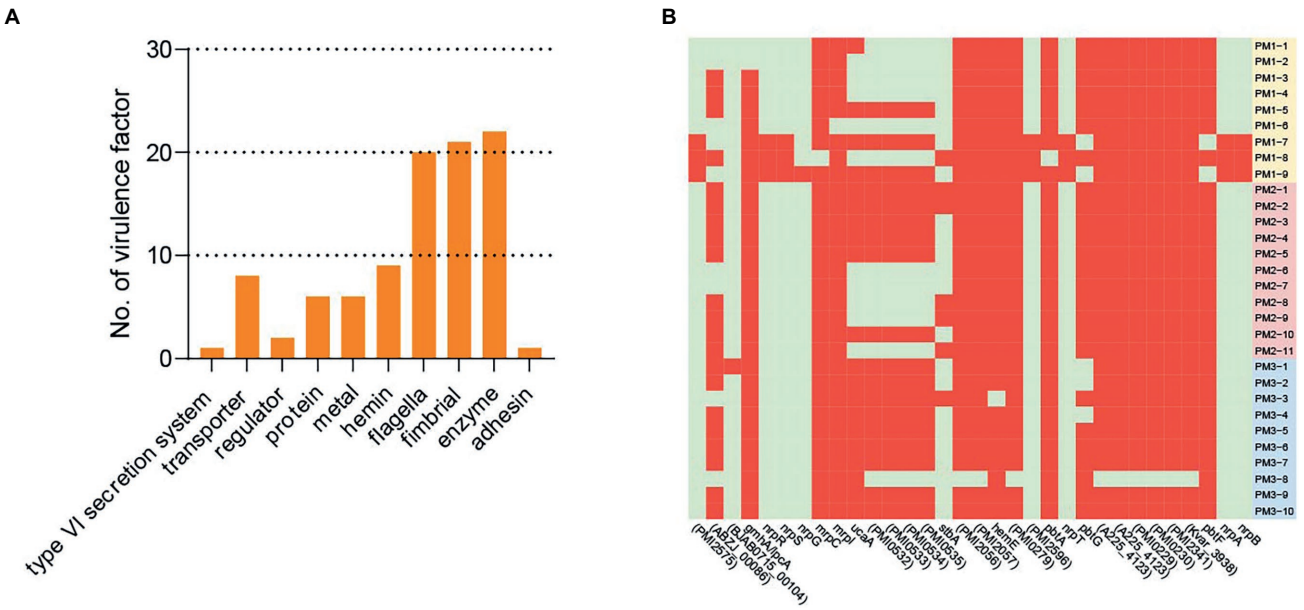


**FIGURE 6 |** Virulence factors. **(A)** Virulence factor categories and the number represented in each category. **(B)** Virulence factors that were variably present among isolates. Red, positive; green, negative.

the 30 isolates. All strains contained at least 8 of the 32 variable virulence genes that included the categories transporter, Proteus-like (MR/P) fimbriae, protein, adhesin, enzyme, fimbrial, metal, and regulator that included ABC transporter permease, hemolysin activator protein, fimbrial adhesin, IS4 family transposase ORF 2, siderophore biosynthesis protein, and non-ribosomal peptide synthase. The PM3 group contained the largest numbers of virulence factors while 10 isolates did not contain any virulence factors related to UCA in the PM1 and PM2 groups (**Figure 6B, Supplementary Table S2**).

The virulence factors for the MR/P and PMF fimbriae and UCA are considered essential in the initial phase of infection and contribute significantly to UTI development (Cook et al., 1995; Jansen et al., 2004; Govindarajan and Kandaswamy, 2022). Aggregation and initial biofilm formation are mediated by MR/P fimbriae that are products of the *mrp* operon (Wasfi et al., 2020). The UCA fimbriae are controlled *via* the *ucaA* gene and PMF confers bladder colonization. All of our isolates contained PMF and 20 contained *ucaA* and most contained 5 other UCA-related virulence genes (**Supplementary Table S3**). Moreover, we found 20 putative fimbrial operons and this is the largest number found in any sequenced bacterial species (Scavone et al., 2016).

*P. mirabilis* is highly mobile and swarm ability allows it to pass through catheters to the urinary tract. Flagella are important to swarmer cell differentiation and are necessary to swarming motility (Belas and Suvanasuthi, 2005). In our study, we found all the isolates carried 20 different flagellar virulence factors including *flgI*, *film*, and *flgC*. Most importantly, 29 isolates except PM3-8 contained the *ZapA* virulence factor (Belas et al., 2004) that enables the bacterium to survive in the urethra *via* degradation of host proteins using the Zap1 metalloproteinase (**Supplementary Table S3**). Our findings indicated the *P. mirabilis* isolated from healthy pigs is a potential threat to public health since its high prevalence and possession of virulence genes and ARGs can be transferred *via* MGEs.

## CONCLUSION

In this study, we conducted the prevalence and characteristics of *P. mirabilis* from healthy farmed pigs in China. Phylogenetic analysis indicated the isolates were closely related and clustered into 2 major groups. Our 30 isolates harbored 91 ARGs and >50% contained MGEs as well as 95 different virulence factors that included the presence of 20 putative fimbrial operons. Most (90%) of the isolates were strong biofilm formers. This is the first study to report the genomic investigation and prevalence of *P. mirabilis* associated with pig farm operations in Zhejiang, China.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

## AUTHOR CONTRIBUTIONS

XX and CX: writing—review and editing. XQ and JZ: investigating and writing—original draft. HH and WW: data

curation. YX, BT, and HL: resources. All authors contributed to manuscript revision, read, and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.952982/full#supplementary-material

## REFERENCES

Algammal, A. M., Hashem, H. R., Alfifi, K. J., Hetta, H. F., Sheraba, N. S., Ramadan, H., et al. (2021). atpD gene sequencing, multidrug resistance traits, virulence-determinants, and antimicrobial resistance genes of emerging XDR and MDR-*Proteus mirabilis*. *Sci. Rep.* 11:9476. doi: 10.1038/s41598-021-88861-w

Belas, R., Manos, J., and Suvanasuthi, R. (2004). *Proteus mirabilis* ZapA metalloprotease degrades a broad spectrum of substrates, including antimicrobial peptides. *Infect. Immun.* 72, 5159–5167. doi: 10.1128/iai.72.9.5159-5167.2004

Belas, R., and Suvanasuthi, R. (2005). The ability of *Proteus mirabilis* to sense surfaces and regulate virulence gene expression involves FliL, a flagellar basal body protein. *J. Bacteriol.* 187, 6789–6803. doi: 10.1128/jb.187.19.6789-6803.2005

Bi, S.-I., Tang, S.-z., Wu, X.-y., and Chen, S.-y. (2013). Quantitative detection of Proteus species by real-time polymerase chain reaction using SYBR green I. *Ann. Microbiol.* 63, 1205–1208. doi: 10.1007/s13213-012-0519-3

Biswas, S., Elbediwi, M., Gu, G., and Yue, M. (2020). Genomic characterization of new variant of hydrogen sulfide (H(2)S)-producing *Escherichia coli* with multidrug resistance properties carrying the mcr-1 gene in China †. *Antibiotics (Basel)* 9:80. doi: 10.3390/antibiotics9020080

Bontron, S., Poirel, L., Kieffer, N., Savov, E., Trifonova, A., Todorova, I., et al. (2019). Increased resistance to Carbapenems in *Proteus mirabilis* mediated by amplification of the Bla(VIM-1)-carrying and IS26-associated class 1 Integron. *Microb. Drug Resist.* 25, 663–667. doi: 10.1089/mdr.2018.0365

Chen, C.-M., Lai, C.-H., Wu, H.-J., and Wu, L.-T. (2017). Genetic characteristic of class 1 integrons in *proteus mirabilis* isolates from urine samples. *Biomedicine* 7:9. doi: 10.1051/bmdcn/2017070202

Chen, L., Yang, J., Yu, J., Yao, Z., Sun, L., Shen, Y., et al. (2005). VFDB: a reference database for bacterial virulence factors. *Nucleic Acids Res.* 33, D325–D328. doi: 10.1093/nar/gki008

CLSI (2017). *Performance Standards for Antimicrobial Susceptibility Testing, 27th Edn.* Wayne, PA: Clinical and Laboratory Standards Institute.

Cook, S. W., Mody, N., Valle, J., and Hull, R. (1995). Molecular cloning of *Proteus mirabilis* uroepithelial cell adherence (uca) genes. *Infect. Immun.* 63, 2082–2086. doi: 10.1128/iai.63.5.2082-2086.1995

Delavat, F., Miyazaki, R., Carraro, N., Pradervand, N., and van der Meer, J. R. (2017). The hidden life of integrative and conjugative elements. *FEMS Microbiol. Rev.* 41, 512–537. doi: 10.1093/femsre/fux008

Drzewiecka, D. (2016). Significance and roles of Proteus spp. bacteria in natural environments. *Microbiol. Ecol.* 72, 741–758. doi: 10.1007/s00248-015-0720-6

Fang, L. X., Deng, G. H., Jiang, Q., Cen, D. J., Yang, R. S., Feng, Y. Y., et al. (2019). Clonal expansion and horizontal transmission of epidemic F2:A1:B1 plasmids involved in co-spread of rmtB with qepA and blaCTX-M-27 in extensively drug-resistant *Salmonella enterica* serovar Indiana isolates. *J. Antimicrob. Chemother.* 74, 334–341. doi: 10.1093/jac/dky441

Gilbert, N. (2012). Rules tighten on use of antibiotics on farms. *Nature* 481:125. doi: 10.1038/481125a

Gong, Z., Shi, X., Bai, F., He, X., Zhang, H., Li, Y., et al. (2019). Characterization of a novel Diarrheagenic strain of *Proteus mirabilis* associated with food poisoning in China. *Front. Microbiol.* 10:2810. doi: 10.3389/fmicb.2019.02810

Govindarajan, D. K., and Kandaswamy, K. (2022). Virulence factors of uropathogens and their role in host pathogen interactions. *Cell Surf.* 8:100075. doi: 10.1016/j.tcsw.2022.100075

Gu, W., Wang, W., Tong, P., Liu, C., Jia, J., Lu, C., et al. (2020). Comparative genomic analysis of Proteus spp. isolated from tree shrews indicated unexpectedly high genetic diversity. *PLoS One* 15:e0229125.

Han, X., Liu, L., Fan, G., Zhang, Y., Xu, D., Zuo, J., et al. (2015). *Riemerella anatipestifer* lacks luxS, but can uptake exogenous autoinducer-2 to regulate biofilm formation. *Res. Microbiol.* 166, 486–493. doi: 10.1016/j.resmic.2015.06.004

Høiby, N., Ciofu, O., Johansen, H. K., Song, Z. J., Moser, C., Jensen, P., et al. (2011). The clinical impact of bacterial biofilms. *Int. J. Oral Sci.* 3, 55–65. doi: 10.4248/ijos11026

Hu, Y. Y., Cai, J. C., Zhang, R., Zhou, H. W., Sun, Q., and Chen, G. X. (2012). Emergence of *Proteus mirabilis* harboring blaKPC-2 and qnrD in a Chinese hospital. *Antimicrob. Agents Chemother.* 56, 2278–2282. doi: 10.1128/aac.05519-11

Jansen, A. M., Lockatell, V., Johnson, D. E., and Mobley, H. L. (2004). Mannose-resistant Proteus-like fimbriae are produced by most *Proteus mirabilis* strains infecting the urinary tract, dictate the in vivo localization of bacteria, and contribute to biofilm formation. *Infect. Immun.* 72, 7294–7305. doi: 10.1128/iai.72.12.7294-7305.2004

Johnson, C. M., and Grossman, A. D. (2015). Integrative and conjugative elements (ICEs): what they do and how they work. *Annu. Rev. Genet.* 49, 577–601. doi: 10.1146/annurev-genet-112414-055018

Kang, Q., Wang, X., Zhao, J., Liu, Z., Ji, F., Chang, H., et al. (2021). Multidrug-resistant *Proteus mirabilis* isolates carrying bla(OXA-1) and bla(NDM-1) from wildlife in China: increasing public health risk. *Integr. Zoo.* 16, 798–809. doi: 10.1111/1749-4877.12510

Klein, E. Y., Van Boeckel, T. P., Martinez, E. M., Pant, S., Gandra, S., Levin, S. A., et al. (2018). Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. *Proc. Natl. Acad. Sci. U. S. A.* 115, e3463–e3470. doi: 10.1073/pnas.1717295115

Knothe, H., Shah, P., Krcmery, V., Antal, M., and Mitsuhashi, S. (1983). Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of Klebsiella pneumoniae and *Serratia marcescens*. *Infection* 11, 315–317. doi: 10.1007/bf01641355

Lajhar, S. A., Brownlie, J., and Barlow, R. (2018). Characterization of biofilm-forming capacity and resistance to sanitizers of a range of *E. coli* O26 pathotypes from clinical cases and cattle in Australia. *BMC Microbiol.* 18:41. doi: 10.1186/s12866-018-1182-z

Larson, C. (2015). Pharmaceuticals. China's lakes of pig manure spawn antibiotic resistance. *Science* 347:704. doi: 10.1126/science.347.6223.704

Lei, C. W., Zhang, A. Y., Wang, H. N., Liu, B. H., Yang, L. Q., and Yang, Y. Q. (2016). Characterization of SXT/R391 integrative and conjugative elements in *Proteus mirabilis* isolates from food-producing animals in China. *Antimicrob. Agents Chemother.* 60, 1935–1938. doi: 10.1128/aac.02852-15

Li, N. (2021). Isolation and identification of swine-sourced *Proteus mirabilis*, and detection of its drug resistance and virulence genes. *Ani. Husb. Vet. Med.* 53, 89–94.

Li, Z., Peng, C., Zhang, G., Shen, Y., Zhang, Y., Liu, C., et al. (2022). Prevalence and characteristics of multidrug-resistant *Proteus mirabilis* from broiler farms in Shandong Province, China. *Poult. Sci.* 101:101710. doi: 10.1016/j.psj.2022.101710

Liu, Q., Chen, W., Elbediwi, M., Pan, H., Wang, L., Zhou, C., et al. (2020). Characterization of Salmonella Resistome and Plasmidome in pork

production system in Jiangsu, China. *Front. Vet. Sci.* 7:617. doi: 10.3389/fvets.2020.00617

Liu, Y., Jiang, J., Ed-Dra, A., Li, X., Peng, X., Xia, L., et al. (2021). Prevalence and genomic investigation of Salmonella isolates recovered from animal food-chain in Xinjiang, China. *Food Res. Int.* 142:110198. doi: 10.1016/j.foodres.2021.110198

Liu, M., Li, X., Xie, Y., Bi, D., Sun, J., Li, J., et al. (2019). ICEberg 2.0: an updated database of bacterial integrative and conjugative elements. *Nucleic Acids Res.* 47, d660–d665. doi: 10.1093/nar/gky1123

Marshall, B. M., and Levy, S. B. (2011). Food animals and antimicrobials: impacts on human health. *Clin. Microbiol. Rev.* 24, 718–733. doi: 10.1128/cmr.00002-11

Mirzaei, A., Habibi, M., Bouzari, S., and Karam, M. R. A. (2019). Characterization of antibiotic-susceptibility patterns, virulence factor profiles and clonal relatedness in *Proteus mirabilis* isolates from patients with urinary tract infection in Iran. *Infect. Drug Resist.* 12, 3967–3979. doi: 10.2147/idr.S230303

Mobley, H. L., and Belas, R. (1995). Swarming and pathogenicity of *Proteus mirabilis* in the urinary tract. *Trends Microbiol.* 3, 280–284. doi: 10.1016/s0966-842x(00)88945-3

Olonitola, O. S., Fahrenfeld, N., and Pruden, A. (2015). Antibiotic resistance profiles among mesophilic aerobic bacteria in Nigerian chicken litter and associated antibiotic resistance genes1. *Poult. Sci.* 94, 867–874. doi: 10.3382/ps/pev069

Partridge, S. R., Kwong, S. M., Firth, N., and Jensen, S. O. (2018). Mobile genetic elements associated with antimicrobial resistance. *Clin. Microbiol. Rev.* 31:e00088-17. doi: 10.1128/cmr.00088-17

Qian, X., Gu, J., Sun, W., Wang, X. J., Su, J. Q., and Stedfeld, R. (2018). Diversity, abundance, and persistence of antibiotic resistance genes in various types of animal manure following industrial composting. *J. Hazard. Mater.* 344, 716–722. doi: 10.1016/j.jhazmat.2017.11.020

Reich, F., Atanassova, V., and Klein, G. (2013). Extended-spectrum β-lactamase- and AmpC-producing enterobacteria in healthy broiler chickens, Germany. *Emerg. Infect. Dis.* 19, 1253–1259. doi: 10.3201/eid1908.120879

Sanchez, C. J. Jr., Mende, K., Beckius, M. L., Akers, K. S., Romano, D. R., Wenke, J. C., et al. (2013). Biofilm formation by clinical isolates and the implications in chronic infections. *BMC Infect. Dis.* 13:47. doi: 10.1186/1471-2334-13-47

Scavone, P., Iribarnegaray, V., Caetano, A. L., Schlapp, G., Härtel, S., and Zunino, P. (2016). Fimbriae have distinguishable roles in *Proteus mirabilis* biofilm formation. *Pathog. Dis.* 74:ftw033. doi: 10.1093/femspd/ftw033

Schultz, E., Haenni, M., Mereghetti, L., Siebor, E., Neuwirth, C., Madec, J. Y., et al. (2015). Survey of multidrug resistance integrative mobilizable elements SGI1 and PGI1 in *Proteus mirabilis* in humans and dogs in France, 2010-13. *J. Antimicrob. Chemother.* 70, 2543–2546. doi: 10.1093/jac/dkv154

Shelenkov, A., Petrova, L., Fomina, V., Zamyatin, M., Mikhaylova, Y., and Akimkin, V. (2020). Multidrug-resistant *Proteus mirabilis* strain with cointegrate plasmid. *Microorganisms* 8:1775. doi: 10.3390/microorganisms8111775

Shi, X., Li, Y., Yang, Y., Shen, Z., Cai, C., Wang, Y., et al. (2021). High prevalence and persistence of carbapenem and colistin resistance in livestock farm environments in China. *J. Hazard. Mater.* 406:124298. doi: 10.1016/j.jhazmat.2020.124298

Stepanović, S., Vuković, D., Hola, V., Di Bonaventura, G., Djukić, S., Cirković, I., et al. (2007). Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *APMIS* 115, 891–899. doi: 10.1111/j.1600-0463.2007.apm_630.x

Stock, I. (2003). Natural antibiotic susceptibility of Proteus spp., with special reference to P. mirabilis and *P. penneri* strains. *J. Chemother.* 15, 12–26. doi: 10.1179/joc.2003.15.1.12

Tabatabaei, A., Ahmadi, K., Shabestari, A. N., Khosravi, N., and Badamchi, A. (2021). Virulence genes and antimicrobial resistance pattern in *Proteus mirabilis* strains isolated from patients attended with urinary infections to tertiary hospitals, in Iran. *Afr. Health Sci.* 21, 1677–1684. doi: 10.4314/ahs.v21i4.22

The Ministry of Agriculture of the People's Republic of China. (2002) Maximum residue limits for veterinary drugs in animal products. Available at: https://www.moa.gov.cn/gk/tzgg_1/gg/200302/t20030226_59300.htm (Accessed December 24, 2002).

Tseng, B. S., Zhang, W., Harrison, J. J., Quach, T. P., Song, J. L., Penterman, J., et al. (2013). The extracellular matrix protects *Pseudomonas aeruginosa* biofilms by limiting the penetration of tobramycin. *Environ. Microbiol.* 15, 2865–2878. doi: 10.1111/1462-2920.12155

Wang, Q., Peng, K., Liu, Y., Xiao, X., Wang, Z., and Li, R. (2021). Characterization of TMexCD3-TOprJ3, an RND-type efflux system conferring resistance to tigecycline in Proteus mirabilis, and its associated integrative conjugative element. *Antimicrob. Agents Chemother.* 65:e0271220. doi: 10.1128/aac.02712-20

Wasfi, R., Hamed, S. M., Amer, M. A., and Fahmy, L. I. (2020). *Proteus mirabilis* biofilm: development and therapeutic strategies. *Front. Cell. Infect. Microbiol.* 10:414. doi: 10.3389/fcimb.2020.00414

Wu, B., Ed-Dra, A., Pan, H., Dong, C., Jia, C., and Yue, M. (2021). Genomic investigation of Salmonella isolates recovered from a pig slaughtering process in Hangzhou, China. *Front. Microbiol.* 12:704636. doi: 10.3389/fmicb.2021.704636

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# Screening and evaluation of lactic acid bacteria with probiotic potential from local Holstein raw milk

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There are massive bacteria in the raw milk, especially the lactic acid bacteria (LABs), which have been considered probiotics in humans and animals for a long time. Novel probiotics are still urgently needed because of the rapid development of the probiotic industry. To obtain new LABs with high probiotic potential, we obtained 26 LAB isolates, named L1~L26, from local Holstein raw milk collected from a farm whose milk had never been used for LAB isolation. We identified them at the species level by biochemical and 16S rDNA sequencing methods. Their antagonistic activities against four target pathogens (*Escherichia coli* ATCC 25922, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* PAO1, and *Salmonella enterica* H9812), co-aggregative ability with these target pathogens, survivability in the simulated gastrointestinal tract conditions and phenol, auto-aggregation and hydrophobicity, hemolytic activity, and antibiotic susceptibility, were evaluated *in vitro*. Five *Lactiplantibacillus plantarum* isolates (L5, L14, L17, L19, and L20) showed more promising probiotic potential than others. Specifically, these five isolates conglutinated with and inhibited all the target pathogens, and survived in the simulated gastric juice (92.55~99.69%), intestinal juice (76.18~83.39%), and 0.4% phenol (76.95~88.91%); possessed considerable auto-aggregation (83.91~90.33% at 24h) and hydrophobicity (79.32~92.70%); and were non-hemolytic, sensitive to kinds of common antimicrobials. Our findings demonstrated that these five isolates could be preliminarily determined as probiotic candidates because they have better probiotic potential than those previously reported. Again, this study highlighted the potential of raw milk for probiotic isolating and screening and provided the probiotic industry with five new LAB candidates.

KEYWORDS

**raw milk, LAB strains, probiotic potential, safety assessment, probiotics, *Lactiplantibacillus plantarum***

# Introduction

Probiotics are defined as a kind of "live microorganisms which when administered in adequate amounts confer a health benefit on the consumer" (Araya et al., 2002). Lactic acid bacteria (LABs), including *Lactococcus*, *Lactobacillus*, *Streptococcus*, and *Enterococcus*, are a group of Gram-positive, cocci or rod, catalase-negative, coagulase-negative, non-spore-forming, and harsh to culture bacteria. LABs possess a high tolerance for low pH (Mokoena, 2017; De Melo Pereira et al., 2018) and have been considered one of the most important probiotics for a long history because of their extensive beneficial effects on humans and animals (Food and Agriculture Organization, 2006; Hill et al., 2014). It has been determined that LABs possess anti-cancer, anti-cholesterol, anti-depression, anti-anxiety, anti-obesity, anti-diabetic, and immunostimulatory activities (Zoumpopoulou et al., 2017; Suez et al., 2019; Mathur et al., 2020). Intriguing, LABs are also considered a role in the respiratory system (De Boeck et al., 2021). The LABs-sourced biofunctional products are still in great need even though diverse functional LABs have been applied in commercial probiotic fermented food worldwide (De Melo Pereira et al., 2018). An approximate 27.9 billion dollars were spent on the purchase of probiotics in 2011, which increased to 44.9 billion dollars in 2018 (Transparency Market Research (TMR), 2013). And the global probiotics demand was expected to increase to 83.5 billion dollars by 2022 T.T.M. Research (2017). Meanwhile, abundant scientific studies involving the selection of LABs with different and specific functional properties have been reported in the last decades, and new probiotic LABs are also being constantly isolated and identified (De Melo Pereira et al., 2018). These new LABs are isolated from multiple sources, such as human raw milk (Shin et al., 2021) and grains (Fiorda et al., 2017), but milk and other dairy products are commonly considered the main sources of LABs (Plessas et al., 2017; Reuben et al., 2020).

Considering the target functions and technological applications, screening, selecting, and evaluating new probiotic LABs require a comprehensive approach consisting of a series of steps (De Melo Pereira et al., 2018). In 2002, the Food and Agriculture Organization of the United Nations and the World Health Organization published the "Guidelines for Evaluation of Probiotics in Food" (Araya et al., 2002), which put an end to the chaos in affirming the efficacy and safety of probiotic microorganisms, and established safety and effectiveness standards for probiotics selection and evaluation. The guidelines proposed several criteria for the selection of probiotics. Firstly, the candidates should possess the ability against the unfavorable conditions imposed by the human body, including the enzymes, adverse pH, mild heat shock, bile acid, phenol, etc. Secondly, the candidates should also possess the ability to colonize the gastrointestinal tract (GIT) epithelial cells, called adhesion ability, consisting of both autoaggregation capacity and hydrophobic properties. Once adhered to the epithelial cells, the candidates should produce extracellular antimicrobial ability by converting carbohydrates, proteins, and other minor compounds into important substances that can inhibit pathogenic bacteria or by competing for nutrients, aggregating with pathogens, and stimulating the immune system (Lebeer et al., 2008). Furthermore, the safety must be assessed when live microbes are introduced to the daily diet (Culligan et al., 2009), including isolation history, taxonomic identification, absence of virulence, infectivity, toxicity, and transferable antibiotic resistance genes (Sanders et al., 2010). Finally, after *in vitro* studies, animal studies and clinical trials should also be executed to validate the safety and efficiency of the final candidates (De Melo Pereira et al., 2018).

Hence, to obtain new LABs with promising probiotic potential, we collected cow milk samples from indigenous Holstein cows raised on a historic farm whose milk had never been sampled for probiotics isolation and isolated and identified the LABs in the milk. Then, the antipathogenic activity, stress tolerance, adhesion activity, safety characteristics, and growth performance were assessed. Our study will provide new probiotic LAB candidates for further probiotic development and industry.

# Materials and methods

## Sample collection

Fresh milk samples were randomly collected from 22 healthy (puerperal period, 524 ± 58 kg, without visible symptoms) Holstein cows belonging to a large-scale farm (over 1,000 puerperal cows, 29.91°N, 103.37°E) with over 50 years of history in Hongya, Sichuan, China. Before the sample collection, the udder and the surrounding area were thoroughly cleaned with 70% ethanol and dried with individual paper towels. The sample was collected into 50 ml sterile corked plastic tubes after discarding the first three drops of raw milk, followed by immediate storage in a 4°C ice-box, transportation to the laboratory for the following experiments.

## Isolation and purification

The isolation method of LABs referred to Reuben et al. (2020). Briefly, 10 ml of each milk sample was enriched with 40 ml of de Man, Rogosa, and Sharpe (MRS) broth (Hopebio, Qingdao, China) and cultured overnight in a 37°C-shaking incubator under aerobic conditions. The fresh cultures with visible turbidity were homogenized in sterile normal saline using a vortex mixer, and 100 µl of each sample after ten-fold of continuous dilution was taken and coated on MRS agar and incubated for 24–72 h at 37°C under aerobic conditions. Then, individual colonies with different morphologies were selected and purified through three continuous passages on MRS agar. According to the standard procedures (Sharpe, 1979), catalase activities and coagulase activities were detected. Together with the Gram staining results (positive), catalase activity (negative), coagulase activity (negative), and cell morphology, 26 purified isolations (with different colony

characteristics) were preliminarily identified as LABs and stored at −80°C in 50% glycerol for the subsequent experiments.

## Species identification

### Biochemical identification

The biochemical characteristics of the 26 purified LAB isolates were simultaneously identified using biochemical tubes (Hopebio, Qingdao, China) according to Bergey's Manual of Determinative Bacteriology (Cummings, 1926), including the ability to ferment different sugars, gelatin liquefaction, and sulfuretted hydrogen production.

### 16S rDNA sequencing and sequences analysis

The DNA of the 26 LAB isolates was extracted by a DNA Extraction Kit (Tiangen, Beijing, China), and the quality of the extracted DNA was measured using an ND-1000 micro UV spectrophotometer (NanoDrop Technologies, United States). Then, the universal primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGACTTAACCCCAATCGC-3′) were used to amplify the 16S rDNA gene. Each PCR reaction (25 μl) contained 12.5 μl PCR Master Mix, 9.5 μl nuclease-free $H_2O$, 1 μl forward primer, 1 μl reverse primer, and 1 μl DNA sample. The PCR procedure was performed as follows: predenaturation at 94°C for 5 min, followed by 30 cycles (30 s of denaturation at 94°C, 30 s of annealing at 55°C, and 1 min of extension at 72°C), with a final extension at 72°C for 7 min. The PCR products were stored at 4°C for subsequently checking on 2% agarose gel electrophoresis stained with the golden view. Part of the checked products was then sent to Sangon Biotech Co.Ltd. (Shanghai, China) for 16S rDNA sequencing. Based on the results of 16S rRNA sequencing, the homology alignment analysis with the nucleic acid sequences of bacteria in GenBank[1] was performed using BLAST.[2] Then, the phylogenetic tree was established by MEGA6 software (Mega Limited, Auckland, New Zealand) and sequences with a demarcation threshold of >99% were classified as the same species. Kimura 2-parameter model and Neighbor-Joining method were used to construct the phylogenetic tree. Briefly, the robustness of individual branches was estimated using bootstrapping with 1,000 replications, and the phylogenetic tree was confirmed by the maximum-parsimony method and maximum-likelihood method. *Lactococcus lactis* strain NBRC 100933 (NR_113960.1), *L. lactis* strain 4,319 (MT544861.1), *Streptococcus lutetiensis* strain CIP 106849 (NR_115719.1), *Weissella hellenica* strain NCFB 2973 (NR_118771.1), *Enterococcus durans* strain 98D (NR_036922.1), *Limosilactobacillus fermentum* strain NBRC 15885 (NR_113335.1), *Limosilactobacillus fermentum* strain CIP 102980 (NR_104927.1), *Enterococcus lactis* strain BT159 (NR_117562.1), *Rummeliibacillus stabekisii* strain KSC-SF6g (NR_043992.1), *Lactobacillus plantarum* strain DKO 22 (NR_042254.1), and *L. plantarum* strain DSM 10667 (NR_025447.1) were used as typical strains to construct the phylogenetic tree.

## Antipathogenic activity detection

### Antagonistic activity

The antimicrobial activities of the 26 LAB isolates against enterotoxigenic *Escherichia coli* ATCC 25922 (ETEC), *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* PAO1, and *Salmonella enterica* H9812 were determined using the Oxford Cup method (Fontana et al., 2015). These four pathogenic bacteria were purchased from the American type culture collection (ATCC). Briefly, the resurgent LAB isolates were inoculated to MRS broth and incubated for 24 h at 37°C under aerobic conditions. Meanwhile, the targeted pathogens were precultured under the same conditions in Luria-Bertani (LB) broth (Hopebio, Qingdao, China). Fresh cultures of the four targeted pathogens (100 μl, $10^7$ CFU/ml) were coated on an LB agar plate and dried. Oxford Cups placed on plates were filled with 100 μl of cell-free supernatant (CFS) obtained from centrifugation of LAB cultures at 4500 r/min for 10 min. The diameters of inhibition zones were measured and recorded after incubating at 37°C for 24 h under anaerobic conditions.

### Co-aggregative ability with pathogens

The co-aggregation abilities of 14 LAB isolates (with inhibitory effect on all four target pathogens) were detected to evaluate their abilities to gather pathogens and facilitate the elimination of pathogens through feces (De Melo Pereira et al., 2018). Briefly, 2 ml of fresh overnight cultures and 2 ml of each pathogen culture were mixed, vortexed, and incubated at 37°C for 2 h. Tubes containing 4 ml of each LAB isolates or each pathogen suspension were used as controls. Then, the absorbance (600 nm) at 2 h of the tubes was measured to calculate the co-agglutination rates followed the formula:

$$\text{co}-\text{agglutination rate}(\%) = 1 - A_{\text{mix}} / \left[\left(A_{\text{LAB}} + A_{\text{pathogen}}\right)/2\right] \times 100$$

in which $A_{mix}$ represents the absorbance of the mixture, $A_{LAB}$ represents the absorbance of the pure LAB cultures, and $A_{pathogen}$ represents the absorbance of the pure pathogen suspension.

## Stress tolerance detection

### Tolerance for simulated GIT conditions

The survivability of the 14 selected LAB isolates to simulated GIT conditions was assessed referred to Zhang's work (Zhang et al., 2016). Firstly, 0.3 g pepsin (Solarbio, Beijing, China) was

1 https://www.ncbi.nlm.nih.gov/genbank/

2 http://blast.ncbi.nlm.nih.gov/Blast.cgi

dissolved in 100 ml 0.9% sterile saline, and the pH was adjusted to 3.0 with 1 M HCL (Hopebio, Qingdao, China) to prepare the simulated gastric juice. And 0.2 g trypsin (Sangon, Beijing, China) and 0.3 g ox-bile salts (Hopebio, Qingdao, China) were dissolved in 100 ml 0.9% sterile saline, and the pH was adjusted to 8.0 with 1 M NaOH (Hopebio, Qingdao, China) to prepare the simulated intestinal juice. The simulated gastric juice and intestinal juice were subsequently filter-sterilized (0.22 μm; Green Union Science Instrument Co., Ltd, Jiangsu, China). After three consecutive passages, the resurgent LAB isolates were incubated in MRS broth for 12 h. Then, 10 ml of the fresh cultures were centrifuged at 8,000 × g for 10 min at 25°C. The pelleted cells were resuspended in an equal volume of sterile normal saline, followed by 10 min of centrifugation with the same parameters. Then, the pelleted cells were resuspended in 10 ml of prepared simulated gastric juice (0 h), followed by incubation at 37°C for 3 h under aerobic conditions (3 h). After that, the pelleted cells obtained by centrifugation from gastric juice were transferred into 10 ml of prepared simulated intestinal juice again and incubated aerobically for 4 h at 37°C (7 h). The viable colonies at 0 h, 3 h, and 7 h were determined using plate counts on MRS agar to calculate the survival rate. All the experiments were repeated three times with three technical replicates each time. The mean value of the results of the three independent experiments was calculated as follows:

$$Survival\ rate\,(\%) = N_1 / N_0 \times 100,$$

in which $N_0$ is the number of viable bacteria at 0 h (CFU/mL) and $N_1$ is the number of viable bacteria in artificial gastrointestinal fluid at 3 or 7 h (CFU/mL).

### Tolerance for phenol

To assess the phenol tolerance of the 14 selected LAB isolates, overnight LAB cultures were transferred to a new MRS broth containing 0.4% phenol (Hopebio, Qingdao, China) at 37°C. After 24 h of incubation, the viable colonies of cultures were measured using plate counts to detect the viability of the LAB isolates.

## Adhesion activity detection

### Auto-aggregation activity

The auto-aggregation abilities of the 14 selected LAB isolates were determined using the spectrophotometer to evaluate the adherence capability to intestinal epithelial cells. Briefly, fresh cultures were centrifuged at 4500 r/min for 10 min to collect the pelleted cells, washed twice with sterile 1 × PBS, and adjusted to $10^8$ CFU/ml in the same buffer. Then, 4 ml of the adjusted cell suspension was vortexed for 10 s and incubated for 24 h at 37°C. To observe the auto-aggregation ability, the absorbance (600 nm) at 0, 3, 6, and 24 h was measured using a spectrophotometer. All the experiments were repeated three times with three technical replicates each time. The mean value of the results of the three independent experiments was calculated as follows:

$$auto-agglutination\ rate\,(\%) = 1 - (A_t / A_0) \times 100,$$

in which $A_t$ represents the absorbance at 3, 6, or 24 h and $A_0$ represents the absorbance at 0 h.

### Cell surface hydrophobicity

To evaluate the adherence ability to hydrocarbons of the selected LAB isolates, the cell surface hydrophobicity was measured. Firstly, fresh overnight LAB cultures were collected by centrifugation at 4,500 r/min for 10 min, and the pelleted cells were washed twice with sterile 1 × PBS and then resuspended in the same buffer. Afterward, 2 ml of cell suspension was mixed with 2 ml xylene (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), and the mixtures were vortexed vibration for 10 min and left at 25°C for 40 min for two phases separation. The lower aqueous phase was carefully absorbed, and its absorbance was measured at 600 nm in triplicate to calculate the cell surface hydrophobicity (%), the formula is as follows:

$$Cell\ surface\ hydrophobicity\,(\%) = 1 - (A_f / A_0) \times 100$$

in which $A_f$ represents final absorbance and $A_0$ represents initial absorbance.

## Safety assessment

### Hemolytic activity

To determine the hemolytic activity, fresh overnight LAB cultures were streaked on blood agar plates, and the phenotype around the colonies was observed after 48 h of incubation at 37°C. *S. aureus* ATCC 25923 was used as the positive control. The hemolytic reaction was evaluated by observing both the partial hydrolysis of red blood cells and the production of a green zone (α-hemolysis), as well as the total hydrolysis of red blood cells producing a clear zone around the bacterial colony (β-hemolysis) or no reaction (γ-hemolysis).

### Antibiotic susceptibility

Disc-diffusion test was used to assess the antibiotic susceptibility of the selected LAB strains, including the following 13 antimicrobials (Lanjun Biotechnology Co., Ltd., Guangzhou, China): penicillin G (P, 10 μg), ampicillin (AMP, 10 μg), ceftriaxone (CRQ, 30 μg), amoxicillin (AML, 25 μg), erythromycin (E, 15 μg), clarithromycin (CLR, 15 μg), tetracycline (TE, 30 μg), gentamicin (C, 10 μg), amikacin (AK, 30 μg), vancomycin (VA, 30 μg), chloramphenicol (C, 30 μg), rifampicin (Rd, 5 μg), and fosfomycin (S, 200 μg). Fresh overnight cultures of each LAB strain were diluted to $10^8$ CFU/ml, 100 μl of which was coated on

MRS agar plates and dried. Then, three homogenous antibiotic discs were manually placed on the surface of the dried MRS plate. After 5 min, the placed plates were turned over and incubated at 37°C for 48 h under anaerobic conditions. The diameters (mm) of the inhibition zones were measured to classify the antibiotic susceptibility as resistance (R), moderate susceptibility (MS), or susceptibility (S) based on the parameters of the Clinical and Laboratory Standards Institute (CLSI; El-Shaer et al., 2017).

## Growth performance evaluation

Referring to the previous study (Liu et al., 2020), we detected the growth performance of the five candidates by constructing their growth curves using MRS broth as the negative control. Briefly, 50 µl (1%) of each LAB culture (the mid-exponential phase) was inoculated into 50 ml of fresh MRS broth and incubated at 37°C for 48 h. The absorbance (600 nm) was measured at a frequency of every 2 h in the 0–24 h and every 6 h in the 25–48 h.

## Statistical analysis

All results were expressed as mean ± SD, and the statistical significance of the differences was evaluated by one-way ANOVA using SPSS 26 (IBM, NYC, United States). Differences were considered significant at $p < 0.05$ and extremely significant at $p < 0.01$. All the graphical presentations were generated by GraphPad Prism 9.0 (GraphPad Software, CA, United States).

# Results

To obtain different LABs, we picked out individual colonies with different morphologies. And a total of 26 isolates with typical morphological characteristics of LAB (Gram-positive *bacilli* and *cocci*, catalase-negative, coagulase-negative, and non-motile) were obtained from the 22 cow milk samples after isolation and purification for subsequent experiments, including species identification, antipathogenic activity detection, stress tolerance detection, adhesion activity detection, safety assessment, and growth performance evaluation.

## Species identification

The biochemical characteristics of the 26 isolates were detected to confirm the type of these isolates preliminarily. The results are shown in Supplementary Table 1. Based on the biochemical characteristics, the 26 isolates were initially identified as *Lactococcus* (L2, L11, L13, L16, and L18), *Streptococcus* (L26), *Lactiplantibacillus* (L1, L3, L4, L5, L6, L7, L10, L14, L17, L19, L20, L21, and L22), *Enterococcus* (L9 and L15), *Rummeliibacillus* (L8 and L12), *Limosilactobacillus* (L23 and L24), and *Weissella* (L25). The 16S rDNA gene sequences of these 26 isolates were used to construct a phylogenetic tree, and the result is shown in Figure 1. L2, L13, and L16 were clustered with *Lactococcus lactis* strain NBRC 100933 (NR_113960.1); L11 and L18 were clustered with *L. lactis* strain 4,319 (MT544861.1); L1, L3, L4, L5, L6, L7, L10, L14, L17, L19, L20, L21, and L22 were clustered with *Lactiplantibacillus plantarum* strain DSM 10667 (NR_025447.1); L23 were clustered with *Limosilactobacillus fermentum* strain NBRC 15885 (NR_113335.1); L24 were clustered with *Limosilactobacillus fermentum* strain CIP 102980 (NR_104927.1); L25 were clustered with *W. hellenica* strain NCFB 2973 (NR_118771.1); L26 were clustered with *S. lutetiensis* strain CIP 106849 (NR_115719.1); L8 and L12 were clustered with *R. stabekisii* strain KSC-SF6g (NR_043992.1); and L15 and L9 were clustered with *Enterococcus lactis* strain BT159 (NR_117562.1) and *E. durans* strain 98D (NR_036922.1), respectively.

## Antipathogenic activity

To evaluate the inhibitory effect of the obtained 26 isolates on the growth of common intestinal pathogens, the antagonistic activities against *E. coli* ATCC 25922 (ETEC), *S. aureus* ATCC 25923, *P. aeruginosa* PAO1, and *S. enterica* H9812 were detected. We observed that 14 of them inhibited the growth of all 4 pathogens. The detailed results of the antipathogenic activities are shown in Table 1. Referring previous study (Reuben et al., 2020), the antipathogenic activities of these LABs were divided into four ranges: I, 8 mm < zone diameters ≤12 mm; II, 12 mm < zone diameters ≤16 mm; III, 16 mm < zone diameters ≤20 mm; and IV, 20 mm < zone diameters. In general, these 14 isolates demonstrated high antagonistic activities against *S. aureus* ATCC 25923, moderate antagonistic activities against *P. Aeruginosa* PAO1, and low antagonistic activities against ETEC and *Salmonella* H9812 ($p < 0.05$). Although the inhibitory effect on the 4 target pathogens was strain-specific, the isolates L17, L1, L21, L14, and L19 exhibited higher inhibitory activities than others ($p < 0.05$).

The co-aggregation abilities of the 14 isolates with the four target pathogens are shown in Table 2. For ETEC, L20 showed the highest co-aggregation ability, followed by L5, L14, L7, and L22. For *S. aureus* ATCC 25923, the isolates with the top five highest co-aggregation abilities were L5, L20, L22, L14, and L7. For *Salmonella* H9812, L22 showed the highest co-aggregation ability, followed by L5, L20, and L14. For *P. Aeruginosa* PAO1, the isolates with the top five highest co-aggregation abilities were L22, L20, L14, L7, and L10.

## Tolerance for simulated GIT conditions and phenol

In order to detect the survival ability of the isolated LABs in a simulated gastrointestinal environment, these 14 isolates were

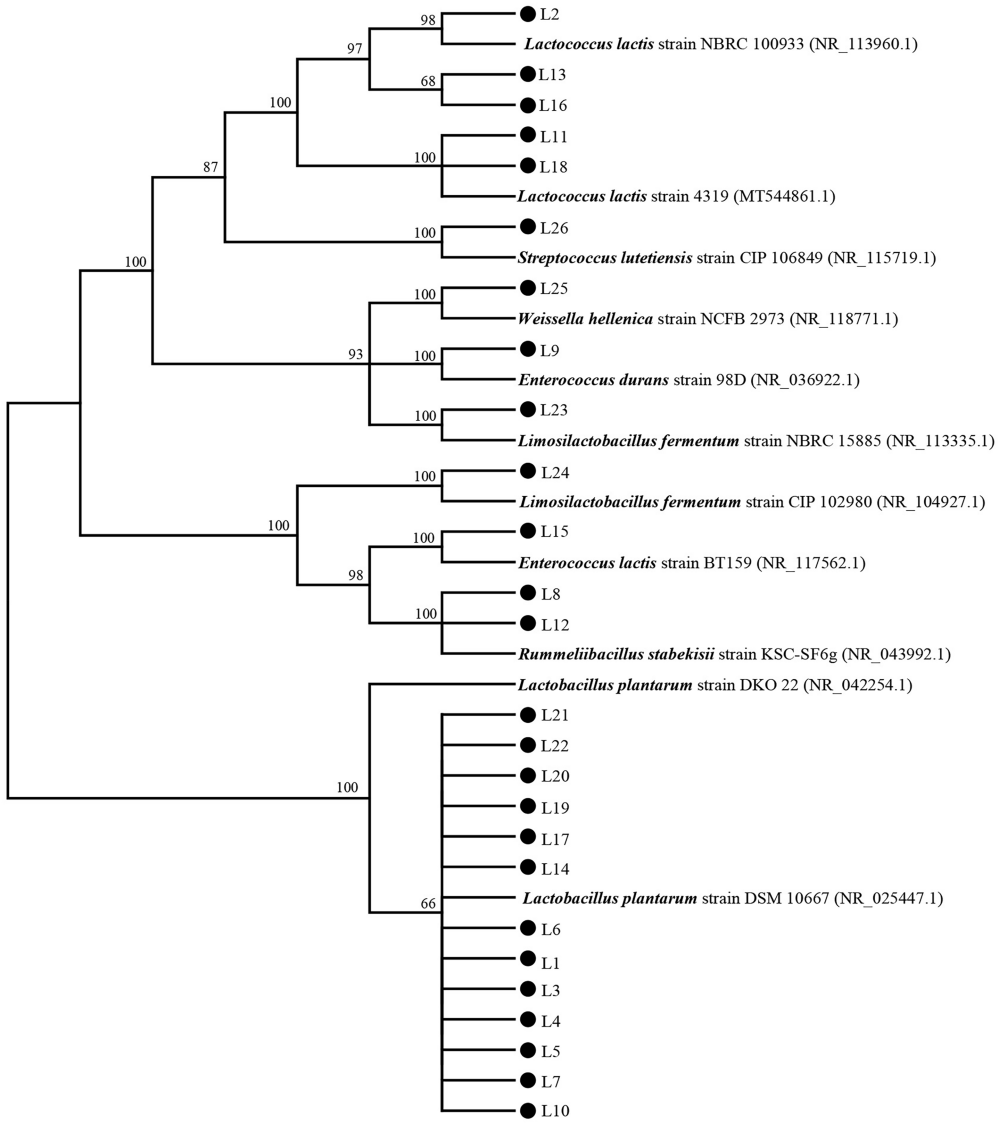


FIGURE 1
The phylogenetic tree based on 16S rDNA genes of the 26 isolates and type strains.

inoculated in artificial gastric juice for 3 h, then transferred to the artificial intestinal juice for 4 h. The results are shown in Table 3. In general, all the isolates showed high survival rates (74.49 ~ 99.69%) in the simulated gastric juice, and most of the isolates (except L2) demonstrated high survival rates (64.95 ~ 84.93%) in the simulated intestinal juice. Specifically, the five isolates with the highest survival rate in gastric juice were L22 (99.69%), L21 (96.92%), L7 (98.05%), L3 (97.82%), and L19 (96.37%), and L21 (84.94%), L22 (83.89%), L14 (83.39%), L4 (82.60%), and L17 (80.48%) were the top five isolates with the highest survival rates in the artificial intestinal juice.

The influence of phenol on the growth of these 14 LAB isolates is shown in Figure 2, in which L14 showed the highest phenol tolerance (88.91%), followed by L6 (87.03%), L4 (84.45%), and L1 (83.57%). Except for L2 (66.50%) and L19 (69.86%), all isolates tolerated 0.4% phenol (>70%).

## Adhesion activity

Different auto-aggregation abilities were revealed for the selected LAB isolates at the 3rd h, 6th h, and 24th h from the beginning of co-culture, and the results are shown in Table 4. In general, the auto-aggregation effect of the identified LAB isolates showed a time-dependent manner. At the 3 h point, L20 showed the highest auto-aggregation ability (76.32%), followed by L14 (62.50%), L7 (49.28%), and L17 (40.73%), while the other tested isolates showed lower auto-aggregation abilities between 1.81 and 27.87%. At 6 h point, the isolates with high auto-aggregation effect were L20 (86.37%), L14

TABLE 1 Antagonistic activity of potential probiotic isolates from cow milk samples against four target pathogenic bacteria by the Oxford cup method.[1]

| Strain | Antagonistic activity (mm) | | | |
|---|---|---|---|---|
| | *E. coli* ATCC 25922 | *Staphylococcus aureus* ATCC 25923 | *Salmonella* H9812 | *P. aeruginosa* PAO1 |
| L1 | 14.57 ± 0.45$^{bc}$ | 27.43 ± 0.51$^{a}$ | 12.57 ± 0.46$^{c}$ | 18.40 ± 0.29$^{b}$ |
| L2 | 11.10 ± 0.26$^{e}$ | 12.43 ± 0.42$^{i}$ | 13.60 ± 0.15$^{b}$ | 14.27 ± 0.20$^{i}$ |
| L3 | 14.20 ± 0.61$^{cd}$ | 22.13 ± 0.51$^{b}$ | 11.07 ± 0.12$^{fg}$ | 15.73 ± 0.25$^{fgh}$ |
| L4 | 14.30 ± 0.78$^{bcd}$ | 21.93 ± 0.72$^{bc}$ | 10.77 ± 0.26$^{g}$ | 16.93 ± 0.15$^{de}$ |
| L5 | 14.50 ± 0.89$^{bc}$ | 21.93 ± 0.50$^{bc}$ | 12.70 ± 0.25$^{c}$ | 18.07 ± 0.87$^{bc}$ |
| L6 | 13.17 ± 0.42$^{d}$ | 21.27 ± 0.90$^{cd}$ | 11.10 ± 0.60$^{fg}$ | 15.63 ± 0.46$^{gh}$ |
| L7 | 13.53 ± 1.04$^{de}$ | 14.40 ± 0.10$^{h}$ | 14.30 ± 0.10$^{a}$ | 16.10 ± 0.70$^{fg}$ |
| L10 | 14.03 ± 0.25$^{cd}$ | 27.43 ± 0.45$^{a}$ | 11.93 ± 0.21$^{de}$ | 15.03 ± 0.15$^{ghi}$ |
| L14 | 12.90 ± 0.82$^{d}$ | 20.97 ± 0.49$^{d}$ | 12.40 ± 0.32$^{cd}$ | 16.13 ± 0.26$^{hi}$ |
| L17 | 13.97 ± 0.45$^{cd}$ | 20.13 ± 0.93$^{e}$ | 14.43 ± 0.50$^{a}$ | 22.03 ± 0.55$^{a}$ |
| L19 | 16.53 ± 0.55$^{a}$ | 19.53 ± 0.64$^{ef}$ | 12.13 ± 0.36$^{cde}$ | 16.50 ± 0.15$^{ef}$ |
| L20 | 14.53 ± 0.21$^{bc}$ | 19.23 ± 0.21$^{f}$ | 11.60 ± 0.15$^{ef}$ | 12.63 ± 0.30$^{j}$ |
| L21 | 15.07 ± 0.15$^{b}$ | 26.87 ± 0.25$^{a}$ | 11.57 ± 0.64$^{ef}$ | 17.47 ± 0.35$^{cd}$ |
| L22 | 16.43 ± 0.75$^{a}$ | 18.10 ± 0.36$^{g}$ | 11.60 ± 0.26$^{ef}$ | 15.60 ± 0.17$^{gh}$ |

[1]*Results of independent experiments (n = 3) of inhibition zones are presented using mean ± SD;*
[a-j]Values in a column with different superscript letters are significantly different (Waller-Duncan, $p < 0.05$).

TABLE 2 Co-aggregation abilities of potential probiotic LABs isolated from cow milk.[1]

| Strain | Co-aggregation (%) | | | |
|---|---|---|---|---|
| | *E. coli* ATCC 25922 | *Staphylococcus aureus* ATCC 25923 | *Salmonella* H9812 | *P. aeruginosa* PAO1 |
| L1 | 35.96 ± 0.64$^{gh}$ | 31.07 ± 0.95$^{i}$ | 30.92 ± 1.22$^{k}$ | 53.49 ± 1.16$^{gh}$ |
| L2 | 38.42 ± 1.53$^{ef}$ | 34.95 ± 0.58$^{g}$ | 36.05 ± 3.15$^{j}$ | 53.40 ± 0.52$^{h}$ |
| L3 | 28.43 ± 1.33$^{i}$ | 29.91 ± 1.14$^{j}$ | 41.97 ± 1.33$^{i}$ | 54.66 ± 0.52$^{fg}$ |
| L4 | 36.71 ± 1.67$^{fg}$ | 41.63 ± 1.01$^{e}$ | 53.70 ± 1.59$^{g}$ | 47.26 ± 0.42$^{i}$ |
| L5 | 66.51 ± 1.01$^{ab}$ | 74.24 ± 0.37$^{a}$ | 94.37 ± 2.12$^{b}$ | 83.48 ± 1.15$^{b}$ |
| L6 | 34.30 ± 0.82$^{h}$ | 29.12 ± 0.67$^{j}$ | 48.37 ± 2.35$^{h}$ | 46.97 ± 0.31$^{i}$ |
| L7 | 52.99 ± 1.37$^{c}$ | 50.49 ± 1.17$^{d}$ | 63.19 ± 1.74$^{e}$ | 65.15 ± 1.02$^{d}$ |
| L10 | 38.74 ± 0.81$^{e}$ | 35.26 ± 0.83$^{g}$ | 52.04 ± 2.18$^{g}$ | 58.14 ± 1.63$^{e}$ |
| L14 | 65.45 ± 1.66$^{b}$ | 56.43 ± 0.22$^{c}$ | 84.91 ± 2.16$^{d}$ | 81.00 ± 0.69$^{c}$ |
| L17 | 50.65 ± 1.46$^{d}$ | 33.33 ± 0.24$^{h}$ | 46.19 ± 1.20$^{h}$ | 54.83 ± 1.06$^{f}$ |
| L19 | 49.61 ± 1.63$^{d}$ | 37.16 ± 0.92$^{f}$ | 57.25 ± 0.92$^{f}$ | 55.31 ± 1.09$^{f}$ |
| L20 | 67.85 ± 1.10$^{a}$ | 73.18 ± 0.48$^{a}$ | 90.75 ± 1.03$^{c}$ | 82.99 ± 0.19$^{b}$ |
| L21 | 21.63 ± 0.92$^{j}$ | 34.60 ± 0.34$^{g}$ | 27.28 ± 1.38$^{m}$ | 37.09 ± 0.15$^{j}$ |
| L22 | 52.95 ± 0.41$^{c}$ | 69.68 ± 0.71$^{b}$ | 97.19 ± 1.04$^{a}$ | 87.41 ± 0.03$^{a}$ |

[1]*Results of independent experiments (n = 3) of co-aggregation rate are presented using mean ± SD;*
[a-m]Values in a column with different superscript letters are significantly different (Waller-Duncan, $p < 0.05$).

(79.61%), L7 (77.64%), L4 (58.62%), and L19 (53.49%). Whereas, at the 24 h point, the auto-aggregation effect of seven isolates (L4, L14, L20, L7, L5, L19, and L17) exceeded 80%, and the others (except L10) also showed high auto-aggregation effects (53.26–65.31%).

The results of the cell surface hydrophobicity of the identified LAB isolates are shown in Figure 3. In general, most of the identified isolates showed high (71 ~ 100%) or medium (36 ~ 70%) cell surface hydrophobicity (the classification standard referred to Ocana et al. (Ocaña et al., 1999)). The hydrophobicity was highest in L14 (94.08%), followed by L20 (92.67%), L7 (92.70%), L5 (91.21%), L19 (84.52%), L17 (79.32%), and L21 (72.95%), and L22 (69.81%) and L2 (64.58%) showed the medium hydrophobic.

## Safety analysis

The hemolytic activity test results showed that these 14 isolates were non-hemolytic (Supplementary Figure 1). The

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TABLE 3 Survival of the potential probiotic isolates in the artificial gastric and intestinal juices.[1]

| Strain | Initial concentration | Artificial gastric juice at pH 3.0 | | Artificial intestinal juice at pH 8.0 | |
|---|---|---|---|---|---|
| | 0 h (log10 CFU ml$^{-1}$) | 3 h (log10 CFU ml$^{-1}$) | Survival rate (%) | 7 h (log10 CFU ml$^{-1}$) | Survival rate (%) |
| L1 | 9.71 ± 0.01$^{a}$ | 7.23 ± 0.10$^{f}$ | 74.49 ± 1.04$^{f}$ | 7.31 ± 0.08$^{e}$ | 75.25 ± 0.85$^{g}$ |
| L2 | 8.90 ± 0.01$^{gh}$ | 7.85 ± 0.06$^{e}$ | 88.20 ± 0.66$^{e}$ | 3.00 ± 0.00$^{j}$ | 33.72 ± 0.00$^{m}$ |
| L3 | 8.88 ± 0.05$^{h}$ | 8.68 ± 0.06$^{bc}$ | 97.82 ± 0.63$^{ab}$ | 6.32 ± 0.03$^{h}$ | 71.19 ± 0.40$^{h}$ |
| L4 | 9.41 ± 0.05$^{cd}$ | 8.05 ± 0.06$^{de}$ | 90.91 ± 0.44$^{de}$ | 7.77 ± 0.06$^{b}$ | 82.60 ± 0.66$^{c}$ |
| L5 | 9.47 ± 0.09$^{c}$ | 8.77 ± 0.56$^{b}$ | 92.55 ± 5.86$^{cd}$ | 7.22 ± 0.02$^{f}$ | 76.18 ± 0.13$^{f}$ |
| L6 | 9.34 ± 0.03$^{de}$ | 8.88 ± 0.08$^{b}$ | 95.09 ± 0.82$^{bc}$ | 6.07 ± 0.02$^{i}$ | 64.96 ± 0.24$^{k}$ |
| L7 | 9.58 ± 0.06$^{b}$ | 9.40 ± 0.06$^{a}$ | 98.05 ± 0.55$^{ab}$ | 7.40 ± 0.03$^{d}$ | 77.20 ± 0.31$^{e}$ |
| L10 | 8.97 ± 0.06$^{g}$ | 8.72 ± 0.08$^{bc}$ | 97.28 ± 0.80$^{ab}$ | 6.00 ± 0.00$^{j}$ | 66.91 ± 0.00$^{j}$ |
| L14 | 9.34 ± 0.03$^{de}$ | 8.88 ± 0.03$^{b}$ | 95.05 ± 0.34$^{bc}$ | 7.79 ± 0.07$^{b}$ | 83.39 ± 0.74$^{bc}$ |
| L17 | 9.35 ± 0.05$^{de}$ | 8.98 ± 0.09$^{b}$ | 96.03 ± 0.95$^{b}$ | 7.53 ± 0.08$^{c}$ | 80.48 ± 0.88$^{d}$ |
| L19 | 9.29 ± 0.01$^{e}$ | 8.95 ± 0.04$^{b}$ | 96.37 ± 0.42$^{b}$ | 6.99 ± 0.09$^{g}$ | 75.25 ± 0.95$^{g}$ |
| L20 | 9.14 ± 0.05$^{f}$ | 8.37 ± 0.06$^{cd}$ | 91.59 ± 0.67$^{d}$ | 6.24 ± 0.03$^{h}$ | 68.22 ± 0.32$^{i}$ |
| L21 | 9.57 ± 0.03$^{b}$ | 8.93 ± 0.05$^{b}$ | 96.92 ± 0.49$^{ab}$ | 8.12 ± 0.01$^{a}$ | 84.94 ± 0.16$^{a}$ |
| L22 | 9.64 ± 0.05$^{ab}$ | 9.60 ± 0.06$^{a}$ | 99.69 ± 0.62$^{a}$ | 8.08 ± 0.03$^{a}$ | 83.89 ± 0.22$^{b}$ |

[1]*Results are expressed as mean ± SD;*

[a-m]*Values in a column with different superscript letters are significantly different (Waller-Duncan, $p < 0.05$).*

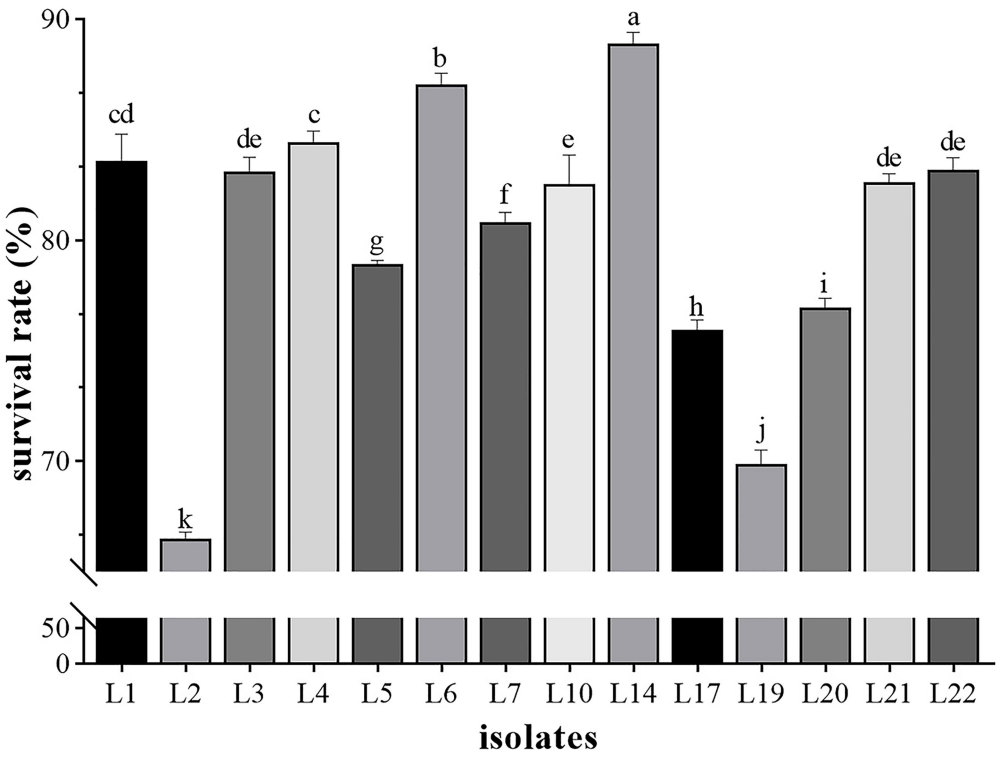


FIGURE 2
The results of phenol tolerance of 14 LAB isolates. Results are expressed as mean±SD of triplicate tests. [a-k] Values in a column with different superscript letters are significantly different (Waller-Duncan, $p$<0.05).

susceptibility profile of all these 14 isolates to 13 commonly used antibiotics was assessed, and the results are shown in Table 5. The resistance rates (includes resistance and intermediate) were 0% (0/14) to penicillin G), 64.28% (9/14) to ceftriaxone, 100% (14/14) to vancomycin, 14.29% (2/14) to chloramphenicol, 100% (14/14) to gentamicin, 64.28% (9/14) to

erythromycin, 85.71% (12/12) to tetracycline, 57.14% (8/14) to rifampicin, 7.14% (1/14) to ampicillin, 100% (14/14) to amikacin, 7.14% (1/14) to amoxicillin, 28.57% (4/14) to clarithromycin, and 100% (14/14) to streptomycin, respectively. L20 showed the highest sensitive rate (76.92%), L3, L5, L7, L14, L19, and L21 showed a higher sensitive rate (69.23%) to these 13 antibiotics.

TABLE 4 Auto-aggregation abilities of potential probiotic LABs from cow milk.[1]

| Strain | Auto-aggregation (%) | | |
|---|---|---|---|
| | 3 h | 6 h | 24 h |
| L1 | 13.00 ± 2.75$^{gh}$ | 26.98 ± 1.50$^{f}$ | 65.31 ± 5.70$^{c}$ |
| L2 | 10.10 ± 2.02$^{h}$ | 18.07 ± 1.90$^{h}$ | 53.26 ± 10.03$^{de}$ |
| L3 | 15.80 ± 2.54$^{g}$ | 23.31 ± 0.49$^{g}$ | 58.70 ± 8.04$^{d}$ |
| L4 | 27.39 ± 5.64$^{e}$ | 58.62 ± 1.86$^{c}$ | 91.62 ± 0.47$^{a}$ |
| L5 | 22.36 ± 1.46$^{f}$ | 38.68 ± 1.47$^{e}$ | 88.51 ± 0.91$^{ab}$ |
| L6 | 13.28 ± 1.92$^{gh}$ | 23.62 ± 1.07$^{g}$ | 55.96 ± 4.39$^{d}$ |
| L7 | 49.28 ± 1.42$^{c}$ | 77.64 ± 1.26$^{b}$ | 88.88 ± 0.58$^{ab}$ |
| L10 | 1.81 ± 0.72$^{i}$ | 13.47 ± 3.16$^{i}$ | 47.83 ± 0.67$^{e}$ |
| L14 | 62.50 ± 0.51$^{b}$ | 79.61 ± 0.98$^{b}$ | 90.33 ± 1.26$^{a}$ |
| L17 | 40.73 ± 2.94$^{d}$ | 39.44 ± 0.81$^{e}$ | 83.91 ± 0.34$^{b}$ |
| L19 | 27.87 ± 0.92$^{e}$ | 53.49 ± 2.72$^{d}$ | 87.76 ± 0.31$^{ab}$ |
| L20 | 76.32 ± 2.27$^{a}$ | 86.37 ± 0.37$^{a}$ | 89.63 ± 0.64$^{ab}$ |
| L21 | 13.79 ± 0.42$^{gh}$ | 26.18 ± 1.22$^{f}$ | 57.19 ± 0.82$^{d}$ |
| L22 | 14.83 ± 1.64$^{g}$ | 25.01 ± 2.20$^{fg}$ | 57.40 ± 1.38$^{d}$ |

[1]*Data are mean ± SD from triplicate experiments;*
*[a-i]Values in a column with different superscript letters are significantly different (Waller-Duncan, $p < 0.05$).*

## Growth curve

Based on the results of the probiotic property test and safety analysis, the growth characteristics of the five most probiotic potential isolates, L5, L14, L17, L19, and L20, were measured, and their growth curves are shown in Figure 4. Except for L20, the other four isolates entered the stationary phase at the 14th h and lasted until the 48$^{th}$ h, indicating their good growth performance. L20 reached the stationary phase at the 30th h. L5 showed the best growth performance, while L20 showed the worst.

# Discussion

The high nutrient content of raw milk provides multiple kinds of bacteria with favorable circumstances (Quigley et al., 2013), and the microbiota profiles from different farms are different (Hornik et al., 2021). LABs, a group of bacteria that ferment lactose to lactate, are a dominant population in cow milk before pasteurization. Hence, raw cow milk was considered an important source of LABs. The raw milk from this farm with 50 years of history had never been sampled for LAB isolation, and we obtained five isolates with promising probiotic potential.

Antipathogenic activity and safety characteristics were considered the most important properties of probiotic LABs (Araya et al., 2002). Hence, we detected the antagonistic activity of these 26 isolates against intestinal pathogens. Four standard common pathogenic strains, ETEC (Fleckenstein and Kuhlmann, 2019), *S. aureus* (Cheung et al., 2021), *Salmonella* (Knodler and Elfenbein, 2019), and *P. aeruginosa* (Bachta et al., 2020), were used as target pathogens for antagonistic activity assay, and 14

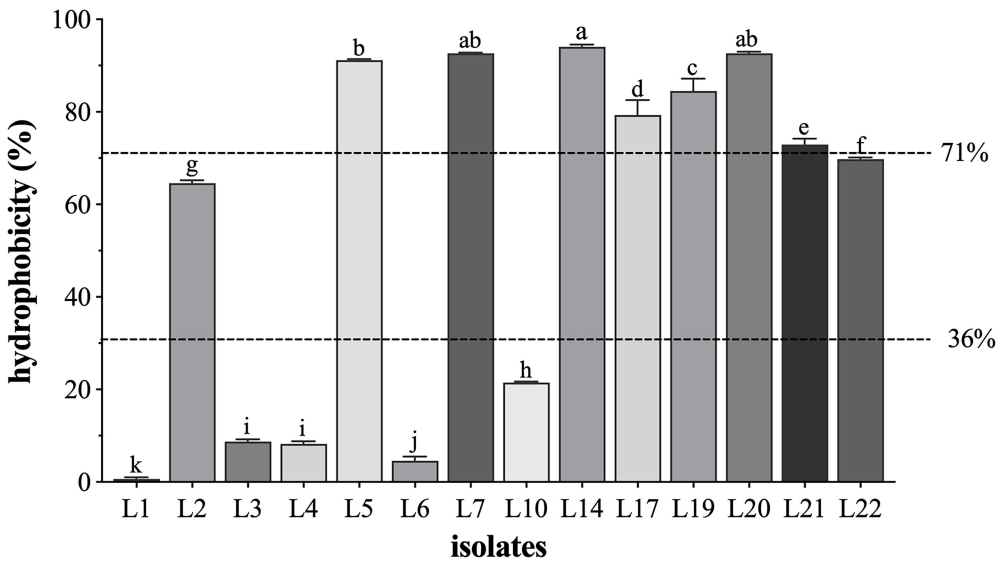


FIGURE 3
The cell surface hydrophobicity of 14 LAB isolates. Results are expressed as mean±SD of triplicate tests. [a-k] Values in a column with different superscript letters are significantly different (Waller-Duncan, $p<0.05$). The dotted lines represent the threshold to distinguish the surface hydrophobicity (high, medium, and low) of the tested isolates.

TABLE 5 Antibiotic susceptibility results of LAB isolates from milk.

| Strain | Antibiotic susceptibility* | | | | | | | | | | | | | Sensitive rate (S + I, %) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | P | CRO | VA | C | CN | E | TE | RD | AMP | AK | AML | CLR | S | |
| L1 | S | R | R | S | R | S | I | S | S | R | S | S | R | 61.54 |
| L2 | S | S | I | I | R | R | S | R | R | R | R | R | R | 38.46 |
| L3 | S | I | R | S | R | I | I | S | S | R | S | S | R | 69.23 |
| L4 | S | S | R | S | R | I | I | R | S | R | S | S | R | 61.54 |
| L5 | S | I | R | S | R | S | I | S | S | R | S | I | R | 69.23 |
| L6 | S | R | R | S | R | I | I | S | S | R | S | S | R | 61.54 |
| L7 | S | I | R | S | R | I | I | I | S | R | S | S | R | 69.23 |
| L10 | S | R | R | S | R | I | I | I | S | R | S | S | R | 61.54 |
| L14 | S | I | R | S | R | I | I | S | S | R | S | I | R | 69.23 |
| L17 | S | S | R | I | R | R | I | R | S | R | S | I | R | 53.85 |
| L19 | S | S | R | S | R | S | S | I | S | R | S | S | R | 69.23 |
| L20 | S | S | R | S | I | S | I | S | S | R | S | S | R | 76.92 |
| L21 | S | I | R | S | R | I | I | I | S | R | S | S | R | 69.23 |
| L22 | S | R | R | S | R | S | I | I | S | R | S | S | R | 61.54 |

*P, penicillin G; CRO, ceftriaxone, VA, vancomycin; C, chloramphenicol; CN, gentamicin; E, erythromycin; TE, tetracycline; RD, rifampicin; AMP, ampicillin; AK, amikacin; AML, amoxicillin; CLR, clarithromycin; and S, streptomycin. *R, Resistance; S, Sensitive; and I, Intermediate.*



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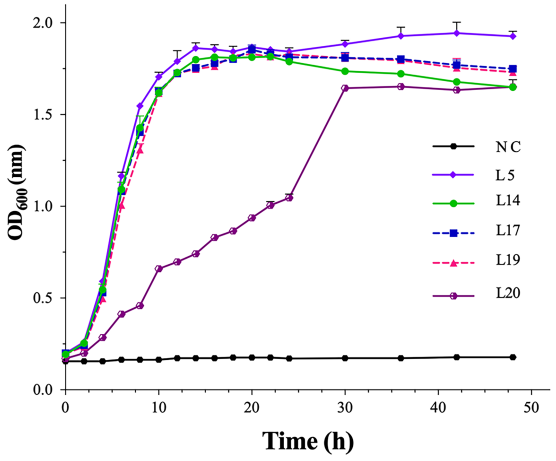


FIGURE 4
The growth curves of the five most probiotic potential isolates. The optical density at 600nm ($OD_{600}$ nm) of the cultures. NC, sterile MRS medium. Error bars refer to the SD of the 3 replicates of each assay.

LAB strains were found to inhibit the growth of all these pathogenic strains (Table 1). In the present study, the inhibition zones against *S. aureus* by the 14 obtained LAB strains were (> 20 mm) much wider than those isolated from human milk (Heikkila and Saris, 2003; Makete et al., 2017; Pellegrino et al., 2019). The strong *S. aureus* antagonistic activities of the LAB strains indicated that the cows might suffer from *S. aureus*-induced mastitis in the past (Pellegrino et al., 2019). Moderate inhibition zones against *P. aeruginosa*, ETEC and *Salmonella* were recorded, indicating a considerable antagonistic activity of these 14 LAB strains, most of which (L1, L5, L10, L14, L17, L19, L20, L21, and L22) showed relative higher antagonistic activities against the target pathogenic strains (Table 1). Intriguing, the antimicrobial ability of LAB strains is mainly produced by the secreted compounds (such as organic acids, hydrogen peroxide, and bacteriocin), and the special microenvironment in the GIT (enzymes, adverse pH, and mild heat shock, et al.) further enhances the antimicrobial potency of these compounds (Gänzle et al., 1999), indicating that they might exhibit higher antimicrobial abilities if they are orally taken. The co-aggregative ability is another antagonistic probiotic activity which boosts pathogen agglomeration with probiotic cells and facilitates its elimination through feces (De Melo Pereira et al., 2018). In our experiment, L5, L7, L14, L19, L20, and L22 showed higher co-aggregation abilities (up to 90%) against all target pathogenic strains (Table 2), indicating that these LAB isolates can easily agglomerate with enteric pathogens and eliminate them. These results proved the considerable antipathogenic activity of these 14 LAB isolates.

After oral administration, the probiotics will have to face all the antimicrobial factors in the stomach (pepsin, gastric acid, and low pH) and intestines (bile salts, trypsin, and high pH), as well as mild heat stimulus caused by the internal body temperature (approximate 37.5°C), which forces the probiotics must have acid and bile tolerance or other exclusion mechanisms to survive in the gut (De Melo Pereira et al., 2018). Previous studies revealed that the tolerant abilities of LAB strains are strain-specific (Liu et al., 2020; Reuben et al., 2020). Similarly, in the present study, the 14 selected LAB isolates showed varying survival rates (33 ~ 84%) after 3 h of low pH gastric acid-containing pepsin, followed by 4 h of high pH intestinal juice-containing ox-bile salts and trypsin, indicating their heterogeneous tolerance for bile salts and acidic gastric (Table 3). It is important to note that the survival rates of our LAB isolates in the simulated gastric acid and intestinal juice were up to 99.69 and 84.94%, respectively, which are higher than those of LABs in recent reports (Liu et al., 2020; Reuben et al., 2020). This high tolerance allows them to survive longer, colonize in GIT environments, and keep effective when administered (Prasad et al., 1998). Phenol, a kind of GIT secreted toxic metabolite that might inhibit the growth of probiotics, is another challenge that poses stresses to the ingested probiotics (Barbour and Vincent, 1950), which means the probiotic candidates must be able to endure the bacteriostatic action of phenol to exert the optimal beneficial effects on the hosts. In the present study, the 14 LAB isolates exhibited varying degrees of tolerance (66 ~ 88%) for 0.4% phenol at 37°C (Figure 2), which are also higher than those in the previous report (Shehata et al., 2016; Reuben et al., 2020). These results indicated that the six LAB isolates, L5, L7, L14, L17, L21, and L22, are able to survive in the GIT, which has a promising probiotic potential.

The ability of adhesion to intestinal cells is considered an essential criterion for probiotic selection (Collado et al., 2006; De Melo Pereira et al., 2018). The adhesion process to epithelial cells is complex, involves the membranes of both microbial and human cells, and depends on the chemical and physicochemical composition of the strain cell's surface, affected by the strain extracellular components and their surrounding composition (Duary et al., 2011). Even though we did not directly explore the adhesion abilities of the 14 LAB isolates to epithelial cells in the present study, the auto-aggregation capacity and hydrophobic properties were evaluated to assess the adhesion abilities indirectly. The auto-aggregation ability ensures that the strains reach a high cell density in the gut, contributing to the adhesion mechanism. Previous studies reported that the LAB strains isolated from raw milk showed no or low auto-aggregation (Espeche et al., 2009, 2012). However, in the present study, we showed that the auto-aggregation at the 3$^{rd}$ h was moderate (< 50%), but they were up to 89% at the 24$^{th}$ h, which is much higher than those in the previous reports, indicating that the auto-aggregation of these LAB isolates increases with time. At the same time, hydrophobicity allows increased interaction between probiotics and host epithelial cells (De Melo Pereira et al., 2018). Hence, we also evaluated the hydrophobicity of the selected LAB strains in our study, and the results showed that L5, L7, L14, L17, L19, L20, and L21 possessed high hydrophobicity (up to 90%; Figure 3), much higher (80%) than those in a previous study (Sirichokchatchawan et al., 2018). Combined with the autoaggregation assessment results, it was

concluded that L5, L7, L14, L17, L19, and L20 had high adhesion activities and were easily adherent to intestinal cells to exert their probiotic effects.

The first step to assessing the safety of probiotics is the identification (Yadav and Shukla, 2017), and strain-level identification was highlighted by the Natural Health Products Regulations (NHPR) for probiotic safety establishment in human health (Coeuret et al., 2004). In the present study, the 26 obtained LAB isolates were identified at the species level based on the biochemical and 16S rDNA sequencing results, and 13 of them were identified as *Lactiplantibacillus plantarum*, an ideal probiotic in the food industry (Seddik et al., 2017). Notably, these isolates showed huge variations in probiotic properties, although all of them belong to *Lactiplantibacillus plantarum*, indicating that they have different gene sequences in their non-16S rDNA gene regions, which needs to be further confirmed by genomic comparison. However, biochemical and genetic analyses are insufficient to compare probiotic bacteria at the strain level (De Melo Pereira et al., 2018). In addition, it was expected that probiotic candidates must not lyse red blood cells when ingested by humans or animals. Hence, we also assessed the hemolytic activities of the 14 LAB isolates. Similar to previous studies (Santini et al., 2010; Reuben et al., 2020), none of them were hemolytic (Supplementary Figure 1). Furthermore, in this study, we detected the antibiotic susceptibilities of the selected 14 LAB isolates against 13 commonly used antimicrobials, and the results showed that the LAB isolates all had high sensitivity (> 60%) except for L2 (Table 5). Intriguing, all isolates were resistant to streptomycin, amikacin, gentamicin (except for 20), and vancomycin (except for L2), which is consistent with previous reports (Liasi et al., 2009; Reuben et al., 2020). It is supposed that the resistance against these four antimicrobials might be associated with LABs' innate resistance caused by the membrane's impermeability, probably through a resistance efflux mechanism (Liasi et al., 2009). On the other hand, the strain-specific molecular mechanism of this intrinsic antimicrobial resistance by these LAB isolates needs further in-depth study because this inherent resistance might promote both preventive and therapeutic outcomes when the probiotics are administered together with antibiotics (Jose et al., 2015). Fortunately, all 14 isolates were sensitive to penicillin G, tetracycline, ampicillin (except for L2), amoxicillin (except for L2), and clarithromycin (except for L2), the five most commonly used antibiotics in humans. Combined with the results of biochemical and genotypic identification, hemolytic activities, and antibiotic susceptibilities, it could be concluded that these selected LAB isolates were safe for use except for L2.

Taken together with the results of these *in vitro* probiotic evaluation tests, five *Lactiplantibacillus plantarum* isolates, L5, L14, L17, L19, and L20, showed promising probiotic potential and were considered the probiotic candidates. These five isolates were isolated from distinct samples and showed varying probiotic properties, indicating their different gene sequences and biological features, which need further study. Mounts of probiotic cells and byproducts can be obtained by the selected LAB strains if they are cultured under a specific, controlled condition with ample nutrient supply (Kuznetsov et al., 2017). In the present study, the growth performance of the five selected probiotic isolates under the common condition (in MRS broth, at 37°C, pH = 6.2), different from the conditions in the gut or under industrial production, was also evaluated (Figure 4). Similarly, they showed different growth performances under the same condition. Interestingly, L20 showed an atypical growth curve, indicating that this condition might not be optimal. Hence, further studies need to be conducted to evaluate the growth performance of the selected isolates under different situations. It is important to note that these *in vitro* evaluations are not adequate to claim these LAB strains as probiotics, and further *in vivo* and clinical trials need to be carried out. In addition, even if the probiotic performance of these five candidates was proved to be relatively better by animal studies than those reported in some other manuscripts, it also needs to be compared with those existing commercialized probiotics to confirm their better application potential in industrialization. Recently, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-Cas 9, a newly developed and very promising gene-editing tool, might enable us to efficiently edit the gene sequences of probiotic candidates to obtain ideal probiotics by knocking down their virulence genes or resistant genes with horizontal transfer capabilities, and overexpressing probiotic effect related genes (Sirichokchatchawan et al., 2018; Goh and Barrangou, 2019).

## Conclusion

Cow milk contains massive bacteria and is important for probiotics isolation, especially LABs. In the present study, we obtained 26 LABs from raw milk collected from a farm whose milk had never been used for LAB isolation. The isolated LAB isolates were identified at the species level by biochemical and 16S rDNA sequencing methods. The probiotic properties of these 26 LABs isolates were screened *via* several *in vitro* experiments, including antagonistic activity, co-aggregation ability with pathogens, tolerance for simulated GIT conditions and phenol, autoaggregation activity, cell surface hydrophobicity, hemolytic activity, and antibiotic susceptibility. Furthermore, four *Lactiplantibacillus plantarum* strains, named L5, L14, L17, and L19, showed comprehensive probiotic properties and considerable growth performance, which need to be further confirmed *via in vivo* studies and clinical trials. This study revealed the probiotic properties of LAB isolates collected from raw milk of a local feedlot and provided five LAB isolates with promising probiotic potential for application in the food and pharmaceutical industries.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

## Author contributions

GP and WZ: conceive and design this study. WZ and SL: sample collection and main experiments. WZ and JY: data analysis and writing—original draft preparation. LD, ZZ, and GP: supervision and writing—reviewing and editing. All authors reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary materials for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.918774/full#supplementary-material

SUPPLEMENTARY FIGURE 1
The results of the hemolytic test of 14 selected isolates.

## References

Araya, M., Morelli, L., Reid, G., Sanders, M., Stanton, C., Pineiro, M., et al. (2002). *Joint FAO/WHO Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food*. London, Canada: World Health Organization, Food and Agriculture Organization of the United Nations.

Bachta, K. E. R., Allen, J. P., Cheung, B. H., Chiu, C.-H., and Hauser, A. R. (2020). Systemic infection facilitates transmission of *Pseudomonas aeruginosa* in mice. *Nat. Commun.* 11, 543. doi: 10.1038/s41467-020-14363-4

Barbour, R. G. H., and Vincent, J. M. (1950). The bacteriostatic action of phenol, benzoic acid and related compounds on bacterium aerogenes. *J. Gen. Microbiol.* 4, 110–121. doi: 10.1099/00221287-4-2-110

Cheung, G. Y. C., Bae, J. S., and Otto, M. (2021). Pathogenicity and virulence of *Staphylococcus aureus*. *Virulence* 12, 547–569. doi: 10.1080/21505594.2021.1878688

Coeuret, V., Gueguen, M., and Vernoux, J. P. (2004). In vitro screening of potential probiotic activities of selected lactobacilli isolated from unpasteurized milk products for incorporation into soft cheese. *J. Dairy Res.* 71, 451–460. doi: 10.1017/S0022029904000469

Collado, M. C., Jalonen, L., Meriluoto, J., and Salminen, S. (2006). Protection mechanism of probiotic combination against human pathogens: in vitro adhesion to human intestinal mucus. *Asia Pac. J. Clin. Nutr.* 15, 570–575.

Culligan, E. P., Hill, C., and Sleator, R. D. (2009). Probiotics and gastrointestinal disease: successes, problems and future prospects. *Gut pathogens.* 1, 1–12. doi: 10.1186/1757-4749-1-19

Cummings, G. D. (1926). Bergey's Manual of Determinative Bacteriology. *Am. J. Pharma. Assoc.* 47:520.

De Boeck, I., Spacova, I., Vanderveken, O. M., and Lebeer, S. (2021). Lactic acid bacteria as probiotics for the nose? *Microb. Biotechnol.* 14, 859–869. doi: 10.1111/1751-7915.13759

De Melo Pereira, G. V., De Oliveira Coelho, B., Magalhães Júnior, A. I., Thomaz-Soccol, V., and Soccol, C. R. (2018). How to select a probiotic? A review and update of methods and criteria. *Biotechnol. Adv.* 36, 2060–2076. doi: 10.1016/j.biotechadv.2018.09.003

Duary, R. K., Rajput, Y. S., Batish, V. K., and Grover, S. (2011). Assessing the adhesion of putative indigenous probiotic lactobacilli to human colonic epithelial cells. *Indian J. Med. Res.* 134, 664–671. doi: 10.4103/0971-5916.90992

El-Shaer, S., Shaaban, M., Barwa, R., and Hassan, R. (2017). Performance Standards for antimicrobial susceptibility testing: twenty-third informational supplement M100-S23. Pennsylvania: Clinical and Laboratory Standards institute. 3-Miller.

Espeche, M. C., Otero, M. C., Sesma, F., and Nader-Macias, M. E. F. (2009). Screening of surface properties and antagonistic substances production by lactic acid bacteria isolated from the mammary gland of healthy and mastitic cows. *Vet. Microbiol.* 135, 346–357. doi: 10.1016/j.vetmic.2008.09.078

Espeche, M. C., Pellegrino, M., Frola, I., Larriestra, A., Bogni, C., and Nader-Macías, M. F. (2012). Lactic acid bacteria from raw milk as potentially beneficial strains to prevent bovine mastitis. *Anaerobe* 18, 103–109. doi: 10.1016/j.anaerobe.2012.01.002

Fiorda, F. A., De Melo Pereira, G. V., Thomaz-Soccol, V., Rakshit, S. K., Pagnoncelli, M. G. B., Vandenberghe, L. P. D. S., et al. (2017). Microbiological, biochemical, and functional aspects of sugary kefir fermentation - A review. *Food Microbiol.* 66, 86–95. doi: 10.1016/j.fm.2017.04.004

Fleckenstein, J. M., and Kuhlmann, F. M. (2019). Enterotoxigenic *Escherichia coli* infections. *Curr. Infect. Dis. Rep.* 21, 9. doi: 10.1007/s11908-019-0665-x

Fontana, C., Cocconcelli, P. S., Vignolo, G., and Saavedra, L. (2015). Occurrence of antilisterial structural bacteriocins genes in meat borne lactic acid bacteria. *Food Control* 47, 53–59. doi: 10.1016/j.foodcont.2014.06.021

Food and Agriculture Organization (2006). Probiotics in food - health and nutritional properties and guidelines for evaluation. Food and Agriculture Organization of the United Nations.

Gänzle, M. G., Weber, S., and Hammes, W. P. (1999). Effect of ecological factors on the inhibitory spectrum and activity of bacteriocins. *Int. J. Food Microbiol.* 46, 207–217. doi: 10.1016/S0168-1605(98)00205-0

Goh, Y. J., and Barrangou, R. (2019). Harnessing CRISPR-Cas systems for precision engineering of designer probiotic lactobacilli. *Curr. Opin. Biotechnol.* 56, 163–171. doi: 10.1016/j.copbio.2018.11.009

Heikkila, M. P., and Saris, P. E. J. (2003). Inhibition of *Staphylococcus aureus* by the commensal bacteria of human milk. *J. Appl. Microbiol.* 95, 471–478. doi: 10.1046/j.1365-2672.2003.02002.x

Hill, C., Guarner, F., Reid, G., Gibson, G. R., Merenstein, D. J., Pot, B., et al. (2014). Expert consensus document. The international scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat. Rev. Gastroenterol. Hepatol.* 11, 506–514. doi: 10.1038/nrgastro.2014.66

Hornik, B., Czarny, J., Staninska-Pięta, J., Wolko, Ł., Cyplik, P., and Piotrowska-Cyplik, A. (2021). The raw Milk microbiota from semi-subsistence farms characteristics by NGS analysis method. *Molecules* 26, 5092. doi: 10.3390/molecules26165029

Jose, N. M., Bunt, C. R., and Hussain, M. A. (2015). Comparison of microbiological and probiotic characteristics of lactobacilli isolates from dairy food products and animal rumen contents. *Microorganisms* 3, 198–212. doi: 10.3390/microorganisms3020198

Knodler, L. A., and Elfenbein, J. R. (2019). *Salmonella enterica*. Trends In. *Microbiology* 27, 964–965. doi: 10.1016/j.tim.2019.05.002

Kuznetsov, A., Beloded, A., Derunets, A., Grosheva, V., Vakar, L., Kozlovskiy, R., et al. (2017). Biosynthesis of lactic acid in a membrane bioreactor for cleaner technology of polylactide production. *Clean Techn. Environ. Policy* 19, 869–882. doi: 10.1007/s10098-016-1275-z

Lebeer, S., Vanderleyden, J., and De Keersmaecker, S. C. (2008). Genes and molecules of lactobacilli supporting probiotic action. *Microbiol. Mol. Biol. Rev.* 72, 728–764. doi: 10.1128/MMBR.00017-08

Liasi, S., Azmi, T., Hassan, M., Shuhaimi, M., Rosfarizan, M., and Ariff, A. (2009). Antimicrobial activity and antibiotic sensitivity of three isolates of lactic acid bacteria from fermented fish product, Budu. *Malaysian J. Microbiol.* 5, 33–37.

Liu, W., Chen, M., Duo, L., Wang, J., Guo, S., Sun, H., et al. (2020). Characterization of potentially probiotic lactic acid bacteria and bifidobacteria isolated from human colostrum. *J. Dairy Sci.* 103, 4013–4025. doi: 10.3168/jds.2019-17602

Makete, G., Aiyegoro, O. A., and Thantsha, M. S. (2017). Isolation, identification and screening of potential probiotic Bacteria in Milk from south African Saanen goats. *Probio.Antimicrob. Proteins* 9, 246–254. doi: 10.1007/s12602-016-9247-5

Mathur, H., Beresford, T. P., and Cotter, P. D. (2020). Health benefits of lactic acid Bacteria (LAB) Fermentates. *Nutrients* 12, 1679. doi: 10.3390/nu12061679

Mokoena, M. P. (2017). Lactic acid Bacteria and their Bacteriocins: classification, biosynthesis and applications against Uropathogens: A Mini-review. *Molecules* 22, 1255. doi: 10.3390/molecules22081255

Ocaña, V. S., Bru, E., Ruiz Holgado, A. A. P. d., and Nader-Macias, M. E. (1999). Surface characteristics of lactobacilli isolated from human vagina. *J. Gen. Appl. Microbiol.* 45, 203–212. doi: 10.2323/jgam.45.203

Pellegrino, M. S., Frola, I. D., Natanael, B., Gobelli, D., Nader-Macias, M. E. F., and Bogni, C. I. (2019). In vitro characterization of lactic acid Bacteria isolated from bovine Milk as potential probiotic strains to prevent bovine mastitis. *Probio. Antimicrob. Proteins* 11, 74–84. doi: 10.1007/s12602-017-9383-6

Plessas, S., Nouska, C., Karapetsas, A., Kazakos, S., Alexopoulos, A., Mantzourani, I., et al. (2017). Isolation, characterization and evaluation of the probiotic potential of a novel Lactobacillus strain isolated from feta-type cheese. *Food Chem.* 226, 102–108. doi: 10.1016/j.foodchem.2017.01.052

Prasad, J., Gill, H., Smart, J., and Gopal, P. (1998). Selection and characterization of Lactobacillus and Bifidobacterium strains for use as probiotic. *Int. Dairy* 8, 993–1002. doi: 10.1016/S0958-6946(99)00024-2

Quigley, L., O'sullivan, O., Stanton, C., Beresford, T. P., Ross, R. P., Fitzgerald, G. F., et al. (2013). The complex microbiota of raw milk. *FEMS Microbiol. Rev.* 37, 664–698. doi: 10.1111/1574-6976.12030

Reuben, R. C., Roy, P. C., Sarkar, S. L., Rubayet Ul Alam, A. S. M., and Jahid, I. K. (2020). Characterization and evaluation of lactic acid bacteria from indigenous raw milk for potential probiotic properties. *J. Dairy Sci.* 103, 1223–1237. doi: 10.3168/jds.2019-17092

Sanders, M. E., Akkermans, L. M. A., Haller, D., Hammerman, C., Heimbach, J., Hörmannsperger, G., et al. (2010). Safety assessment of probiotics for human use. *Gut Microbes* 1, 164–185. doi: 10.4161/gmic.1.3.12127

Santini, C., Baffoni, L., Gaggia, F., Granata, M., Gasbarri, R., Di Gioia, D., et al. (2010). Characterization of probiotic strains: an application as feed additives in poultry against *Campylobacter jejuni*. *Int. J. Food Microbiol.* 141, S98–S108. doi: 10.1016/j.ijfoodmicro.2010.03.039

Seddik, H. A., Bendali, F., Gancel, F., Fliss, I., Spano, G., and Drider, D. (2017). Lactobacillus plantarum and its probiotic and food potentialities. *Probiotics and Antimicrob. Prot.* 9, 111–122. doi: 10.1007/s12602-017-9264-z

Sharpe, M. E. (1979). *Identification of the lactic acid bacteria*. London, UK: Academic Press.

Shehata, M. G., El Sohaimy, S. A., El-Sahn, M. A., and Youssef, M. M. (2016). Screening of isolated potential probiotic lactic acid bacteria for cholesterol lowering property and bile salt hydrolase activity. *Ann. Agric. Sci.* 61, 65–75. doi: 10.1016/j.aoas.2016.03.001

Shin, M., Ban, O. H., Jung, Y. H., Yang, J., and Kim, Y. (2021). Genomic characterization and probiotic potential of *Lactobacillus casei* IDCC 3451 isolated from infant faeces. *Lett. Appl. Microbiol.* 72, 578–588. doi: 10.1111/lam.13449

Sirichokchatchawan, W., Pupa, P., Praechansri, P., Am-In, N., Tanasupawat, S., Sonthayanon, P., et al. (2018). Autochthonous lactic acid bacteria isolated from pig faeces in Thailand show probiotic properties and antibacterial activity against enteric pathogenic bacteria. *Microb. Pathog.* 119, 208–215. doi: 10.1016/j.micpath.2018.04.031

Suez, J., Zmora, N., Segal, E., and Elinav, E. (2019). The pros, cons, and many unknowns of probiotics. *Nat. Med.* 25, 716–729. doi: 10.1038/s41591-019-0439-x

T.T.M. Research (2017). Digestive Health Products Market (Product Type – Dairy Products, Bakery Products and Cereals, Non-alcoholic Beverages; Ingredient – Probiotics, Prebiotics, Food Enzymes; Sales Channel – Modern Trade, Convenience Stores, Online Retailers) – Global Industry Analysis, Size, Share, Growth, Trends and Forecast 2017–2022. [Online]. Available at: https://www.transparencymarketresearch.com/digestive-health-products-market.html (Accessed July 4, 2022).

Transparency Market Research (TMR) (2013). Probiotics Market (Dietary Supplements, Animal Feed, Foods & Beverages) – Global Industry Analysis, Market Size, Share, Trends, Analysis, Growth and Forecast, 2012–2018. [Online]. Available at: https://www.transparencymarketresearch.com/probiotics-market.html (Accessed July 4, 2022).

Yadav, R., and Shukla, P. (2017). An overview of advanced technologies for selection of probiotics and their expediency: a review. *Crit. Rev. Food Sci. Nutr.* 57, 3233–3242. doi: 10.1080/10408398.2015.1108957

Zhang, B., Wang, Y., Tan, Z., Li, Z., Jiao, Z., and Huang, Q. (2016). Screening of probiotic activities of lactobacilli strains isolated from traditional Tibetan Qula, A raw yak Milk cheese. *Asian-Australas J. Anim. Sci.* 29, 1490–1499. doi: 10.5713/ajas.15.0849

Zoumpopoulou, G., Pot, B., Tsakalidou, E., and Papadimitriou, K. (2017). Dairy probiotics: Beyond the role of promoting gut and immune health. *Int. Dairy J.* 67, 46–60. doi: 10.1016/j.idairyj.2016.09.010

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# Molecular characterization and phylogeny of Shiga toxin-producing *Escherichia coli* derived from cattle farm

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Shiga toxin-producing *Escherichia coli* (STEC) is an important food-borne pathogen, which can cause diseases such as diarrhea, hemorrhagic enteritis, and hemolytic uremic syndrome in humans. Twelve STEC isolates were collected from beeves and feces of commercial animals in China between 2019 and 2020 for this study. In addition to the determination of serotype and Shiga toxin subtype, whole-genome sequencing (WGS) was used for determining phylogenetic relationships, antimicrobial resistance (AMR), virulence genes, and sequence type (ST) of isolates. A total of 27 AMR genes were detected, and each STEC isolate carried more than 10 AMR genes. Eight STEC isolates from ground beef and four STEC isolated from feces were screened. A total of seven serotypes were identified, and one isolate ONT:H10 was undetermined by SeroTypeFinder. Three O157:H7 strains were confirmed and the remaining five serogroups were confirmed as O26:H11, O81:H31, O105:H8, O178:H19, and O136:H12. The phylogenetic analysis showed that STEC isolates of the same serotype or ST were clustered together based on cgMLST. The comparison of the genomes of 157 STEC reference isolates worldwide with our local STEC isolates showed that STEC isolates screened in China represented various collections and could not form a separate cluster but were interspersed among the STEC reference collection, which suggested that several STEC isolates shared a common ancestor irrespective of STEC serotype isolates. cgMLST revealed that isolates of the same O serotype clustered irrespective of their H type. Further investigation is required to determine the pathogenic potential of other serotypes of STEC, particularly in regard to these rare serotypes.

KEYWORDS

**STEC, food, cgMLST, serogroups, phylogenetic relationships**

# Introduction

Shiga toxin-producing *Escherichia coli* (STEC) is an important food-borne pathogen causing zoonotic diseases. As a subpopulation of diarrheagenic *E. coli* (DEC), STEC can result in severe cases of disease such as hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS). The pathogenicity of STEC is associated with the production of Shiga-like toxins (*Stx*) encoding either one or both of the $stx_1$ and $stx_2$ genes (Gonzalez and Cerqueira, 2020). Since STEC was first described in 1982, more than 400 different serotypes of STEC have been identified, and research hotspots have focused on O157:H7 (Karmali et al., 2003). Moreover, several non-O157 serotypes have been associated with sporadic cases and outbreaks, such as O26, O45, O103, O111, O121, and O145, which were considered as the top six non-STEC serogroups (Mathusa et al., 2010; Amézquita-López et al., 2018).

In China, the outbreak of STEC was traced back in Xuzhou, Jiangsu province in 1999, which led to 195 hospitalized HUS patients and 177 deaths (Wang et al., 2011). The traceability analysis revealed that the food was contaminated by the fecal shedding of courtyard animals carrying STEC O157:H7 (Xiong et al., 2012). Although the main reservoir of STEC was considered to be cattle, there has been an increase in non-bovine food-related outbreaks worldwide (Smith et al., 2014). Some researches show that the STEC was transmitted mainly through foods, such as raw poultry, fresh vegetables, fruits, water, ground beef, and dairy products, to humans (Smith et al., 2014). Various transmission routes of STEC have been proposed, and the primary one is presumably *via* consumption of contaminated food or water (Parsons et al., 2016). However, some transmissions are not clear. For epidemiologic purposes, several genetic fingerprinting methods have been used to classify, trace, and prevent the dissemination of STEC (Heir et al., 2000). Pulsed-field gel electrophoresis (PFGE) and multilocus variable-number of tandem-repeat (MLVA) have mostly been applied in analyzing the molecular epidemiology of STEC and proved to be reliable among the sequence-based methods. However, low discriminatory power is exposed in the methods of complex workflows, expensive reagents, and time-consuming process.

The advances in next-generation sequencing in the past decade have made it true to perform WGS of organisms, including STEC at affordable costs (Joensen et al., 2014, 2015), and there was an ever-increasing evidence demonstrating that WGS was not only epidemiologic typing to detect and support outbreak investigations but also to define transmission pathways of pathogens and to reveal laboratory cross-contamination (Keoser et al., 2012; Chattaway et al., 2016; Baha et al., 2019). WGS data have the potential to provide powerful, high-level phylogenetic analysis and to show insight into the evolutionary background of the outbreak strains by using quantifiable genetic differences (Baltasar et al., 2014; Mikhail et al., 2018). For example, the comparison of 62 STEC local isolates from Chile with STEC isolated from the rest of the world by core genome multilocus sequence typing (cgMLST) typing method showed Chile STEC did not cluster with genomes of the rest of the world, suggesting local STEC isolates and STEC isolated from worldwide were not phylogenetical (Smith et al., 2014; Gutierrez et al., 2021) and indicating STEC phylogeny was affected by the origin of geographical isolation.

An earlier study described the diversity of Chinese *E. coli* O157 obtained from different sources such as mutton, beef, chicken, pork, and vegetable salad. Twenty-two pulsotypes by PFGE and 23 types by MLVA were found, and this study demonstrated the diversity among 30 STEC O157 isolates (Li et al., 2015). However, other STEC serotypes have not been studied in China. In this study, non-O157 STEC and O157 STEC were mainly isolated from cattle feces and ground beef in China, and serotype, sequence type (ST), and virulence genes of these strains were characterized. Furthermore, based on WGS data, cgMLST analysis from STEC isolates screened by our lab and National Center for Biotechnology Information (NCBI) confirmed phylogenetic relationships among different STEC serotypes found and suggested possibly high-risk foods causing STEC. The comparison of these characteristics with those of foodborne and clinical isolates around the worldwide could provide some information for food safety risk assessment.

# Materials and methods

## Collection of STEC strains

Between 2019 and 2020, the STEC isolates used in this study were isolated from animal food and feces in Shanghai. All samples were collected in sterile sample bags and transported in ice as soon as possible to the laboratory for immediate processing. The enrichment method was modified from the GB4789.6-2016 food microbiological examination of *Escherichia coli* (National Food Safety Standards of China). Briefly, 225 ml of sterile Tryptone Soya Broth was added to a sterile sample bag with 25 g of each sample. Then, incubated at 37°C for 16–22 h on a shaking platform (200 rpm). Enrichment solutions were inoculated into CHROMagar™ STEC plates (CHEOMagar, Pairs, France). Discrete, strongly mauve colonies were picked and streaked out on MacConkey agar (MAC) and sorbitol MacConkey agar (CT-SMAC; Hopebio, Qingdao, China) for 18–22 h (John et al., 2018). STEC was identified by using PCR for the targeted genes *wzxO157*, $stx_1$, and $stx_2$ screening, as shown in Table 1. All positive samples were further processed to obtain pure *stx*-positive isolates (Boer et al., 2015).

TABLE 1 Sequences of all primers and annealing temperature used in this work.

| Primers | Direction | Sequence (5′-3′) | Product size (base) | Annealing temperature (°C) | Reference |
|---|---|---|---|---|---|
| $stx_1$ | Forward | AAATCGCCATTCGTTGACTACTTCT | 370 | 58 | GB4789.6-2016 |
| | Reverse | TGCCATTCTGGCAACTCGCGATGCA | | | |
| $stx_2$ | Forward | CAGTCGTCACTCACTGGTTTCATCA | 283 | 58 | |
| | Reverse | GGATATTCTCCCCACTCTGACACC | | | |
| *wzxO157* | Forward | CGGACATCCATGTGATATTGG | 259 | 28 | |
| | Reverse | TTGCCTCTGTACAGCTAATCC | | | |

## DNA extraction

Genomic DNA of STEC isolates was extracted from overnight cultures using boiling. Briefly, 0.5 g of feces and 25 g of food samples were added together into 225 ml of lysogeny broth (LB) and incubated at 37°C for 18–24 h. The enrichment broth (1 ml) was centrifuged at 4,000 rpm for 2 min, then centrifuged at 12,000 rpm for 5 min, and supernatant was removed. Finally, 200-μl sterile deionized water was added and boiled at 100°C for 15 min.

## Whole-genome sequencing

Whole-genome sequencing (WGS) using a 400-bp paired end was performed on the STEC genomic DNA on an Illumina Novaseq according to the manufacturer's instructions. The genomic DNA libraries were prepared using the TIANamp Bacteria DNA Kit (Tiangen Biotech Beijing Co., Ltd., China). The raw data were assembled using SPAdesv3.11.1 software to obtain scaffolds sequences with default parameters, and raw data were cleaned by Fastp (v0.19.4; number of bases to average across: 4, average quality required: 20, fold coverage was required to be >30 for the cleaned data) (Bolger et al., 2014; Shifu et al., 2018).

## Data analysis and molecular characterization

The serotype of each STEC isolate was determined by uploading the assembled genomes to the SerotypeFinder 2.0 (https://cge.cbs.dtu.dk/services/SerotypeFinder/) of the Center for Genomic Epidemiology (CGE) website: the threshold of identity was set to 85% and the minimum length was set to 60%, VirulenceFinder 2.0 web-based tool (https://cge.cbs.dtu.dk/services/VirulenceFinder/) was used to determine virulence genes of each STEC isolates in this study with the following parameters: the 90% threshold identity and 60% minimum length. ResFinder 4.1 web-based tool (https://cge.food.dtu.dk/services/ResFinder/) was used to determine AMR genes for each STEC isolate with default parameters. The sequence types (STs) were identified by uploading assembled genomes to the MLST Finder (https://cge.cbs.dtu.dk/services/MLST/) (Jaureguy et al., 2008).

## Phylogenetic analysis of STEC isolates based on cgMLST

To determine the phylogenetic relationship of the isolates, a gene-by-gene approach was performed by SeqSphere (v3.1.1-rc04, Ridom) and BLAST (v2.2.12) (Michaela et al., 2021). The key parameters identity was 90% and aligned was 100%. Briefly, a core genome MLST scheme was developed using the genome of *E. coli* O157:H7 strain Sakai (accession no. NC_002695; https://www.cgmlst.org/ncs/schema/8896773/) as a reference genome and an additional eleven *E. coli* as query genomes to extract open reading frames (ORFs) from the genome of each isolate using MLST+ (v2.11.0+) of SeqSphere (v3.1.1-rc04, Ridom). The genes shared by all isolates analyzed were defined as the core genome for phylogenetic analysis. Loci were detected by chewBBACA (https://github.com/B-UMMI/chewBBACA), BLAST Score Ratio threshold was 0.8, and the number of loci present in genomes was 95% (Jagadesan et al., 2019). By default, a minimum spanning tree (MST) was calculated based on loci, which were carried out using SeqSphere (v3.1.1-rc04, Ridom).

The phylogenetic relationship of the STEC isolates of this study with isolates of the STEC reference collection ($n = 157$; Supplementary File 1) from NCBI was determined by cgMLST method. The download criteria for raw data of reference collection were as follows: (1) sequence depth cannot be lower than 200×, and (2) sequence length in the range of 4.7–5.5 M.

TABLE 2 Prevalence of STEC isolates in cattle feces, lettuce, and ground beef samples.

| Year | Method | Cattle feces (%) | Lettuce (%) | Ground beef (%) | Total (%) |
|---|---|---|---|---|---|
| 2019–2020 | PCR[a] | 9/204 (4.41%) | 1/55 (1.81%) | 0/30 (0.00%) | 10/289 (3.46%) |
| | Culture[b] confirmed | 4/204 (1.96%) | 0/55 (0.00%) | 0/30 (0.00%) | 4/289 (1.38%) |

[a]Samples consists of $stx_1$ or $stx_2$ gene identified by multiplex PCR were considered to be positive.
[b]PCR positive samples were further cultured and at least one isolate was isolated by CHROMagar$^{TM}$ STEC agar.

# Results and discussion

## STEC detection and isolation

As shown in Table 2, 289 samples from cattle feces, lettuce, and ground beef were tested during 2019–2020, 4.41% (9/204) of cattle feces, 1.81% (1/55) of lettuce, and 0.00% (0/30) of ground beef were $stx_1$ and $stx_2$-positive. Further, 4 STEC strains were isolated from nine STEC-positive samples of cattle feces, whereas no STEC strains were found in any samples of lettuce that tested positive for STEC. The prevalence of STEC in cattle feces was higher than the other samples, with a PCR-positive rate of 4.41% and an isolation rate of 1.96%. Eight STEC strains were isolated from ground beef earlier, and 12 STEC strains were collected for this study. STEC has emerged as an important food-borne pathogen, several reports indicated that STEC cases have been transmitted to humans *via* food (Smith et al., 2014; Jenkins et al., 2019; Mohammad et al., 2021). In this study, STEC isolates were collected from cattle feces and ground beef in Shanghai, China. The findings in this study supported that cattle carry various serotypes (Monaghan et al., 2012).

## The analysis of molecular characterization

The serotypes of 12 STEC isolates were identified by Serotypefinder software. In addition to the common serotypes such as O157:H7 and O26:H11, other serotypes such as O81:H31, O136:H12, and O105:H8 were isolated. However, an unknown STEC serotype ONT:H10 was found, which may be due to the incomplete coverage of the area involved in O-antigen determination, resulting in the generation of unknown serotype STEC. As a result, three O157:H7 strains were confirmed and the remaining five serogroups were O26:H11, O81:H31, O105:H8, O178:H19, and O136:H12. In this study, seven housekeeping (*adk*, *fumC*, *gyrB*, *icd*, *mdh*, *purA*, and *recA*) genes were used for MLST analysis, and seven STs were identified, as shown in Table 3. Three O157:H7 were ST 11, four O136:H12 belong to ST 329. O26:H11 clustered in ST 21, ONT:H10 clustered in ST 441, O178:H19 clustered in ST 192, O81:H31 clustered in ST101, and O105:H8 clustered in ST 13.

A total of 20 virulence genes were detected among the 12 STEC strains. The distribution of virulence genes among strains of different serogroups is shown in Table 3. Glutamate decarboxylase-encoding *gad* gene and tellurium ion resistance protein-encoding *terC* were the most widespread gene and were detected in all strains. Other well-known virulence genes found were as follows, with their distribution noted in parentheses: *eae* which encodes intimin (33.3%, $n = 4$), *astA* which encodes heat-stable enterotoxin (66.7%, $n = 8$), and *ompT* which encodes outer membrane protease (75%, $n = 9$). Four isolates with the combination of $stx_1$ and $stx_2$ as the most frequently detected type were found. Three isolates O157:H7 and one O26:H11 with $stx_1$ and $stx_2$ virulence genes were acquired, and the non-O157 serotype STEC isolated from ground beef with single $stx_2$ was found. This result was consistent with previous studies in China, which reported that $stx_1$ and $stx_2$ were common in retail meat or slaughterhouses (Leung et al., 2001; Li et al., 2015, 2016). All the STEC obtained from 204 cattle feces were O136:H12 serotype and only contained $stx_1$ virulence gene. WGS characterization of the isolates revealed that 12 isolates possessed AMR genes that can confer resistance to at least six classes of antimicrobials. As shown in Table 4, 27 AMR genes were detected, and each STEC isolate carried more than 10 AMR genes, suggesting that 12 STEC isolates may be multidrug-resistant. According to the health outcome of reported confirmed human STEC cases, serogroups O81:H31, ONT:H10 were classified to group E indicating non-human only disease, serogroups O178:H19, O105:H8 serotype were classified to group A/B/C indicating a HUS-associated serotype (Hazards, 2013). All those STEC isolates distributions indicated a higher diversity of serotypes in cattle and revealed a potential threat to consumers.

## cgMLST scheme

The cgMLST scheme including 3,152 cgMLST targets, 1,485 accessory targets (Supplementary File 2), and 567 low-quality genes were filtered out. According to the cgMLST typing scheme, based on genome typing with PanGen.py in the chewBBACA tool, which uses Prodigal annotation, a total of 2,514 loci were selected as cgMLST targets shared by 12 STEC and 2,586 loci were selected as cgMLST targets shared by 169 STEC

TABLE 3 Sequence types and virulence genes detected by WGS in 12 STEC strains in this study.

| | MRL380001 Ground beef | MRL380002 Ground beef | MRL380003 Ground beef | MRL380004 Ground beef | MRL380005 Ground beef | MRL380006 Ground beef | MRL380007 Ground beef | MRL380008 Ground beef | MRL380009 Cattle feces | MRL380010 Cattle feces | MRL380011 Cattle feces | MRL380012 Cattle feces |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Serotype | O157:H7 | O26:H11 | O157:H7 | O157:H7 | ONT:H10 | O81:H31 | O178:H19 | O105:H8 | O136:H12 | O136:H12 | O136:H12 | O136:H12 |
| Sequence type | ST11 | ST21 | ST11 | ST11 | ST441 | ST101 | ST192 | ST13 | ST329 | ST329 | ST329 | ST329 |
| *astA* | + | + | + | + | – | – | – | + | – | + | + | + |
| *chuA* | + | – | + | + | – | – | – | – | – | – | – | – |
| *eae* | + | + | + | + | – | – | – | – | – | – | – | – |
| *ehxA* | + | + | + | + | – | + | – | – | – | – | – | – |
| *espF* | + | + | + | + | – | – | – | – | – | – | – | – |
| *espP* | – | + | + | + | + | + | + | – | – | – | – | – |
| *etpD* | + | – | + | + | – | – | – | – | – | – | – | – |
| *gad* | + | + | + | + | + | + | + | + | + | + | + | + |
| *hra* | – | + | + | + | – | – | – | – | – | – | – | – |
| *iss* | + | + | + | + | – | – | + | + | + | + | + | + |
| *nleA* | + | + | + | + | – | – | – | – | – | – | – | – |
| *nleB* | + | + | + | + | – | – | – | – | – | – | – | – |
| *nleC* | + | + | + | + | – | – | – | – | – | – | – | – |
| *ompT* | + | + | + | + | – | – | + | – | + | + | + | + |
| $stx_1$ | + | + | + | + | – | – | – | + | + | + | + | + |
| $stx_2$ | + | + | + | + | + | + | + | – | – | – | – | – |
| *terC* | + | + | + | + | + | + | + | + | + | + | + | + |
| *tir* | + | – | + | + | – | – | – | – | – | – | – | – |
| *traT* | + | + | + | + | + | + | – | – | + | + | + | + |
| *IpfA* | – | – | – | – | – | – | – | – | + | – | + | – |

+, detected; –, not detected.





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(Supplementary Files 3, 4). The MST was constructed based on the loci for the analysis of phylogenetic relationship with default.

## Phylogenetic comparison of STEC isolates and reference collections

The number of locus differences in core genome MLST minimum spanning tree ranged from 1 to 2,437 between 12 STEC isolates (Figure 1). Isolates of the same serotype are clustered together, four O136:H12 (MRL380009, MRL380010, MRL380011, and MRL380012) can be considered homologous because of their small allelic differences. Figure 2 shows a minimum spanning tree representing 12 STEC isolates from our lab and 157 STEC reference collections (Supplementary File 1) by cgMLST. In this study, STEC isolates representing various serotypes collections and without forming a separate cluster suggested that STEC isolates were phylogenetically related to STEC reference collections, on the one hand. On the other hand, epidemiologically related strains grouped together or were even part of a clonal cluster as shown for strains concurrently isolated from the farm. The top six non-STEC serotypes O145:H25 and four local isolated STEC strains O136:H12 clustered together, suggesting O136:H12 may be pathogenic. In order to determine the relatedness between different serotype STEC strains, the present study calculated the allelic distances between strains ranging from 1 to 2532. The greatest distances were observed between the O136:H12 and O145:H25. In addition, the same somatic antigen (O antigen) and the different flagella antigen (H antigen) STEC were divided into same lineages, such as O121:HNT, O121:H19 and O111:NM, O111:H8.

TABLE 4 Summary of resistance genes carried by STEC isolates.

| Description | AMR genes |
|---|---|
| β-Lactam resistance genes | *ompC*, *mdrA*, *oppA*, *ampG*, *arcB*, *rtcB*, *mppA*, *arcA*, *ampH* |
| Vancomycin resistance genes | *ddl*, *murG*, *mraY*, *murF*, *alr*, *vanX* |
| Fluoroquinolones resistance genes | *emrB*, *MdtH*, *emrK*, *EvgA*, *MdtE*, *AcrE* |
| Nitroimidazole resistance genes | *MsbA* |
| Peptide resistance genes | *PmrF* |
| Aminoglycoside resistance genes | *BaeS*, *MdtB*, *tolC*, *cpxA* |

Based on sequencing data and *in silico* analysis, STEC isolates from our lab and STEC reference collection strains revealed that the STEC isolates represented a heterogeneous group. A minimum spanning tree was constructed by all 169 STEC strains based on cgMLST method illustrated that STEC of the same serotype or ST were clustered together. However, STEC strains of the same O serogroup were located in the same phylogenetic clusters, isolates of the same H type,

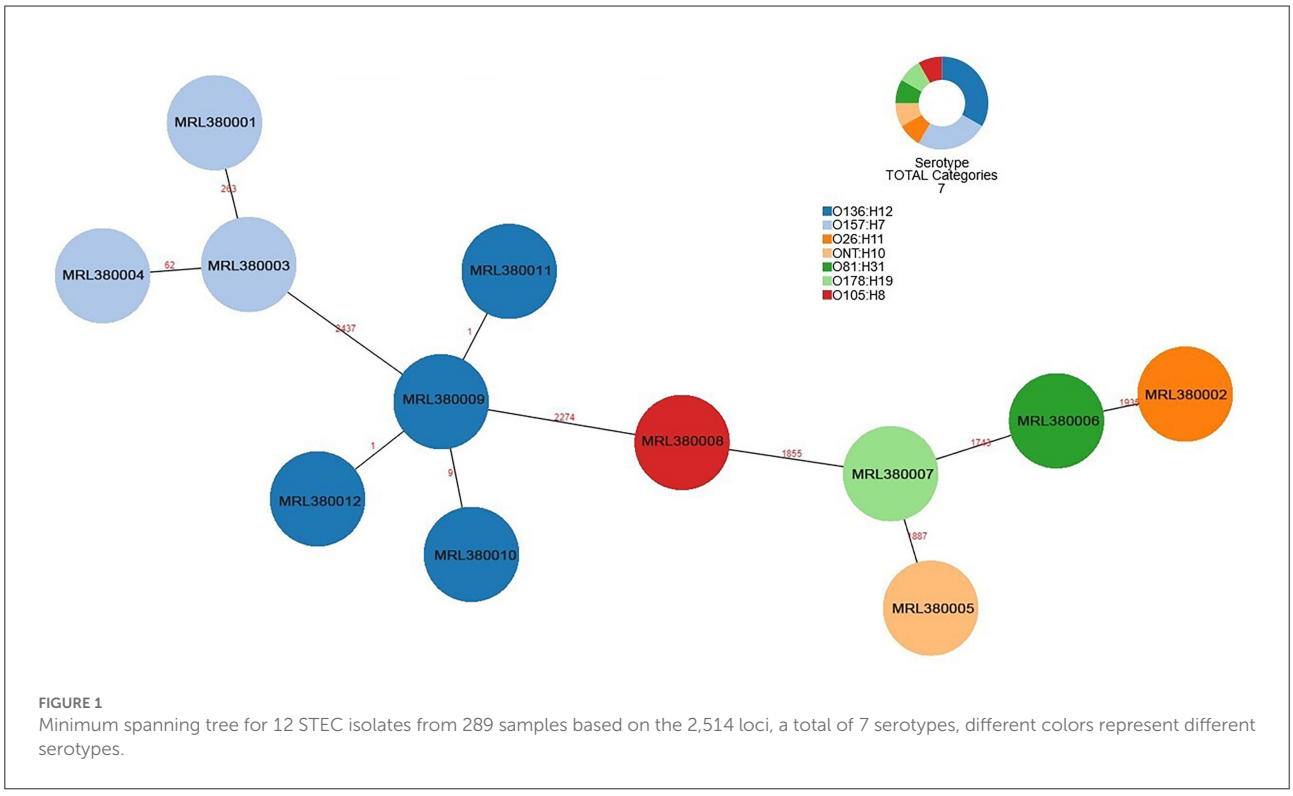


FIGURE 1
Minimum spanning tree for 12 STEC isolates from 289 samples based on the 2,514 loci, a total of 7 serotypes, different colors represent different serotypes.

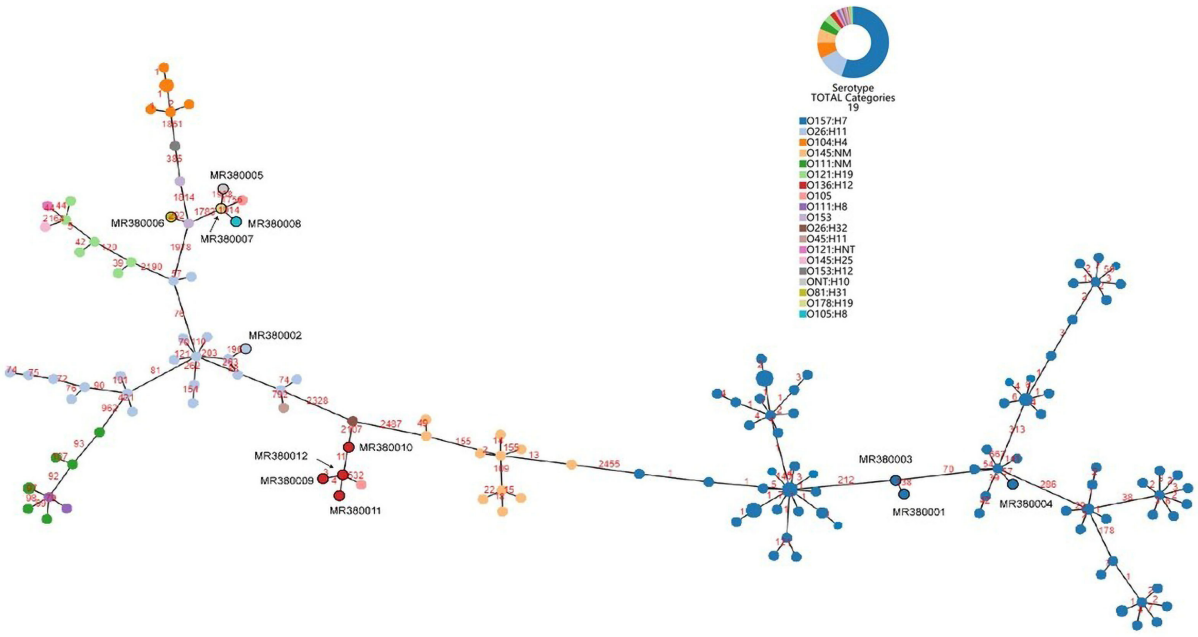


FIGURE 2
Minimum spanning tree for 12 STEC isolates from 289 samples and 157 STEC strains from NCBI based on the 2,586 loci, a total of 19 serotypes, different colors represent different serotypes.

irrespective of their O serotype may not share a common ancestor. This finding was not consistent with the results from cgMLST typing analysis revealing H serogroups were described as monophyletic, while O serogroups were described as polyphyletic (Ju et al., 2012; Steyert et al., 2012; Ferdous et al., 2016).

Several limitations existed in this study. Firstly, STEC isolation and identification methods should be further improved, because parasites in cow manure can cause false positives during the initial screening process (Ferdous et al., 2016). STEC strain false positives can be decreased but not entirely eliminated by choosing CHROMagar$^{TM}$ STEC plates, MacConkey agar, and sorbitol MacConkey agar. The limited local STEC isolates and lacking clinical isolates might have influenced the reported diversity.

## Conclusion

In conclusion, the results analyzing 12 isolates from food sources and feces of commercial animals, indicated a low prevalence of STEC in Shanghai, China. The capability of WGS providing rapid data for identification, serotyping, sequence typing, and virulence genes of STEC strains compared with traditional methods was further confirmed. STEC strains of the same O serogroup were located in the same phylogenetic clusters, isolates of the same H type, irrespective of their O serotype may not share a common ancestor by cgMLST. The study revealed that cgMLST typing method could be useful for outbreak investigations of STEC strains. In addition, the data stemmed from wide-ranging molecular characteristics with WGS resolution could be used as an effective approach for comparing with similar human, food, or animal isolates at the international level.

## Data availability statement

The data presented in the study are deposited in the Genome Sequence Archive (Genomics, Proteomics & Bioinformatics 2017) in National Genomics Data Center (Nucleic Acids Res 2021), accession number CRA007022.

## Author contributions

QD and HL conceived of the study and modified the first draft of the article. SZ is responsible for the experimental work, article writing, and data analysis. ZB and ZW isolated laboratory strains. XW and WW provided help with research ideas. All authors reviewed and approved the final manuscript.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.950065/full#supplementary-material

## References

Amézquita-López, B. A., Soto-Beltrán, B. G. L., and Jaszemyn, C. (2018). Isolation, genotyping and antimicrobial resistance of Shiga toxin-producing *Escherichia coli*. *J. Microbiol. Immunol.* 51, 425–434. doi: 10.1016/j.jmii.2017.07.004

Baha, M., Bouska, A. C. B., Zhang W., and Brianna L. S. H., (2019). Whole genome sequencing to characterize shiga toxin-producing *Escherichia coli* O26 in a public health setting. *J. Infect. Public Health* 12, 884–889. doi: 10.1016/j.jiph.2019.06.008

Baltasar, M., Caio, T. C. C., Raquel, S. P., and Susana, D. (2014). Impact of next generation sequencing techniques in food microbiology. *Curr. Genomics*. 15, 293–309. doi: 10.2174/1389202915666140616233211

Boer, R. F., Ferdous, M., Ott,. A., and Scheper, H. R. (2015). Assessing the public health risk of Shiga toxin-producing *Escherichia coli* by use of a rapid diagnosticscreening algorithm. *J. Clin. Microbiol.* 53, 1588–1598. doi: 10.1128/JCM.03590-14

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Chattaway, M. A., Dallman, T. J., Gentle, A., Michael, J. W., and Sophie, E. L., P. M. A. (2016). Whole genome sequencing for public health surveillance of shiga toxin-producing *Escherichia coli* other than serogroup O157. *Front. Microbiol.* 7, 251–264. doi: 10.3389/fmicb.2016.00258

Ferdous M, Friedrich AW, Grundmann H, de Boer RF, Croughs PD, Islam MA, et al. (2016). Molecular characterization and phylogeny of Shiga toxin–producing *Escherichia coli* isolates obtained from two Dutch regions using whole genome sequencing. *Clin. Microbiol. Infec.* 22, 1.e1–1.e9. doi: 10.1016/j.cmi.2016.03.028

Gonzalez, A. G. M., and Cerqueira, A. M. F. (2020). Shiga toxin-producing *Escherichia coli* in the animal reservoir and food in Brazil. *J. Appl. Microbiol.* 128, 1268–1282. doi: 10.1111/jam.14500

Gutierrez, S., Díaz, L., Reyes-Jara, A., Yang, X., Meng, J., González-Escalona, N., et al. (2021). Whole-genome phylogenetic analysis reveals a wide diversity of non-O157 STEC isolated from ground beef and cattle feces. *Front. Microbiol.* 11, 622663. doi: 10.3389/fmicb.2020.622663

Hazards, E. (2013). Scientific opinion on VTEC-seropathotype and scientific criteria regarding pathogenicity assessment. *EFSA J.* 11, 3138. doi: 10.2903/j.efsa.2013.3138

Heir, E., Lindstedt, B. A., Vardund, T., Wasteson, Y., and Kapperud, G. (2000). Genomic finger printing of shiga toxin-producing *Escherichia coli* (STEC) strains: comparison of Pulsed-field gel electrophoresis (PFGE) and fluorescent amplified-fragment-length polymorphism (FAFLP). *Epidemiol. Infect.* 125, 537–548. doi: 10.1017/S0950268800004908

Jagadesan, S., Udayakumar, S. V., Paramasamy, G., and Jeyaprakash, R. (2019). Development and evaluation of a core genome multilocus sequence typing (cgMLST) scheme for *Brucella* spp. *Infect. Genet. Evol.* 67, 38–43. doi: 10.1016/j.meegid.2018.10.021

Jaureguy, F., Landraud, L., Passet, V., Diancourt, L., Frapy, E., Guigon, G., et al. (2008). Phylogenetic and genomic divers-ity of human bacteremic *Escherichia coli* straints. *BMC Genomic.* 9, 550–564. doi: 10.1186/1471-2164-9-560

Jenkins, C., Dallman, T. J., and Grant, K. A. (2019). Impact of whole genome sequencing on the investigation of food-borne outbreaks of Shiga toxin-producing Escherichia coli serogroup O157:H7, England, 2013 to 2017. *Euro Surveill.* 24:1800346. doi: 10.2807/1560-7917.ES.2019.24.4.1800346

Joensen, K. G., Scheutz, F., Lund, O., Hasman, H., Nielsen, E. M., and Aaretrup, F. M. (2014). Real-time whole-genome sequencing for routine typing, surveillance, and outbreak detection of verotoxigenic *Escherichia coli*. *J. Clin. Microbiol.* 52, 1501–1510. doi: 10.1128/JCM.03617-13

Joensen, K. G., Tetzschner, A. M., Iguchi, A., Aarestrup, F. M., and Scheutz, F. (2015). Rapid and easy *in silico* serotyping of *Escherichia coli* isolates by use of whole genome sequencing data. *J. Clin. Microbiol.* 53, 2410–2426. doi: 10.1128/JCM.00008-15

John, B. K., Suereta, F., Bridget, C., and Lourens, R. (2018). Proteomic comparison of three clinical diarrhoeagenic drug-resistant *Escherichia coli* isolates grown on CHROMagar™ STEC media. *J. Proteomics*. 180, 25–35. doi: 10.1016/j.jprot.2017.09.003

Ju, W. T., Cao, G., Timme, R., Errol, S., Yan, L., and Meng, J. L. (2012). Phylogenetic analysis of non-O157 Shiga toxin-producing *Escherichia coli* strains by whole-genome sequencing. *J. Clin. Microbiol.* 50, 4123–4127. doi: 10.1128/JCM.02262-12

Karmali, M. A., Mascarenhas, M., Shen, S., Ziebell, K., Clarl, C., Rahn, K., et al. (2003). Association of genomic O island 122 of *Escherichia coli* EDL 933 with verocytotoxin-producing *Escherichiacoli* seropathotypes that are linked to epidemic and/or serious disease. *J. Clin. Microbiol.* 41, 4930–4940. doi: 10.1128/JCM.41.11.4930-4940.2003

Keoser, C. U., Ellington, M. J., Gillespie, S. H., Brown, N. M., and Farrington, M. (2012). Routine use of microbial whole genome sequencing in diagnostic and public he-alth microbiology. *PLoS Pathog.* 8, e1002824. doi: 10.1371/journal.ppat.1002824

Leung, P. H., Yam, W. C., and Ng, W. W. (2001). The prevalence and characterization of verotoxin-producing *Escherichia coli* isolated from cattle and pigs in an abattoir in Hong Kong. *Epidemiol. Infect.* 126, 173–179. doi: 10.1017/S0950268801005210

Li, B., Yunchang, G., Ruiting, L., Yingping, D., Wei, W., Yujie, H., et al. (2015). Genotypic characterization of Shiga toxin-producing *Escherichia coli* O157:H7 isolates in food products from China between 2005 and 2010. *Food Control* 50, 209–214. doi: 10.1016/j.foodcont.2014.08.045

Li, R., Xiao, T., Xiao, J., Hongxun, W., Zhiguo, L., Min, Z., et al. (2016). Molecular screening and characterization of Shiga toxin-producing *Escherichia coli* in retail foods. *Food Control* 60, 180–188. doi: 10.1016/j.foodcont.2015.07.045

Mathusa, E. C., Chen, Y., Enache, E., and Lloyd, H. (2010). Non-O157 Shiga toxin-producing *Escherichia coli* in foods. *J. Food Protect.* 73, 1721–1736. doi: 10.4315/0362-028X-73.9.1721

Michaela, P., Marina, C. L., Petra, G., Andr,é, G., Sandra, C., Lorenz, W., et al. (2021). Genetic diversity and pathogenic potential of Shiga toxin-producing *Escherichia coli* (STEC) derived from German flour. *Int. J. Food Microbiol.* 347, 109197. doi: 10.1016/j.ijfoodmicro.2021.109197

Mikhail, A. F. W., Jenkins, C., Dallman, T. J., Inns, T., Douglas, A., Martin, A. I. C., et al. (2018). An outbreak of Shiga toxin-producing *Escherichia coli* O157:H7 associated with contaminated salad leaves: epidemiological, genomic and food trace back investigations. *Epidemiol. Infect.* 146, 87–196. doi: 10.1017/S0950268817002874

Mohammad, M., Masoumeh, D., Abbas, R. F., and Rahimeh, S. (2021). Molecular characterization and prevalence of virulence factor genes of Shiga toxin-producing *Escherichia coli* (STEC) isolated from diarrheic children. *Gene Rep.* 25, 101379. doi: 10.1016/j.genrep.2021.101379

Monaghan, Á., Byrne, B., Fanning, S., Sweeney, T., McDowell, D., and Bolton, D. J. (2012). Serotypes and virulotypes of non-O157 Shiga-toxin producing *Escherichia coli* (STEC) on bovine hides and carcasses. *Food Microbiol.* 32, 223–229. doi: 10.1016/j.fm.2012.06.002

Parsons, B., Zelyas, N., Berenger, B., and Chui, L. (2016). Detection, characterization, and typing of Shiga toxin-producing *Escherichia coli*. *Front. Microbiol.* 07, 478–490. doi: 10.3389/fmicb.2016.00478

Shifu, C., Yanqing, Z., Jia, G., and Yaru, C. (2018). fastp: an ultra-fast all-in-one FASTQ pre-processor. *Bioinformatics* 34, 884–890. doi: 10.1093/bioinformatics/bty560

Smith, J. L., Fratamico, P. M., and Gunther, N. (2014). Shiga toxin-producing *Escherichia coli*. *Adv. Appl. Microbiol.* 10, 145–197. doi: 10.1016/B978-0-12-800262-9.00003-2

Steyert, S. R., Sahl, J. W., Fraser, C. M., Fraser, C. M., Teel, L. D., Scheutz, F., et al. (2012). Comparative genomics and stx phage characterization of LEE-negative Shiga toxin-producing *Escherichia coli*. *Front. Cell. Infect. Microbiol.* 2, 133. doi: 10.3389/fcimb.2012.00133

Wang, P., Xiong, Y., Lan, R. T., Changyun, Y., Hua, W., Ren, J., et al. (2011). pO157_Sal, a novel conjugative plasmid detected in outbreak isolates of *Escherichia coli* O157: H7. *J. Clin. Microbiol.* 49, 1594–1597. doi: 10.1128/JCM.02530-10

Xiong, Y., Wang, P., Lan, R., Changyun, Y., Hua, W., Ren, J., et al. (2012). A novel *Escherichia coli* O157:H7 clone causing a major hemolytic uremic syndrome outbreak in China. *PLoS ONE.* 7, e36144. doi: 10.1371/journal.pone.0036144

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# First report on the metabolic characterization of Sterigmatocystin production by select *Aspergillus* species from the *Nidulantes* section in *Foeniculum vulgare*

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Spices are typically grown in climates that support the growth of toxigenic fungi and the production of mycotoxins. The *Aspergilli* described in this study, as well as the sterigmatocystin (STC) detected, are causes for concern due to their potential to induce food poisoning. One of the most well-known producers of the carcinogenic STC is *Aspergillus nidulans*. This research explores the occurrence of STC-producing fungi in *Foeniculum vulgare*, a spice that is marketed in India and other parts of the world. This innovative study details the mycotoxigenic potential of five *Aspergilli* belonging to Section *Nidulantes*, namely *Aspergillus latus* (02 isolates), *Emericella quadrilineata* (02 isolates), and *Aspergillus nidulans* (01 isolate), with respect to STC contamination. These five isolates of *Aspergilli* were screened to produce STC on yeast extract sucrose (YES) medium in a controlled environment with regard to light, temperature, pH, and humidity, among other variables. The expression patterns of regulatory genes, namely, *aflR*, *laeA*, *pacC*, *fluG*, *flbA*, *pksA*, and *mtfA* were studied on the Czapek–Dox agar (CDA) medium. STC biosynthesis by the test isolates was done in potato dextrose broth (PDB) under optimum conditions, followed by the extraction and purification of the broth using ethyl acetate. High-performance liquid chromatography (HPLC) with an ultraviolet (UV) detector was utilized to detect compounds in eluted samples. *F. vulgare* contains *Aspergilli* that have been shown to have mycotoxigenic potential, which can accumulate in the spice during its active growth and thereby cause the elaboration of mycotoxins.

KEYWORDS

***Aspergillus*, sterigmatocystin (STC), food spoilage, mycotoxigenic, regulatory genes, HPLC**

## Introduction

*Aspergillus* is a genus of filamentous fungi that has grown to comprise more than 250 different mold species (Houbraken and Samson, 2011) across the world since it was first described approximately 300 years ago. Some species of these mitosporic, conidial fungi reveal teleomorphic forms and are hence classified as part of the phylum Ascomycota (Bennett, 2010). *Aspergillus* species are extensively employed by the medical (Bladt et al., 2013) and industrial sectors (Polizeli et al., 2016) for the applications of their beneficial secondary metabolites. However, certain *Aspergillus* species are pathogenic to humans (Alshehri and Palanisamy, 2020), animals, and birds (Arné et al., 2021) and are also prolific producers of secondary metabolites known as mycotoxins (Navale et al., 2021).

As the name suggests, mycotoxins are highly toxic and produced predominantly by fungi such as *Aspergillus*, *Penicillium*, and *Fusarium* (Liew and Mohd-Redzwan, 2018). They are frequently discovered in various foods or feedstuffs, thereby posing a significant risk to human and animal health, as well as economic losses caused by STC contamination (Cardwell et al., 2001). This is a major food safety issue for underdeveloped countries, where detection, monitoring, and regulating procedures are often lacking to protect the food supply from mycotoxins. The genes required for secondary metabolite production in fungi are arranged in gene clusters that encode key enzymes (Keller et al., 2005), such as polyketide synthases, non-ribosomal polypeptide synthetases, terpene cyclases, dehydrogenases, esterases, and methyltransferases (Brakhage, 2013). Though gene clusters are of enormous biological and economic importance, information about their structure, function, and regulation remains unclear.

The flowering plant *Foeniculum vulgare* Mill, often known as fennel or saunf in Hindi, is a member of the Umbelliferae (Apiaceae) family. It grows in the United States, Northern Europe, Southern Canada, Asia, and Australia. It is a good source of protein, fiber, vitamin A, thiamin, vitamin C, and minerals, namely, calcium, iron, magnesium, manganese, and fatty acids. Essential oils from fennel are utilized in beverages, bread, pickles, pastries, and cheese. Well known is their antioxidant, cryoprotective, estrogenic, hyperglycemic, and hyper-protective properties (Javidnia et al., 2003; Samadi-Noshahr et al., 2021). In addition, they contain anti-inflammatory and anti-tumor properties (Malini et al., 1985) and are used to treat bacterial, viral, fungal, mycotic, and protozoan infections (Rather et al., 2016). Furthermore, there is evidence that fennel may offer protection against cardiovascular disease (Osman et al., 2017).

The annual fennel production in India was 58,265 tons in 2010–2011 (Abubacker, 2011) and has increased to 139,760 tons during 2019–2020 (Anonymous, 2020a), indicating that consumption is soaring. Fennel is grown in India and exported to other nations. In 2018 and 2020, the range of exports was between 2,35,62,460 and 2,02,95,380 million metric tons (Anonymous, 2020b). The Ministry of Food Processing estimates that poor scientific conditions, unhygienic harvesting practices, and post-harvest procedures cost 93,000 crores of Indian rupees (INR) in economic losses, of which fennel is one (Moloney, 2019).

*F. vulgare*, like the majority of cereals and grains, is susceptible to infection by a variety of mycoflora, some of which can severely reduce the crop's economic worth. However, fungi from the *Aspergillus* and *Penicillium* genera can thrive in low humidity and cause significant damage to crops. Infections caused by *Aspergillus* spp. and several other species have been of particular concern in recent years, as all these fungal phytopathogens release hazardous mycotoxins that cause substantial contamination of food (Navale et al., 2021), feed (Variane et al., 2018), and agricultural goods, namely, spices (Garcia et al., 2018). Spices (e.g., fennel seeds) serve as an important ingredient in Indian cuisine (Siruguri and Bhat, 2015). Toxic secondary metabolites produced by contaminating mycoflora have a negative influence on their quality.

Aflatoxins (AFs) are the most extensively researched mycotoxins, and are primarily synthesized by *Aspergillus flavus* and *Aspergillus parasiticus*. It has been shown to be harmful to fetuses (Lauer et al., 2019), possibly teratogenic, and the most carcinogenic substance in the world, according to the International Agency for Research on Cancer (IARC). STC is a highly mutagenic chemical that, in substantial doses, is also a recognized liver carcinogen. The IARC classified STC as a group 2B carcinogen, indicating that it may cause cancer in people. No country has defined a maximum permissible level for it because of its infrequent natural occurrence, even though it is exceedingly hazardous.

Studies on the model organism *Aspergillus nidulans*, which produces the precursor of aflatoxin, namely STC, have led to a greater understanding of the metabolic pathways involved in the formation of AF (Brown et al., 1996). *A. nidulans* reproduces predominantly through the formation of asexual spores called conidia and produces the mycotoxin STC, the penultimate precursor in the AF biosynthetic process, which is found in related organisms like *A. parasiticus*, *A. flavus*, and *Aspergillus nomius* (Cole and Cox, 1981). Both polyketides are known for causing mammalian hepatocarcinoma (Bressac et al., 1991), animal toxicity (Anderson et al., 1990; Balogh et al., 2019) and are believed to be immune impairing to infants as well as the aged (Cardwell and Miller, 1996). It has been proposed that the STC potentially plays a part in the development of chronic liver disease in human beings in Africa (Cole et al., 2003). The toxic effect of STC is modest, but the primary issue is that it can cause cancer; the carcinogenicity is approximately one-tenth that of AFB1, which

is a significant difference. STC structure is similar to AFs, in that it is made up of a xanthone nucleus that is connected to a bisfuran ring structure. STC is soluble in acrylonitrile, benzene, chloroform, ethyl acetate, and acetone. However, it is insoluble in water and petroleum ether. Many *Aspergillus* species, namely, *A. versicolor*, *A. nidulans*, *A. sydowii*, and various species of *Bipolaris*, generate STC. Mycotoxin gene clusters from *A. nidulans* and *A. parasiticus* exhibit a high degree of homology (Yu et al., 2004), which aids in the study of AF/STC synthesis and regulation measures. In *A. nidulans*, the expression of STC is controlled by an intricate network of genes that are clustered together on chromosome IV around a 60 kb DNA region. The cluster contains 25 genes that are involved in the intricate processes necessary for the STC pathways to function properly (Brown et al., 1996).

*Aspergillus* diversity, phylogeny, and mycotoxin-producing potential in *F. vulgare* have not been previously reported by researchers. In a recent research article (Mahata et al., 2022), a detailed morphological identification of *Aspergilli*, supported by molecular barcoding using ITS 1 and ITS 4 as well as β-tubulin as genetic markers (Tam et al., 2014), has been published. Accurate identification of *Aspergilli* has bridged the information gap on misidentification and determining the STC-producing potential, to recognize the significant danger associated with the consumption of toxin-laden food and feed in humans and animals, respectively.

This study aims to provide knowledge on the prevalence of *Aspergillus* species producing STC, associated with fennel. *Aspergilli* were examined on a mycological medium to identify STC-producing isolates in the preliminary screening procedure. The identification of genes involved in the synthesis of toxins and the purification of mycotoxin in the laboratory allowed us to assess the potential toxigenicity of these species. As a result, this article includes an inventory of potentially STC-producing *Aspergillus* species belonging to the *Nidulantes* Section of the *Aspergillus* taxonomy.

# Materials and methods

## Isolates of fungal species

In our analyses (Mahata et al., 2022), we procured 70 samples of an Indian spice, *F. vulgare* from several retail marketplaces around India, namely, New Delhi, Lucknow, Pinjore, West Midnapore, and Puducherry. The mycofloral tests of Indian fennel samples were carried out using standard agar plating techniques defined by the International Seed Testing Association (ISTA). *Aspergillus* isolates were screened by plating *F. vulgare* spice samples on mycological media that included potato dextrose agar (PDA) and Czapek–Dox agar (CDA) (HiMedia®, Mumbai, India) by adopting techniques described by researchers (Xie et al., 2007; Hamzah et al., 2018). All the fungal flora of fennel samples were isolated and identified using standard fungal manuals and keys (Thom and Raper, 1945; Refai et al., 2014). A single-point or three-point inoculation technique was used to inoculate the isolates into glass Petri plates (100 × 15 mm, S-line, Borosil®, Mumbai, India). After 7 days of incubation, colony sizes, coloration, textures, spore germination pattern, obverse and reverse colonial color schemes, and the presence or absence of ascomata (in older cultures kept for 15–25 days) were observed. Besides this, the study of hyphal features such as septation, vesicle morphology, conidiophore architecture, sterigmata arrangement, and conidia development received special attention. These microscopic characteristics of individual *Aspergilli* were studied consecutively using light microscopy (Olympus CH20i), scanning electron microscopy (SE; Hitachi, Model E-1010), and differential interference contrast (DIC; Nikon Upright Motorized Microscope, ECLIPSE Ni series, Nikon Corporation, Tokyo) microscopy. By examining microscopic characteristics as described in identification keys and manuals, standard protocols were adopted (Thom and Raper, 1945; Varga and Samson, 2008). Light and Scanning electron micrographs (SEM) were acquired at the Fungal Genetics and Mycotoxicology Laboratory, Department of Microbiology and Central Instrumentation Facility (CIF), Pondicherry University, while DIC micrographs were documented at the Fungal Biotechnology Laboratory, Department of Biotechnology, School of Life Sciences, Pondicherry University.

Isolates of *A. nidulans* (**Figure 1**), *E. quadrilineata* (**Figure 2**), and *A. latus* (**Figure 3**) were subjected to in-depth research pertaining to the properties of their macromorphological and micromorphological structures as required in fungal taxonomic studies. Fungal stock cultures were prepared and maintained at −20°C. These macromorphological features include 7-day colony development, coloration, and textures on PDA and CDA media. Micromorphological considerations, moreover, included microscopic analysis of asexual and sexual phases of the *Aspergillus* life cycle.

The five *Aspergillus* isolates (**Table 1**) included in this study (*A. nidulans*-01 isolate; *E. quadrilineata*-02 isolates; *A. latus*-02 isolates) were isolated and identified by morphological and molecular methods using the internal transcribed spacer (ITS) and β-tubulin marker genes. The isolates inspected were from one of our previously published research studies (Mahata et al., 2022). Additionally, cultural features of *E. quadrilineata* and *A. latus* on the Czapek–Dox agar medium, as well as microscopic images of *A. nidulans*, *E. quadrilineata*, and

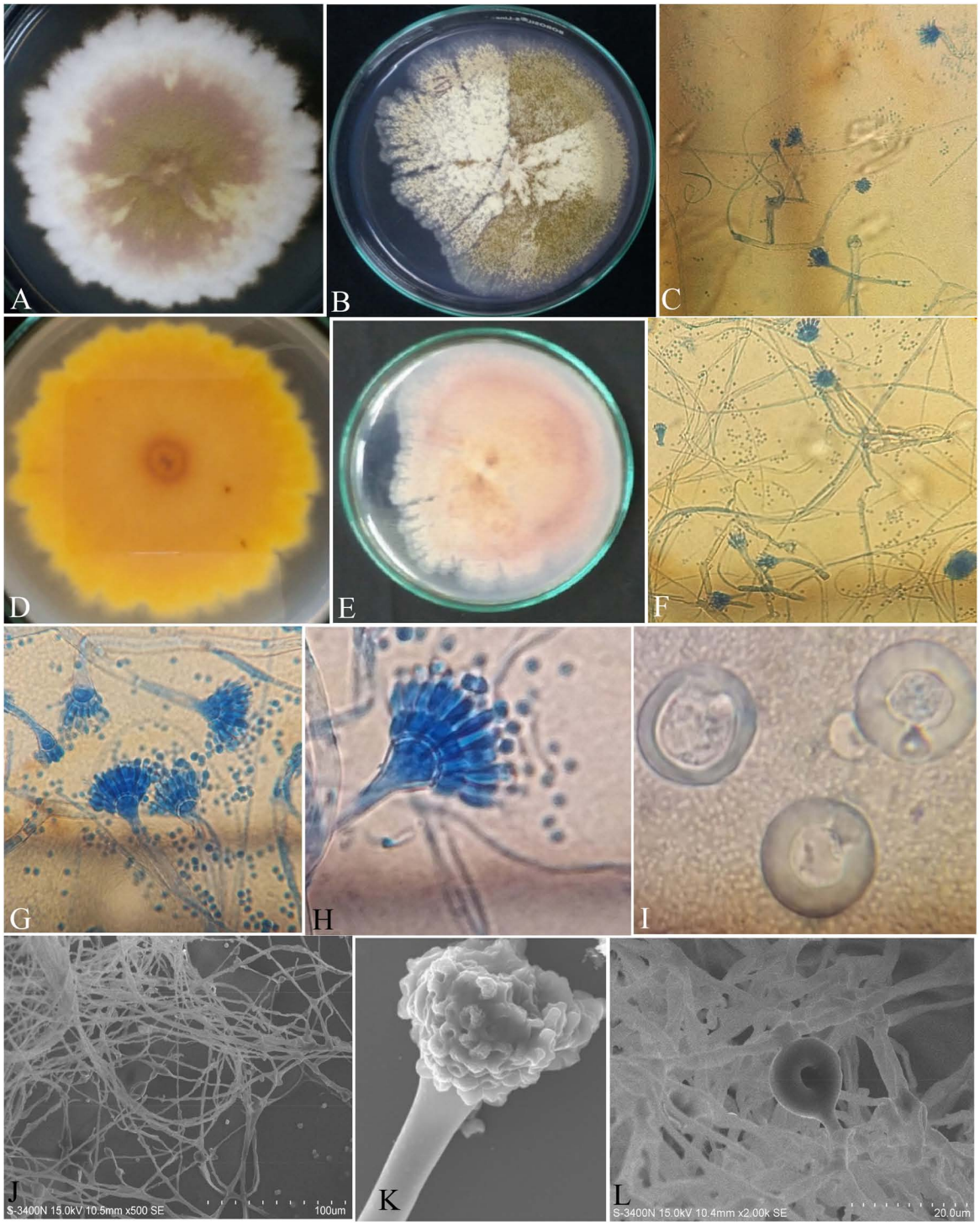


FIGURE 1
*(Continued)*

*A. latus*, have been shown to aid in the morphological identification process.

The ITS gene represents the Internal Transcribed Spacer.

FGM Laboratory-Fungal Genetics and Mycotoxicology Laboratory; Pranab Kumar Mahata and Regina Sharmila Dass identified, cultivated, and analyzed all fungal isolates.

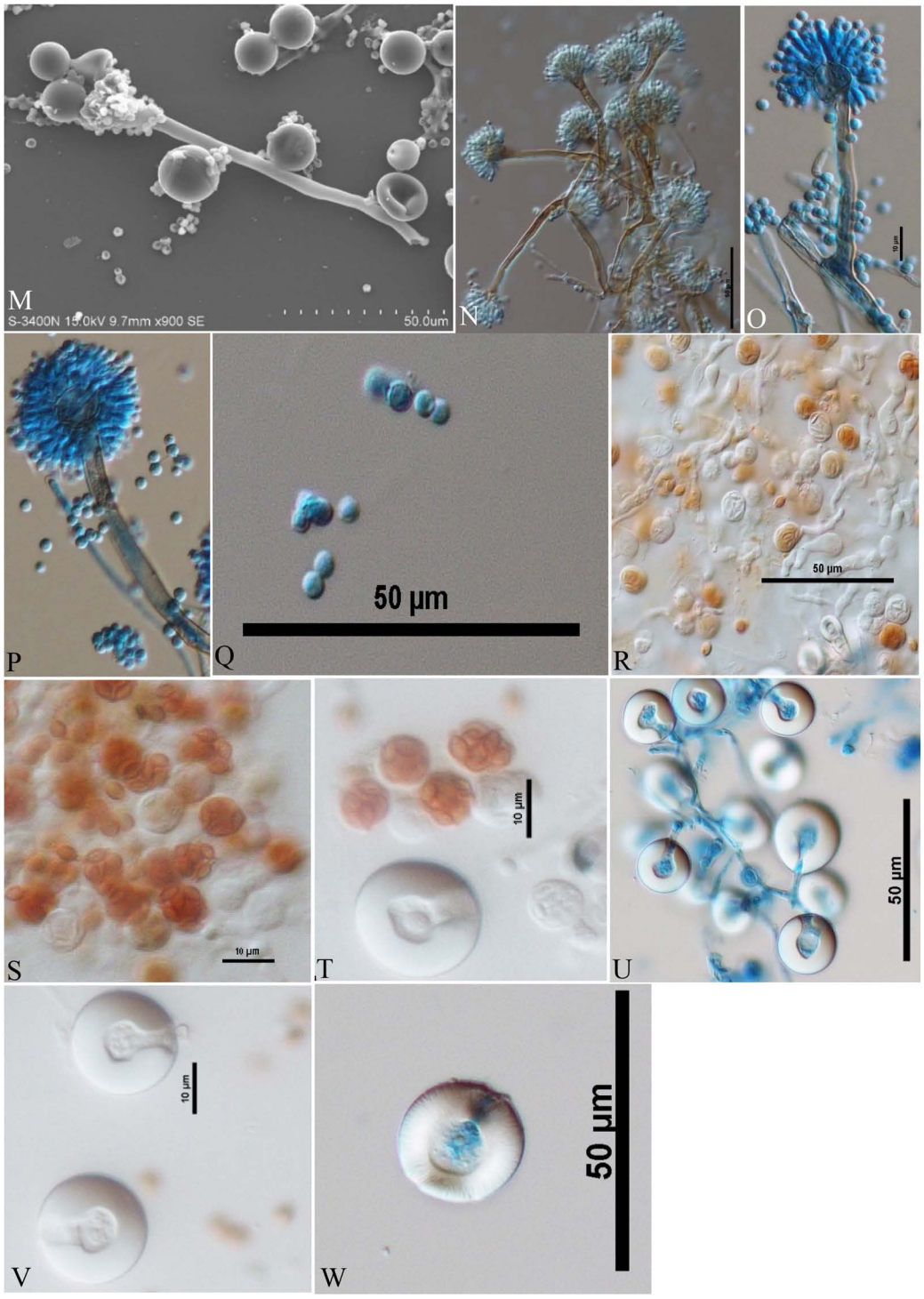


FIGURE 1
*Aspergillus nidulans* (Acc. No. MN791101) colonies, conidiophores, vesicles, metulae, phialides, conidia, asci and Hülle cells. **(A,B)** (Obverse) Colonies cultured at 25°C on PDA and CDA, respectively; **(C)** (CLM) hyphae and conidiophores; **(D,E)** (Reverse) colonies on PDA and CDA, respectively; **(F,G)** (CLM) magnified view of conidiophores in development; **(H)** (CLM) conidiophore enlarged; **(I)** (CLM) Hülle cells; **(J)** (SEM) mycelium, conidiophores and developing spores; **(K)** (SEM) young developing conidial head; **(L)** (SEM) mycelium and Hülle cell development; **(M)** (SEM) Hülle cells; **(N–P)** (DIC) smooth walled, sinuate conidiophores and columnar conidial heads with small, hemispherical vesicles, metulae, phialides and conidia; **(Q)** (DIC) rough, globose conidia; **(R–T)** (DIC) asci; and **(U–W)** (DIC) Hülle cells.—Scale bars: **(J)** = 100 µm; **(M,N,P–R,U,W)** = 50 µm; **(K,L)** = 20 µm; **(O,S,T,V)** = 10 µm. **(A–D,H,I,K–M,P,Q,T,W)** were published in the first and correspondence authors' recent research article (Mahata et al., 2022).

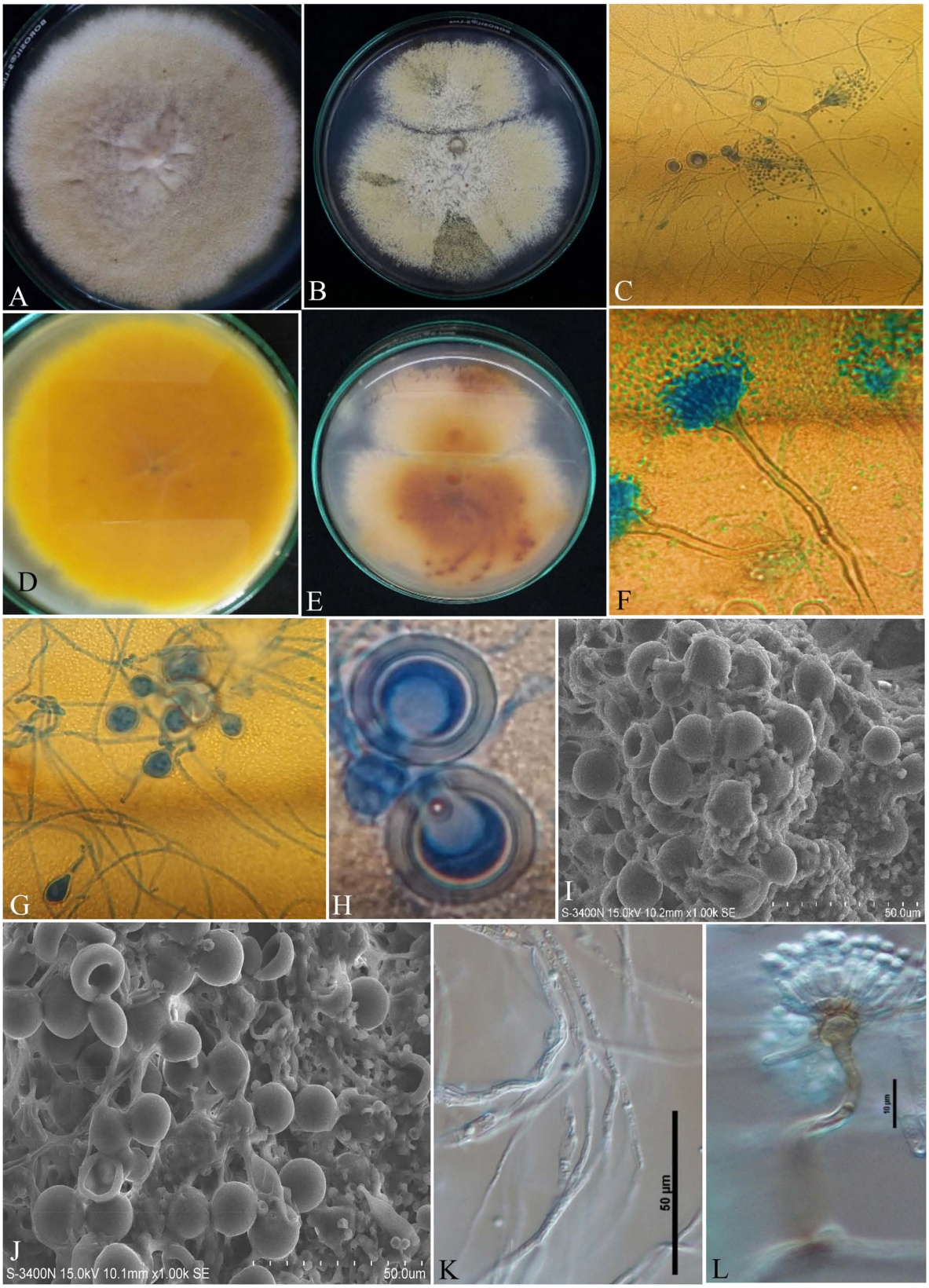


FIGURE 2
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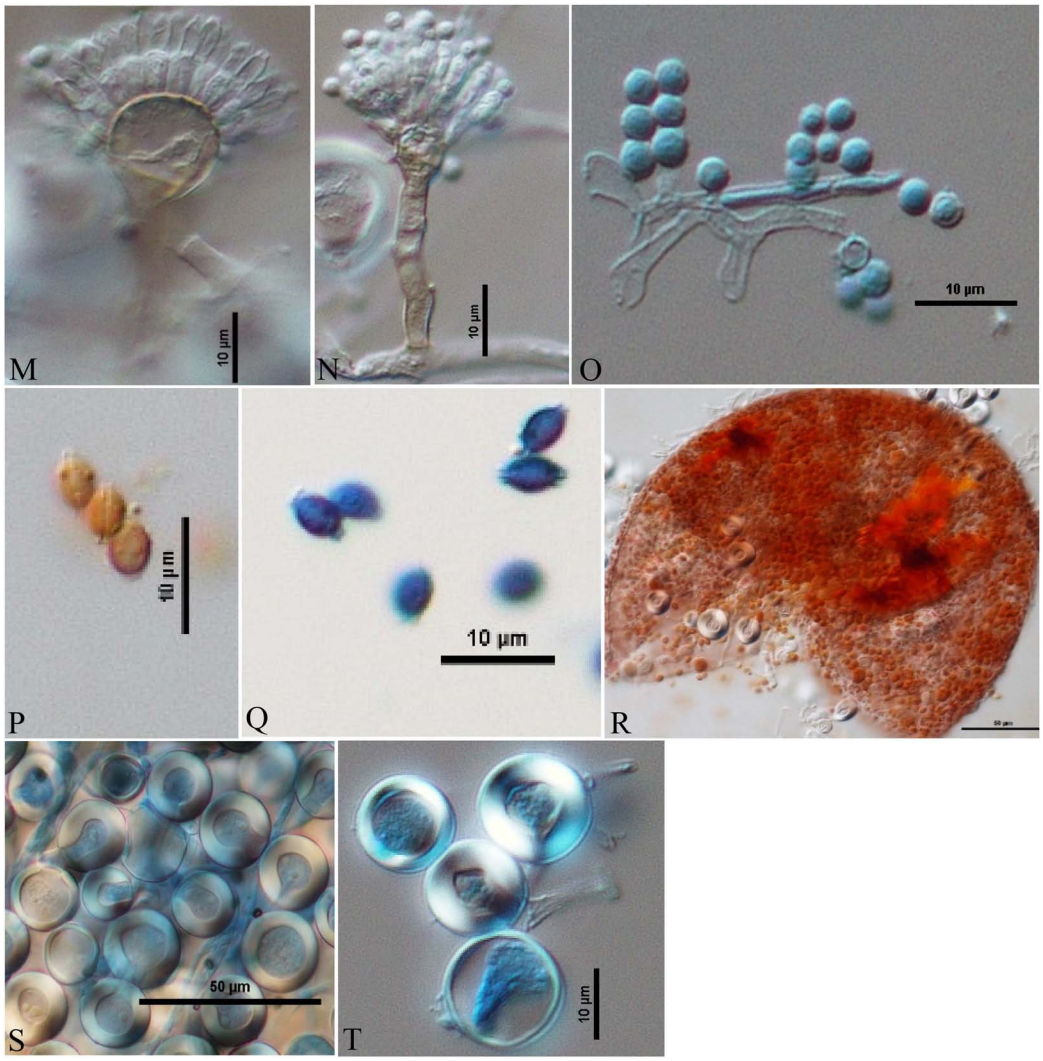


FIGURE 2
*Aspergillus quadrilineatus/Emericella quadrilineata* (Acc. No. MN791105) colonies, conidiophores, vesicles, metulae, phialides, conidia, cleistothecia, ascospores and Hülle cells. **(A,B)** (Obverse) Colonies were cultured at a temperature of 25°C on PDA and CDA; **(C)** (CLM) conidiophores and development of spores; **(D,E)** (Reverse) colonies on PDA and CDA, respectively; **(F)** (CLM) conidiophores; **(G,H)** (CLM) the formation of Hülle cells and Hülle cells; **(I,J)** (SEM) formation of Hülle cells; **(K)** (DIC) mycelium growth; **(L,M)** (DIC) conidiophores with smooth, sinuate margins and short, columnar conidial heads with hemispherical vesicles, metulae, phialides, and conidia; **(O)** (DIC) conidia; **(Q)**, (DIC) ascospores having lenticular, smooth-walled ascospores; **(P)** (DIC) smooth-walled lenticular (unstained with cotton-blue) ascospores; **(R)** (DIC) cleistothecium ruptured; and **(S,T)** (DIC) Hülle cells.—Scale bars: **(I–K,R,S)** = 50 µm; **(L–Q,T)** = 10 µm. **(A,D,F,H,J,M,O,P,R–T)** Were published in the first and correspondence authors' recent research article (Mahata et al., 2022).

## Screening for secondary metabolites in *Aspergillus nidulans, Emericella quadrilineata,* and *Aspergillus latus*

### Media

In the preliminary, each *Aspergillus* isolate was inoculated in yeast extract sucrose (YES) (HiMedia®, Mumbai, India) agar culture medium, and a secondary metabolite production assay for STC was performed.

### Cultivation and fluorescence detection

All of the *Aspergillus* isolates that had been evaluated for STC production were cultured in YES medium, incubated for the appropriate incubation time, and assessed by UV fluorescence detection (Fente et al., 2001) to confirm the presence of STCs. The experiment was carried out by adding 2% β-cyclodextrin (HiMedia®, Mumbai, India) to the medium. The plates were incubated at 28°C for 4 days. Upon examination using ultraviolet radiation (365 nm), the development of fluorescence in the agar medium around the *Aspergillus* colonies

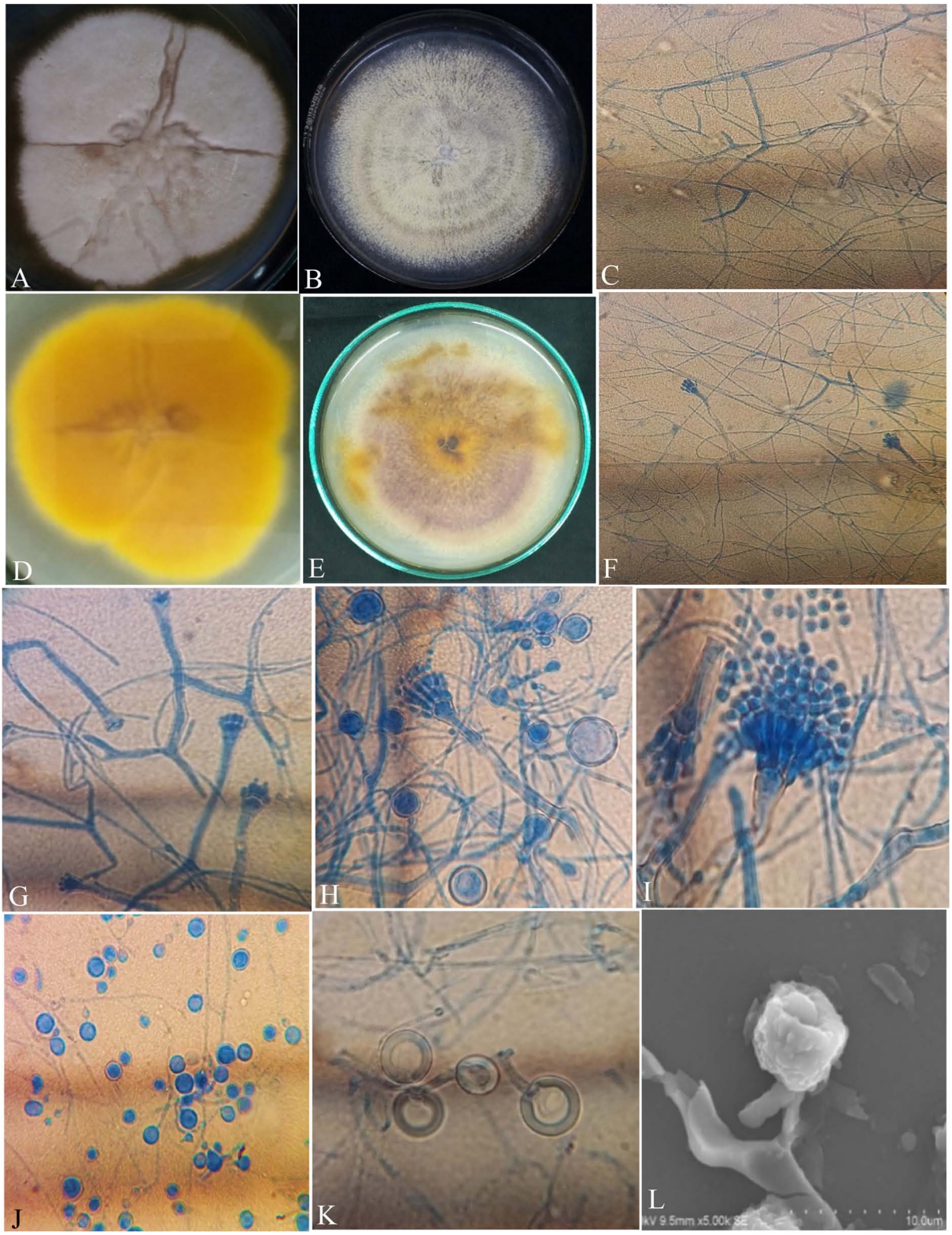


FIGURE 3
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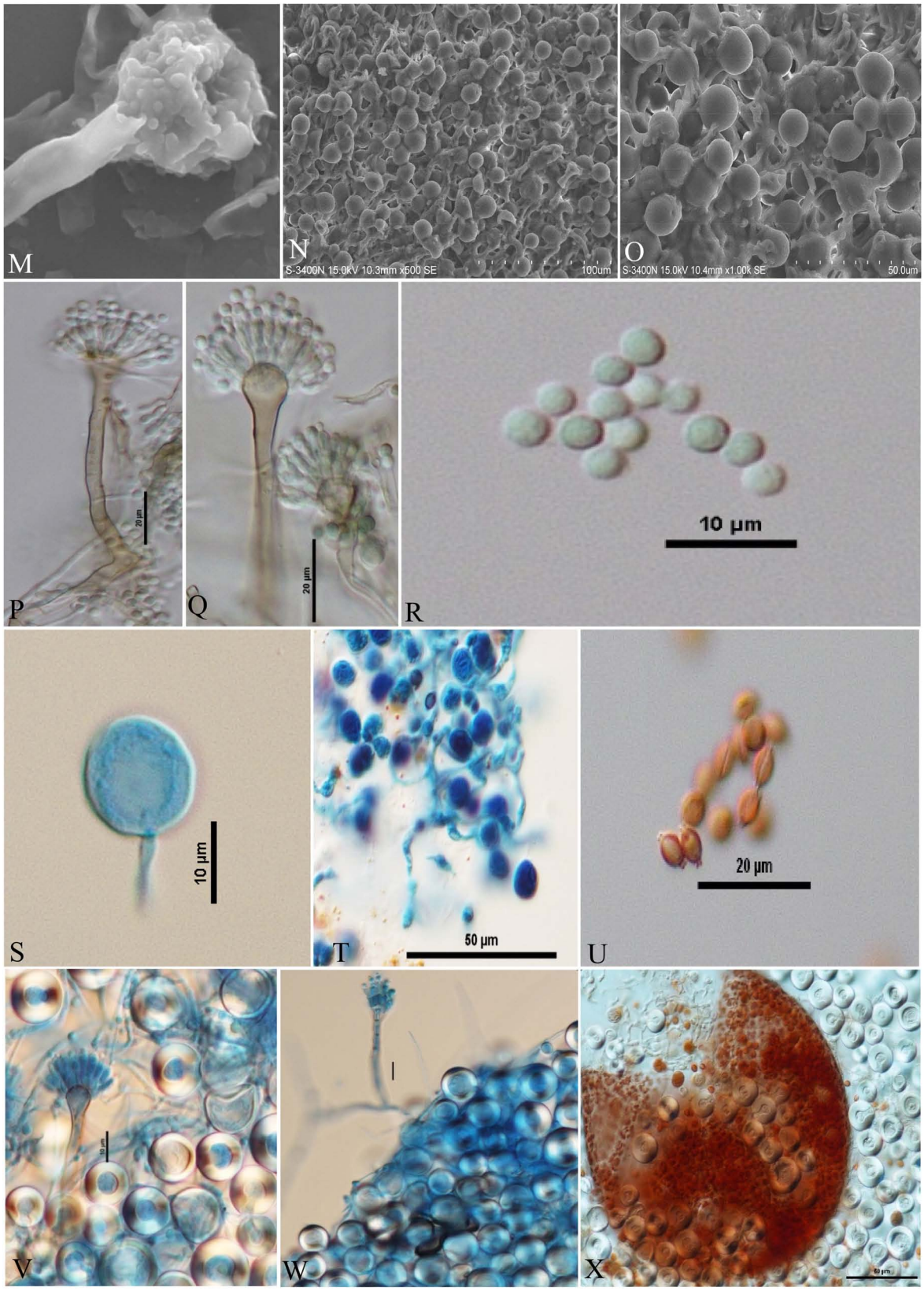


FIGURE 3
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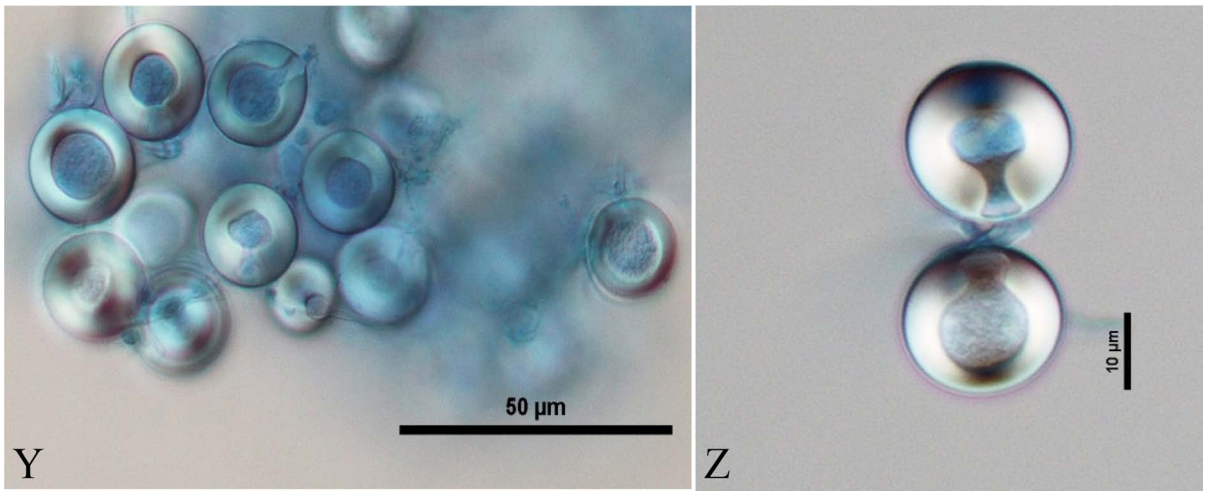


FIGURE 3
*Aspergillus latus* (Acc. No. MN791110) colonies, conidiophores, vesicles, metulae, phialides, conidia, cleistothecia, asci, ascospores and Hülle cells. **(A,B)** (Obverse) Colonies cultured at 25°C on PDA and CDA, respectively; **(C)** (CLM) hyphae and magnified view of conidiophores in development; **(D,E)** (Reverse) colonies on PDA and CDA, respectively; **(F–H)** (CLM) the growth of conidiophores and spores; **(I)** (CLM) conidiophore; **(J,K)** (CLM) Hülle cells; **(L,M)** (SEM) conidiophores; **(N,O)** (SEM) Hülle cells; **(P,Q)** (DIC) smooth walled, sinuate conidiophores and columnar conidial heads with small, hemispherical vesicles, metulae, phialides and conidia; **(R,S)** (DIC) conidia with a globose appearance; **(T)** (DIC) asci bearing ascospores; **(U)** (DIC) ascospores lenticulares; **(V,W)** (DIC) Hülle cells; **(X)** (DIC) cleistothecium ruptured, having asci, and enclosed by Hülle cells; and **(Y,Z)** (DIC) Hülle cells.—Scale bars: **(L–N)** = 100 µm; **(O,T,X,Y)** = 50 µm; **(P,Q,U)** = 20 µm; **(L,R,S,V,W,Z)** = 10 µm. **(A,D,I,K,M,Q,R,T,U,V,X,Z)** Were published in the first and correspondence authors' recent research article (Mahata et al., 2022).

TABLE 1 *Aspergilli* used in this study, ITS and β-tubulin gene sequences GenBank accession numbers.

| Sl. No. | FGM lab isolate code | Genus/species | Accession number (ITS) | Accession number (β-tubulin) |
|---|---|---|---|---|
| 1 | 45 | *A. nidulans* | MN309877 | MN791101 |
| 2 | 49 | *E. quadrilineata* | – | MN791104 |
| 3 | 58 | *E. quadrilineata* | – | MN791105 |
| 4 | 4 | *A. latus* | – | MN791110 |
| 5 | 11 | *A. latus* | – | MN791111 |

TABLE 2 Primers sequences used.

| Sl. No. | Gene | Primer sequence (5′–3′) | Amplicon size (bp) | References |
|---|---|---|---|---|
| 1 | *aflR* | F: GCCATCCTGTCTCCGAATAC<br>R: CGAACCTCTACGACTGTCTTG | 450 | Delgado-Virgen and Guzman-de-Peña, 2009 |
| 2 | *laeA* | F: GGTGACGATTTGTATAGTCC<br>R: CTCTTCATGAAACTGGTTTC | 200 | Delgado-Virgen and Guzman-de-Peña, 2009 |
| 3 | *pacC* | F: GACTGACGGTATGACTTCTG<br>R: GTTGGCAATGTAGTTACGTA | 250 | Delgado-Virgen and Guzman-de-Peña, 2009 |
| 4 | *fluG* | F: ACCCTAATGTTTATTTGGAT<br>R: TGGATAGGTCTGGTATAAGG | 250 | Delgado-Virgen and Guzman-de-Peña, 2009 |
| 5 | *flbA* | F: TCCCTCAAATTCTCTCAATCGAACCGG<br>R: GTAGAATGACAGGTTTTCTTCGCAGA | 250 | Delgado-Virgen and Guzman-de-Peña, 2009 |
| 6 | *pksA* | F: TTCTGCATGGGTTCCTTGGC<br>R: CCATTGTGGGCCGGTAAACA | 300 | Leema et al., 2011 |
| 7 | *mtfA* | F: GCCCTCACCCTCATCGGCAATG<br>R: GGTCGTGGTTCTGCTGGTAGGGTGT | 250 | Ramamoorthy et al., 2013 |

was indicative of a preliminary positive result. All experiments were carried out in triplicates. For the demonstration of their STC-producing abilities, all isolates were grown in the CDA medium to determine their gene expression profiles.

## RNA-Seq and gene expression analysis

### Conditions of growth

Each *Aspergillus* isolate was cultivated using the CDA medium designed for STC-induction for a set period of time, typically 7 days.

### RNA total extraction

High-quality RNA was isolated using the Qiagen RNeasy kit (QIAGEN India Pvt. Ltd., New Delhi, India) method (following the manufacturer's instructions). In order to avoid genomic DNA contamination, DNase treatment and purification processes were performed. RNA was measured using UV-Vis, and its quality was tested using 260/280, which showed that the purity was satisfactorily good (1.8–2). The RNAs were also run on a 1% agarose gel, to assess their purity.

## Polymerase chain reaction primers

### Reverse transcription-polymerase chain reaction and polymerase chain reaction-amplified gene expression

cDNA was produced utilizing the Bio-Rad iScript cDNA synthesis kit (Bio-Rad Laboratories India Pvt. Ltd., Haryana, India) (per the manufacturer's instructions) using random hexamers and oligo(dT) primers as follows: 5X Mix 10, 18 μL of nuclease-free water, 2 μg RNA in 10 μL, and 2 μL of reverse transcriptase. In a PCR cycler, the sample was incubated as follows: 5 min of priming at 25°C, 20 min of RT at 46°C, and 1 min of RT inactivation at 95°C.

The polymerase chain reaction (PCR) was performed on a Bio-Rad thermocycler (Bio-Rad Laboratories India Pvt. Ltd., Haryana, India) using all seven primers (**Table 2**) for the *Aspergillus* isolates. The reaction volumes for real-time polymerase chain reaction (real-time PCR) were fixed at 25 μL, which contained 2X PCR Master Mix (12.5 μL; Thermo Scientific™, Vilnius, Lithuania), forward primer (1 μL), reverse primer (1 μL), cDNA (1 μL; 50 ng RNA equivalent cDNA was used), and 9.5 μL of molecular biology grade water (9.5 μL; HiMedia®, Mumbai, India). The cycle protocol included a 5 min denaturation step at 95°C, 40 cycles of denaturation at 95°C for 10 s, annealing at 55°C for 20 s, and extension at 72°C for 1 min, following the final extensions step for 10 min at 72°C. To validate adequate PCR amplification (**Figures 4–8**), the sizes of select isolates related to STC-expression genes were determined using a 1% agarose gel and a 100 bp DNA ladder (Thermo Fisher Scientific, Vilnius, Lithuania).

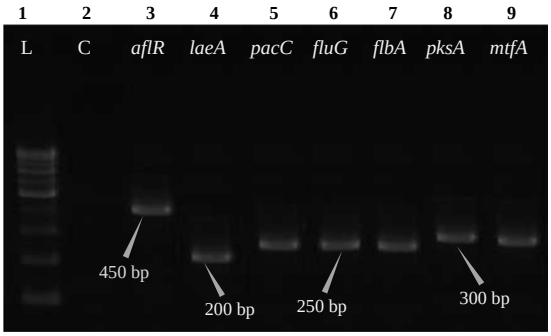


FIGURE 4
Electrophoretogram showing PCR amplification of transcribed genes in *A. nidulans* (MN791101). Lane 1–100 bp DNA ladder, Lane 2-Negative control PCR, Lane 3 through 9 includes amplicons for the *aflR*, *laeA*, *pacC*, *fluG*, *flbA*, *pksA*, and *mtfA* genes, sequentially.

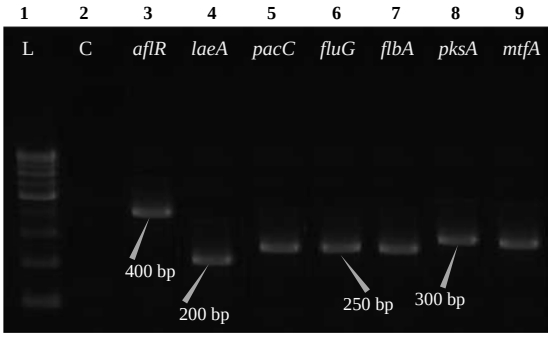


FIGURE 5
Electrophoretogram showing PCR amplification of transcribed genes in *E. quadrilineata* (MN791104). Lane 1–100 bp DNA ladder, Lane 2-Negative control PCR, Lane 3 through 9 includes amplicons for the *aflR*, *laeA*, *pacC*, *fluG*, *flbA*, *pksA*, and *mtfA* genes, sequentially.

### Sterigmatocystin purification

All *Aspergillus* isolates were grown in a carbohydrate-rich medium (containing D-glucose at a concentration of 20 g/L and potato infusion at a concentration of 200 g/L) at 180 rpm on a rotary shaker (VDRL, Delhi, India) for 13 days, with a temperature of 23°C. The cultures (2L) were filtered in order to separate the mycelium from the broth. The extraction and purification methods were adopted as given by Han et al. (2020). The ethyl acetate was added to the filtered broth and mycelium, followed by evaporation in a fume hood (Ganapathy Industries, Karnataka, India) to maintain the requisite level of dryness. The crude ethyl acetate extract of broth (12.8 g) was then separated through

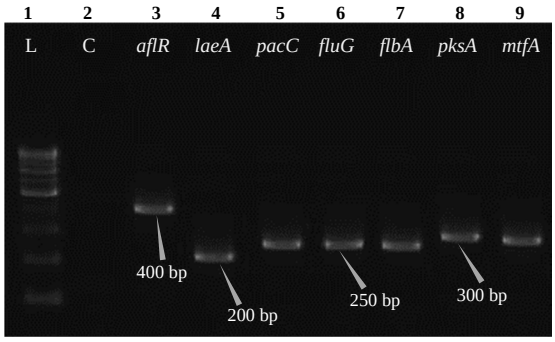


FIGURE 6
Electrophoretogram showing PCR amplification of transcribed genes in *E. quadrilineata* (MN791105). Lane 1–100 bp DNA ladder, Lane 2-Negative control PCR, Lane 3 through 9 includes amplicons for the *aflR*, *laeA*, *pacC*, *fluG*, *flbA*, *pksA*, and *mtfA* genes, sequentially.

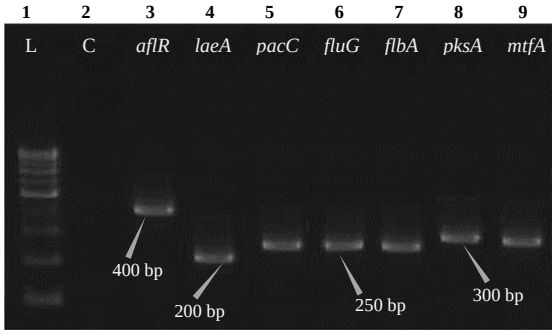


FIGURE 7
Electrophoretogram showing PCR amplification of transcribed genes in *A. latus* (MN791110). Lane 1–100 bp DNA ladder, Lane 2-Negative control PCR, Lane 3 through 9 includes amplicons for the *aflR*, *laeA*, *pacC*, *fluG*, *flbA*, *pksA*, and *mtfA* genes, sequentially.

a silica gel column and eluted using a gradient of petroleum ether/acetone (in 50:1–1:1, v:v) and dichloromethane (from 50:1 to 1:1, v:v). The eluted samples were pooled for HPLC analysis.

### High-performance liquid chromatography with ultraviolet detection

The use of reverse phase high-performance liquid chromatography (RP–HPLC) was employed in order to investigate the production of STC. The HPLC system with an ultra-violet (UV) detector at 325 nm wavelength (Shimadzu Corporation in Kyoto, Japan) and the stationary phase, C18 reverse phase column (Thermo Fisher Scientific, Vilnius, Lithuania), 150 mm × 4.6 mm, 3 μm particle size, was utilized. Each sample, including the STC standard, was produced in an acetonitrile solution. The separation was achieved by

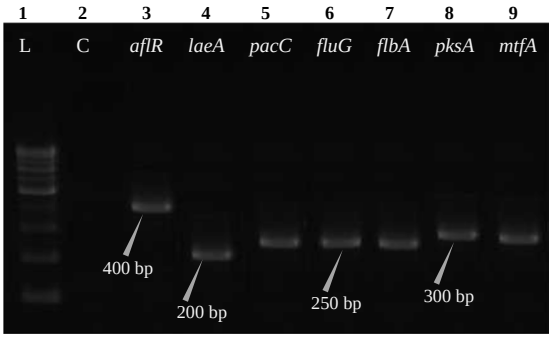


FIGURE 8
Electrophoretogram showing PCR amplification of transcribed genes in *A. latus* (MN791111). Lane 1–100 bp DNA ladder, Lane 2-Negative control PCR, Lane 3 through 9 includes amplicons for the *aflR*, *laeA*, *pacC*, *fluG*, *flbA*, *pksA*, and *mtfA* genes, sequentially.

chromatographic conditions, i.e., an isocratic mobile phase of acetonitrile: Water (60:40) with a flow rate of 1.0 mL $min^{-1}$, column oven temperature of 30°C, an injection volume of 10 μl, and a total run time of 30.0 min. The retention time ($t_R$) of the STC standard was determined to be 9.982 min. The STC in samples was confirmed by comparing $t_R$ values with those of the standard.

## Results

The analysis of 70 different samples of *F. vulgare* led to the isolation and identification of 14 distinct species of *Aspergilli,* from across a number of geographical regions in India. A very high incidence of *Aspergillus niger* was recorded, followed by other *Aspergilli* species, namely *A. flavus, A. terreus*, *A. nidulans*, *A. tamarii*, *A. species*, *Emericella quadrilineata*, *A. fumigatus*, *A. latus*, *A. aureoterreus, A. awamori*, *A. brasiliensis*, *A. ochraceus*, and *A. sydowii*, in decreasing order. The above findings of a detailed phenotypic and anatomical characterization of *Aspergilli* and authentication using a molecular fungal barcode have been previously published (Mahata et al., 2022).

## Morphology and molecular biomarkers of *Aspergillus* species

Following the phenotypic studies, five *Aspergillus* isolates belonging to three distinct species categories were subjected to detailed genetic analysis using the ITS gene of the ribosomal DNA (rDNA) and for β-tubulin gene sequences for molecular identification. The *Aspergilli* with their GenBank accession numbers are presented (**Table 1**).

TABLE 3 UV-visualization of *Aspergillus* isolates on β-cyclodextrin supplemented baseline culture media on the fourth day of incubation at 28°C.

| Genus/species | Isolate/lab code | Fluorescence on 2% β-cyclodextrin supplemented media |
|---|---|---|
| *A. nidulans* | FOEVPRB45 | Bright blue fluorescence |
| *E. quadrilineata* | FOEVPRB49 | Bright blue fluorescence |
| *E. quadrilineata* | FOEVPRB58 | Bright blue fluorescence |
| *A. latus* | FOEVPRB4 | Bright blue fluorescence |
| *A. latus* | FOEVPRB11 | Bright blue fluorescence |

## Toxicity assessment

All isolates were tested under UV light (365 nm) and the incubation duration was 4 days on YES agar supplemented with 2% β-cyclodextrin at 28°C. The result was evidently decisive, as fluorescence was detected after 4 days of incubation for all sterigmatocystin isolates that were tested (**Table 3**). The fluorescence displayed a light blue glow (**Figure 9**), which served as a preliminary tool for toxicity assessment. In parallel to this mycotoxin, other compounds produced by *Aspergillus* isolates can exhibit blue fluorescence when exposed to UV light; hence, in order to validate this hypothesis in our investigations, additional genomic profiling and STC purification tests were carried out.

## Gene profiling

Total RNA was obtained from all five test isolates of *Aspergillus* isolates. Agarose gel (1%) electrophoresis was performed in order to detect 28SrRNA and 18SrRNA as distinct bands (**Figure 10**). Seven genes of the STC biosynthesis cluster were detected using PCR to examine their role in toxin production. *A. latus* (02 isolates), *E. quadrilineata* (02 isolates), and *A. nidulans* (01 isolate) are all known to have the potential ability to produce STC among the species that were investigated. A positive amplification pattern (**Table 4**) for all seven genes tested was obtained for the *A. nidulans* FOEVPRB45 (**Figure 4**), *E. quadrilineata* FOEVPRB49 (**Figure 5**), *E. quadrilineata* FOEVPRB58 (**Figure 6**), *A. latus* FOEVPRB4 (**Figure 7**), and *A. latus* FOEVPRB11 (**Figure 8**) isolates in our inquiry. This led us to infer that a portion of the *Aspergillus* population has a significant potential to contaminate *F. vulgare* with STC in India.

## *Aspergillus* species sterigmatocystin assay

Sterigmatocystin was detected by an HPLC-UV assay at 325 nm. The retention time with the names of the peaks for the test isolates of *Aspergilli* is provided (**Table 5**). The retention time ($t_R$) was 10.120 min for *A. nidulans* FOEVPRB45 and 9.688 min for *A. latus* FOEVPRB4 (**Figures 11–15**).

# Discussion

The culinary preparations in India place a significant emphasis on the use of various spices (Siruguri and Bhat, 2015). Ever since the beginning of time, people have been seasoning their food with various spices. It is well known that *F. vulgare* possesses pharmacological activities such as antibacterial, anti-inflammatory, apoptotic, and anticancer activities, among others. Despite these diverse roles, fennel is susceptible to mycological degradation, continually being colonized by mycoflora like *Aspergilli*, *Curvularia*, and *Fusaria* (Mahata et al., 2022) during pre-harvest procedures, post-harvest losses in the field and storage. The occurrence of *Aspergillus* species on *F. vulgare* is the first sign of possible mycotoxin exposure. Specifically, in India, fennel seeds are a widespread spice used by the populace, which raises the risk of mycotoxin consumption and adds to health hazards and complications. The study is the first of its kind to detect the toxigenic gene clusters using DNA-based assays in addition to STC detection and purification. A total of 14 different types of *Aspergilli* have been isolated and reported from *F. vulgare* (Mahata et al., 2022), and yet, another study reported mycotoxigenic *Aspergilli* produced at varying humidities and storage periods (Amadi and Adeniyi, 2009) to produce AFs, ochratoxins, and fumonisins. This seems to be the issue with the toxigenic potential of these species and environmental exposures that cause toxicity.

Mycotoxin contamination of seed crops caused by *Aspergillus* spp. has been a challenge in all types of agricultural commodities for a long time. Generally speaking, most spices appear to be susceptible to fungal infection and consequently mycotoxin contamination. Aflatoxin and STC contamination of grain crops is a matter of concern as these mycotoxins are carcinogenic in nature (Anonymous, 2013) and have been detected in a variety of agricultural products (Kang et al., 2022). Section *Nidulantes* encompasses numerous species like *A. nidulans*, *A. quadrilineatus*, *A. latus*, and *A. rugulosus* that are routinely encountered in the ecosystem, primarily inhabiting the soil or decomposing plant debris (Domsch et al., 2007). Closely related species of *A. nidulans*, *A. versicolor*, and certain *A. sydowii* isolates have often been isolated from stored grains, nuts, seeds, dried medicinal herbs, and other foods and feeds (Pitt and Hocking, 2009; Hong et al., 2015). In addition, homothallic species of *A. nidulans*, *A. quadrilineatus*,

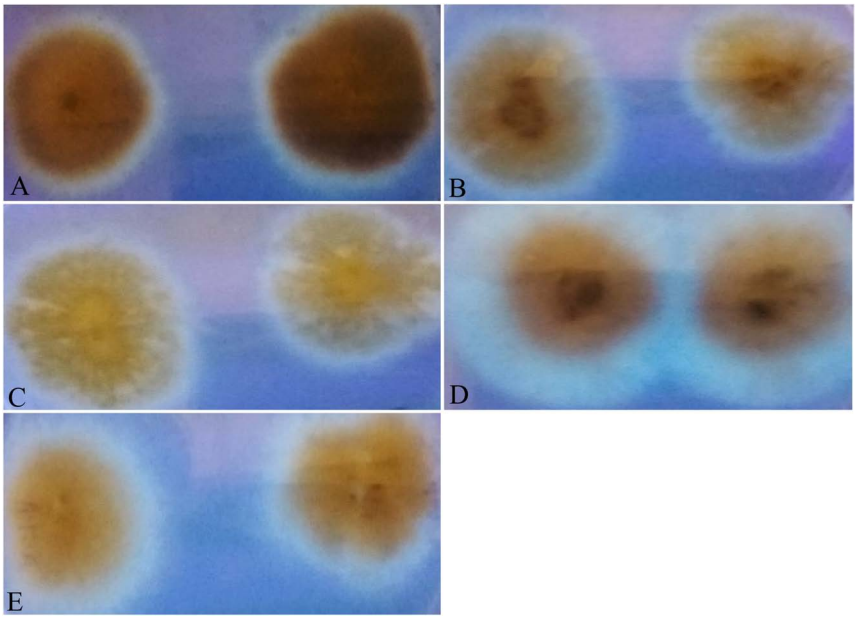


FIGURE 9
Fungal cultures of *Aspergilli* on yeast sucrose medium (YES), supplemented with β-methyl cyclodextrin, incubated at 25°C and photographed on the fourth day. The colonies of **(A)** *A. nidulans* (MN791101), **(B)** *A. latus* (MN791110), **(C)** *E. quadrilineata* (MN791105), **(D)** *E. quadrilineata* (MN791104), and **(E)** *A. latus* (MN791111) fluoresced with a light blue glow under long wavelength UV light.

*A. spinulosporus* (*Emericella echinulata*), and *A. rugulosus* have been mostly implicated in invasive infections (Yu et al., 2013; Uhrin et al., 2015). In individuals with chronic granulomatous illness, *A. nidulans* is more prevalent and virulent than in other immunocompromised patients (Segal, 2009). *A. nidulans* and *A. quadrilineatus* have also been linked to sinusitis, endophthalmitis, and onychomycosis (Hubka et al., 2012; Sharma et al., 2015). Cases of aspergillosis have been reported in dogs and horses (Seyedmousavi et al., 2015), but these diseases are rather rare in animals.

Ascomycete genomes encode an average of 40 biosynthetic gene clusters that are essential for secondary metabolites (SM) biosynthesis (Pusztahelyi et al., 2015). Genomic investigations of *Aspergilli* indicate that each species contains about 39 and 80 biosynthesis gene clusters (Inglis et al., 2013). The gene cluster required for STC biosynthesis in *A. nidulans* (Brown et al., 1996) has been identified and is known to share similarities with those of *Podospora anserina* (Slot and Rokas, 2011).

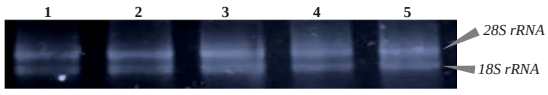


FIGURE 10
The agarose gel electrophoresis images show that the total RNA extracted from five different *Aspergilli* is comprised of two bright bands that correspond to *28S rRNA* and *18S rRNA*. Lane 1- *A. latus* (MN791111), Lane 2- *A. latus* (MN791110), Lane 3- *A. nidulans* (MN791101), Lane 4- *E. quadrilineata* (MN791104), and Lane 5- *E. quadrilineata* (MN791105).

The generation of natural products as a result of the secondary metabolism of microbes and plants is affected by a variety of environmental factors, namely, temperature, humidity, light, pH, and the source of nutrients, among other parameters. Consequently, this has been described as temperature and nitrogen supply influence AF production in *A. parasiticus* and STC synthesis in *A. nidulans*, but in distinct ways (Feng and Leonard, 1998).

The fungal isolates used in our experiment were chosen due to the fact that there is a serious cause for concern regarding their toxigenicity and STC production. STC-producing *Aspergilli*, namely *A. latus* (02 isolates), *E. quadrilineata* (02 isolates), and *A. nidulans* (01 isolate) were isolated from the *F. vulgare*. Identification of the various species of *Aspergillus* is an arduous task and has become even more challenging as a result of the close species association. Data from the selective media used for *Aspergillus* species cultivation, mycotoxin processing, and various molecular tools are brought together here for our better understanding. In our analysis, *Aspergilli* characterization was derived from a combination of detailed morphology (**Figures 1–3**) and molecular techniques (**Table 1**) that is composed of two DNA barcode genes, ITS and β-tubulin.

Fungi from the *Nidulantes* section are commonly present in foods and feeds destined for human and animal consumption. Though some isolates produce STC, not all isolates are producers, and this has motivated researchers to use screening

TABLE 4 PCR amplification data for STC biosynthetic gene cluster.

| Genus/species | *aflR* | *laeA* | *pacC* | *fluG* | *flbA* | *pksA* | *mtfA* |
|---|---|---|---|---|---|---|---|
| *A. nidulans* FOEVPRB45 | + | + | + | + | + | + | + |
| *E. quadrilineata* FOEVPRB49 | + | + | + | + | + | + | + |
| *E. quadrilineata* FOEVPRB58 | + | + | + | + | + | + | + |
| *A. latus* FOEVPRB4 | + | + | + | + | + | + | + |
| *A. latus* FOEVPRB11 | + | + | + | + | + | + | + |

+: Amplicon positive, with the expected size of the target gene.

methods to identify fungal isolates that can produce mycotoxins. In these processes, culture conditions with additives are used to enhance the growth and detect fluorescence that is potential STC producers. Once fluorescence was detected under UV light on the fourth day of incubation, it seemed as a preliminary determination of the toxigenic potential and unmistakably decisive for every sterigmatocystin isolate tested. Further, the addition of 2% β-cyclodextrin to the basal medium improved the efficiency of fluorescence and STC detection (**Figure 9**) on the fungal culture plates. Also, it has been established through well-known studies that genes involved in secondary metabolite production tend to cluster in fungal genomes (Keller and Hohn, 1997). Formerly, methods for determining the extent of actively transcribed loci included northern blotting or reverse transcription-polymerase chain reaction (RT-PCR) and evaluating the expression in secondary metabolite-inducing vs. non-inducing settings. This strategy is viable for individual clusters, as demonstrated for trichothecene toxins (Kimura et al., 2003; Brown et al., 2004), lolitrem (Young et al., 2006), fumonisins (Proctor et al., 2003), gliotoxin (Gardiner and Howlett, 2005), sirodesmin (Gardiner et al., 2004), and others.

The AF and STC biosynthesis pathways are primarily regulated by the *aflR* gene (regulatory gene for aflatoxin biosynthesis) in *A. flavus*, *A. parasiticus*, and *A. nidulans* (Yu et al., 1996b; Yu and Keller, 2005). The *A. nidulans aflR* gene cluster is comprised of approximately 24 additional genes that are directly or indirectly involved in the production of the AF-related mycotoxin STC. It has been revealed that the protein known as *A. nidulans* AflR (AnAflR) interacts with the promoter regions of a number of AF and STC cluster genes (*stc* genes) (Fernandes et al., 1998). Moreover, it was demonstrated that the *laeA* gene, which is responsible for the loss of *aflR* expression, is implicated in the universal regulation of AF, STC, penicillin, and gliotoxin in numerous fungi.

A conserved filamentous fungus protein known as laeA (loss of *aflR* expression A) is also known as a conserved virulence factor in every pathogenic fungus that has been explored to this date (Wiemann et al., 2010; López-Berges et al., 2013). LaeA is a transcription factor that regulates the formation of gliotoxin, fumagillin, fumigatin, and helvolic acid metabolites in *A. fumigatus* (Keller et al., 2006). In *A. nidulans*, protein laeA, a potential methyltransferase, is a master regulator of secondary metabolism, which is essential for the expression of *aflR* and other secondary metabolite biosynthesis genes undergo chromatin remodeling (Bok et al., 2006; Patananan et al., 2013). LaeA expression is negatively controlled in a unique feedback loop by aflR, a STC transcription factor, as well as by two signaling components, protein kinase A and RasA (Bok et al., 2005; Sugui et al., 2007). They established that when *laeA* is deficient, secondary metabolism and pathogenicity are impaired. LaeA isolates contain hyphal pigments and play a minor role in spore production, indicating that its primary function is to regulate metabolic gene clusters (Bok and Keller, 2004). Additionally, laeA is necessary for the production of light-dependent conidia, which requires the veA protein to be in its native state (Sarikaya Bayram et al., 2010).

An important component of the circuit is pacC (pH-responsive transcription factor) (Tilburn et al., 1995), a zinc finger protein that activates alkaline-expressed genes while inhibiting the expression of acid-expressed genes. An alkaline-expressed gene in *A. nidulans*, isopenicillin N synthase (ipnA), has been used to study the binding of pacC to the promoter. In *A. nidulans*, a transcription factor called pacC modulates the expression of acidic and alkaline-structural genes based on the pH of the extracellular medium (Caddick et al., 1986; Tilburn et al., 1995). Sequential proteolysis of pacC forms the active form of the protein, PacC27, at an alkaline pH, which positively influences the activation of alkaline genes and negatively affects the regulation of acidic genes (Díez et al., 2002; Arst and Peñalva, 2003). Proteolysis of pacC during constitutive processing increases pathogenicity and promotes invasive development (Bignell et al., 2005). It demonstrates that both the pacC process and Pal-mediated pH signaling

TABLE 5 Detection of sterigmatocystin using high-performance liquid chromatography (HPLC) with UV light at 325 nm and retention time ($t_R$) in minutes for the investigated isolates.

| Genus/species | Name of peak | Retention time (min) |
|---|---|---|
| *A. nidulans* FOEVPRB45 | S1* | 10.120 |
| *E. quadrilineata* FOEVPRB49 | S2* | 09.982 |
| *E. quadrilineata* FOEVPRB58 | S3* | 09.823 |
| *A. latus* FOEVPRB4 | S4* | 09.688 |
| *A. latus* FOEVPRB11 | S5* | 09.785 |

*Represents the S1–S5 peaks of *Aspergillus* STC at their retention times (**Figures 11–15**).

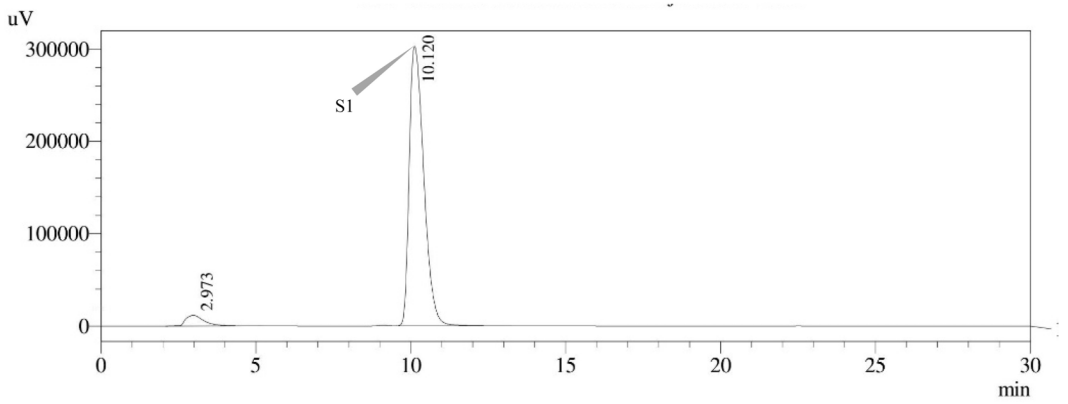


FIGURE 11
HPLC chromatogram of *A. nidulans* (MN791101) producing sterigmatocystin, detected using UV light at 325 nm with retention time ($t_R$) of 10.120 min.

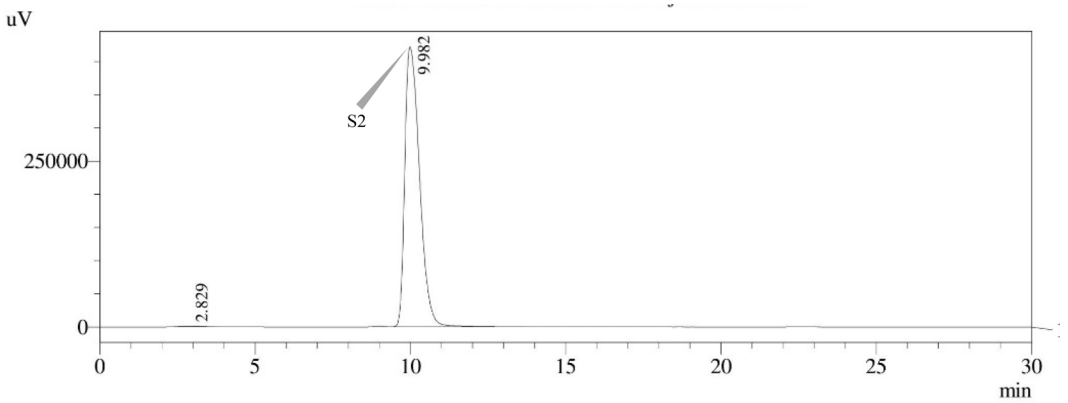


FIGURE 12
HPLC chromatogram of *E. quadrilineata* (MN791104) producing sterigmatocystin, detected using UV light at 325 nm with retention time ($t_R$) of 9.982 min.

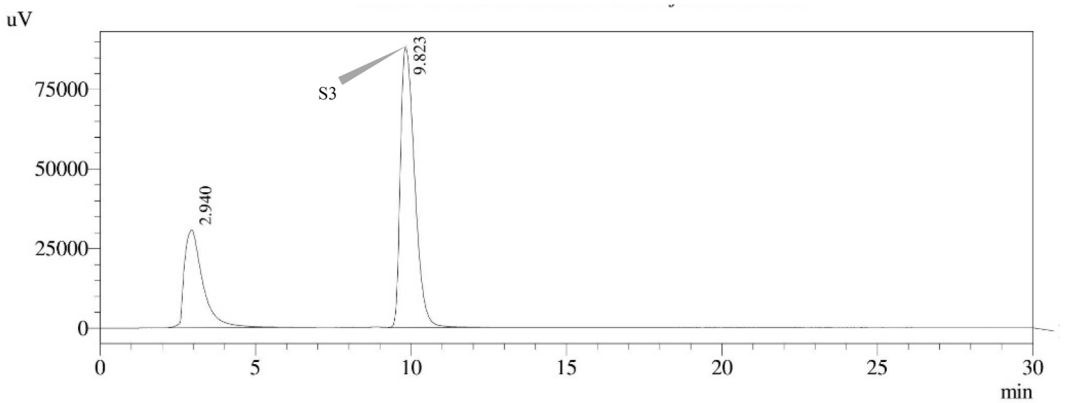


FIGURE 13
HPLC chromatogram of *E. quadrilineata* (MN791105) producing sterigmatocystin, detected using UV light at 325 nm with retention time ($t_R$) of 9.823 min.

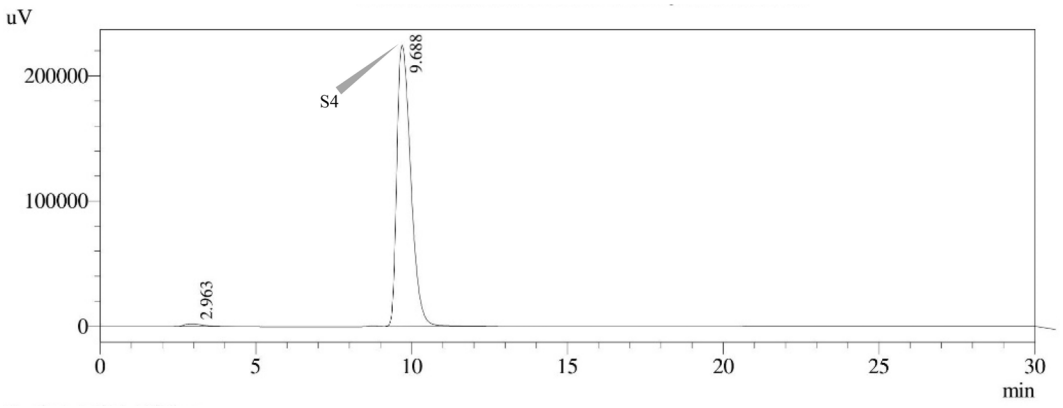


FIGURE 14
HPLC chromatogram of *A. latus* (MN791110) producing sterigmatocystin, detected using UV light at 325 nm with retention time ($t_R$) of 9.688 min.

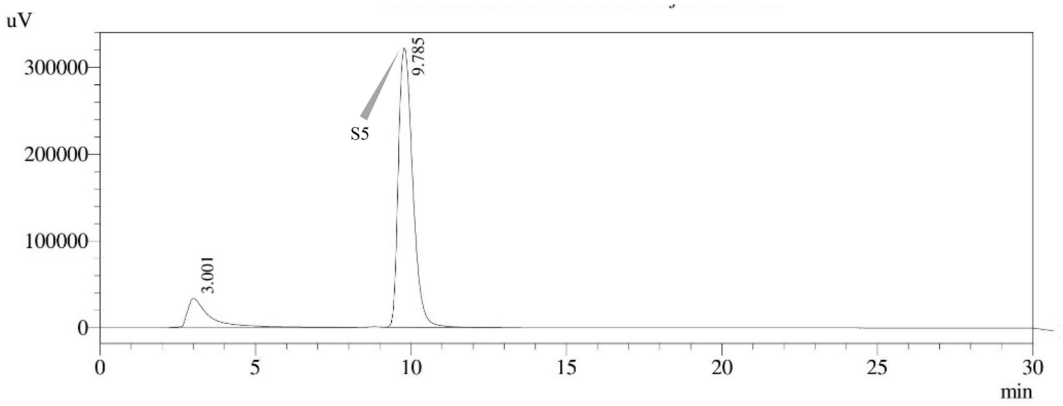


FIGURE 15
HPLC chromatogram of *A. latus* (MN791111) producing sterigmatocystin, detected using UV light at 325 nm with retention time ($t_R$) of 9.785 min.

are essential for the promotion of pathological processes and that constitutive *pacC* activation promotes virulence (Bignell et al., 2005). The pathogenicity of *A. nidulans* is dependent on pH signaling and the pH-responsive transcription factor pacC (Bignell et al., 2005).

The *fluG* (fluffy) gene encodes a cytoplasmically localized protein that is maintained at relatively constant levels throughout the entire life cycle of *A. nidulans* (Lee and Adams, 1994), synthesizes an extracellular factor, and drives both development-specific processes and the stimulation of flbA (fluffy with low brlA expression), which then inhibits fadA (fluffy-autolytic dominant) signaling (Yu et al., 1996b). Researchers looking into the early events that lead to conidiation activation and STC synthesis in *A. nidulans* discovered six genes, *fluG* (Adams et al., 1992; Seo et al., 2006), and *flbA–flbE*, that are necessary for normal *brlA* activation (Wieser et al., 1994). Each one of these genes can be mutated to produce undifferentiated, fluffy colonies (Wieser et al., 1994). *A. nidulans fluG* mutants are incapable of producing the secondary metabolite STC (Hicks et al., 1997). The carbon and nitrogen sources, as well as the pH of the medium, are among the most critical physiological factors of AF/STC production in *Aspergilli*. In *A. parasiticus*, simple carbohydrates such as glucose and sucrose promote AF production, but in *A. nidulans*, STC is produced (Payne and Brown, 1998). Additionally, while ammonium promotes the biosynthesis of AF in *A. parasiticus*, it inhibits the formation of STC in *A. nidulans* (Guzman-de-Peña et al., 1998).

FlbA and FadA were the first RGS-Gα (regulator of G protein signaling-G protein α subunits) pair found in a filamentous fungus. Together, they are essential for the upstream control of hyphal growth, maturation, and secondary metabolite biosynthesis (Yu et al., 1996a; Yu and Keller, 2005). When *flbA* function is impaired, a fluffy-autolytic phenotype similar to that generated by $FadA^{d+}$ (fluffy-autolytic phenotype) mutant alleles is produced (Yu et al., 1996a). A first *A. nidulans* regulator of G protein signaling (RGS) protein, *flbA*, was identified by the analysis of a fluffy-autolytic mutant. FlbA and FadA are necessary for the expression of *aflR*, which encodes a

transcription factor specific to the STC biosynthetic pathways. Conidiation is genetically connected to STC biosynthesis in *A. nidulans* (Hicks et al., 1997). When the *flbA* gene is deleted, the *aflR* and *brlA* genes, as well as the consequent STC synthesis and conidiation, are all negatively regulated (Hicks et al., 1997).

Two fatty acid synthases (FAS) enzymes and polyketide synthase (pksA) combine to make the polyketide from a hexanoyl beginning unit (NR-PKS, PksA). It was discovered that fatty acid and polyketide synthases are responsible for controlling the early steps of AF production (Watanabe et al., 1996; Watanabe and Townsend, 2002). In *A. nidulans*, STC production was also found to be dependent on FASs, which were named *stcJ* and *stcK* (Brown et al., 1996). Gene disruption experiments on *A. parasiticus* revealed that AF synthesis requires the *pksA* genome (Chang et al., 1995).

Mutagenesis screening for novel VeA-dependent STC regulators in *A. nidulans* led to the discovery of the nucleus-based *mtfA* (master transcription factor A) transcription factor. A recent transcriptome investigation of *mtfA* in *A. nidulans* and *A. fumigatus* revealed its role in controlling the expression of hundreds of genes, including secondary metabolite gene clusters. In *A. nidulans*, *mtfA* regulates the production of STC, penicillin, and terriquinone A (Ramamoorthy et al., 2013), and other gliotoxins in *A. fumigatus*. This regulatory section includes clusters essential for mycotoxin production (Lind et al., 2015), some of which are known virulence factors (Coméra et al., 2007; Kwon-Chung and Sugui, 2009). MtfA exerts its effect on STC synthesis by modulating the expression of the STC gene cluster activator *aflR*. Along with affecting secondary metabolism, *mtfA* also has an impact on the development of *A. nidulans* both asexually and sexually. Additionally, *mtfA* is needed to maintain the proper development of sclerotia during conidiation.

Seven STC-linked genes were detected in the isolates tested (**Figures 4–8**) in this investigation, indicating that these isolates had positive amplification patterns for the STC gene cluster (**Table 4**). With a flow rate of 1 mL $min^{-1}$ during HPLC analysis, the STC retention times ($t_R$) for the *A. nidulans* FOEVPRB45, *E. quadrilineata* FOEVPRB49, *E. quadrilineata* FOEVPRB58, *A. latus* FOEVPRB4, and *A. latus* FOEVPRB11 isolates were 10.120 (**Figure 11**), 9.982 (**Figure 12**), 9.823 (**Figure 13**), 9.688 (**Figure 14**), and 9.785 (**Figure 15**) min. Quantification was accomplished by analyzing the peak areas of the STC and comparing them to those of the standard (**Supplementary Figure 1**); in this particular instance, STC employed for commercial purposes was purchased. The STC was detected and confirmed from five different isolates of *Aspergillus* using the HPLC-UV method. We were able to achieve separate STC peaks as shown in the chromatograms (**Figures 11–15**). Consequent to HPLC analysis, all five isolates tested positive and were evaluated for their potential ability to produce STC under optimal conditions of fungal growth. The HPLC-UV detection method was utilized in a prior experiment that was carried out by Marley et al. (2015) to look for STC contamination in a variety of foods and beverages, namely, cereals, seeds, livestock feed, dairy, and beer. This particular study reported HPLC chromatograms of STC, with the UV detector configured to 325 nm. STC was proven to have been detected in two samples of light and dark beer by the HPLC-UV analysis, and the presence of STC in the samples was validated by the HPLC chromatograms. This discovery was made in a study that was published in Veršilovskis et al. (2008). The level of STC found in beer was proportional to the amount found in barley. A first and effective HPLC-UV approach for determining STC production by *Aspergilli* is suitable for the efficient, specific, and routine analysis of STC production.

There are only a few accounts of STC occurring spontaneously, despite the fact that fungi capable of making STC are ubiquitous all over the world. In a focused study by Tabata (2011), STC was not detected in harvested grains or cheese but was found to be present in preserved products. STC has been detected in oregano, up to 28.0 μg/kg from Turkey, in thyme up to 14.0 μg/kg from Poland (Reinholds et al., 2017), and in paprika, 18.0 μg/kg from South Africa (Motloung et al., 2018). In another investigation, moderate quantities of STC in black pepper and chili were detected in Sri Lanka at 49.0 and 32 μg/kg, respectively (Jacxsens and De Meulenaer, 2016). The discovery of these potentially toxic variants may point to a possible presence of STC contamination of fennel grown in India. The data suggest an enormous danger of STC contamination in fennel, which can be worsened by unscientific methods, poor technologies applied by small farmers, and lack of temperature and humidity control during storage. Nonetheless, *Aspergillus* species from the *Nidulantes* Section are found in the Indian fennel analyzed in this study, highlighting the necessity to monitor or implement measures to limit the population's STC intake. Accurate and timely identification of toxigenic fungi and their respective toxin may open doors for further research on STC contamination in fennel grown in India. The study could serve as a template for further research inquiries spanning different food groups. To this day, there has been no research conducted on the sterigmatocystin potential of *Aspergillus* produced from *F. vulgare*.

## Conclusion

In conclusion, the various outcomes of our study indicated that five *Aspergillus* isolates from the *Nidulantes* Section isolated from *F. vulgare* in India were STC producers. This demonstrated that fennel spice could harbor carcinogenic compounds, in particular STC. Therefore, the potential cumulative toxicities and synergistic action from these mycotoxins are major concerns in terms of risk assessment. This report gives a preliminary evaluation of the degree to which the spices may be contaminated with *Aspergilli*, as well as their vulnerability to fungal infection and STC production. In future, more research

with larger, diverse samples of fennel, and other foodstuffs should be conducted to ascertain the contamination levels of STC and other possible mycotoxins present in spices, as well as their associated consumer concerns. After harvest, advancements in drying and storing procedures might restrict fungal contamination and mycotoxin exposure, but consumers' safety also relies on suitable storage environments and quality standards at every stage of processing, packaging, or marketing. In order to limit the risk of disease in humans and animals in complex food chains, we emphasize the significance of reliable tests and strict government policies and regulations on permissible levels of mycotoxin, especially for spices.

## Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/ (MN791101; MN791104; MN791105; MN791110; and MN791111).

## Author contributions

PM conceptualized, designed, performed, executed the research experiments and written the manuscript. RD conceptualized, guided the research experiments and written the manuscript. LG and PT helped in manuscript preparation and assisted in experimentation. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.958424/full#supplementary-material

## References

Abubacker, A. T. N. (2011). *Market survey*. Available online at: https://efy.efymag.com/admin/issuepdf/fennel_ffy-oct11.pdf (accessed August 11, 2017).

Adams, T. H., Hide, W. A., Yager, L. N., and Lee, B. N. (1992). Isolation of a gene required for programmed initiation of development by *Aspergillus nidulans*. *Mol. Cell. Biol.* 12, 3827–3833. doi: 10.1128/mcb.12.9.3827-3833.1992

Alshehri, B., and Palanisamy, M. (2020). Evaluation of molecular identification of *Aspergillus* species causing fungal keratitis. *Saudi J. Biol. Sci.* 27, 751–756. doi: 10.1016/j.sjbs.2019.12.030

Amadi, J. E., and Adeniyi, D. O. (2009). Mycotoxin production by fungi isolated from stored grains. *Afr. J. Biotechnol.* 8, 1219–1221.

Anderson, J. A., Chung, C. H., and Cho, S. H. (1990). Versicolorin A hemiacetal, hydroxydihydrosterigmatocystin, and aflatoxin G2 alpha reductase activity in extracts from *Aspergillus parasiticus*. *Mycopathologia* 111, 39–45. doi: 10.1007/BF02277300

Anonymous (2013). Scientific Opinion on the risk for public and animal health related to the presence of sterigmatocystin in food and feed. *EFSA J.* 11:3254. doi: 10.2903/j.efsa.2013.3254

Anonymous (2020a). *Major fennel seed producing states in India–production and area under cultivation [2019-20 Provisional]*. Available online at: https://numerical.co.in/numerons/collection/60a5d842190ebb1700cdd92e (accessed February 18, 2021).

Anonymous (2020b). *Connect2india*. Available online at: https://connect2india.com/global/Fennel-Seed-export-from-india/1 (accessed February 18, 2021).

Arné, P., Risco-Castillo, V., Jouvion, G., Le Barzic, C., and Guillot, J. (2021). Aspergillosis in wild birds. *J. Fungi* 7:241. doi: 10.3390/jof7030241

Arst, H. N. Jr., and Peñalva, M. A. (2003). pH regulation in *Aspergillus* and parallels with higher eukaryotic regulatory systems. *Trends Genet.* 19, 224–231. doi: 10.1016/S0168-9525(03)00052-0

Balogh, K., Kövesi, B., Zándoki, E., Kulcsár, S., Ancsin, Z., Erdélyi, M., et al. (2019). Effect of sterigmatocystin or aflatoxin contaminated feed on lipid peroxidation and glutathione redox system and expression of glutathione redox system regulatory genes in broiler chicken. *Antioxidants* 8:201. doi: 10.3390/antiox8070201

Bennett, J. W. (2010). "An overview of the genus *Aspergillus*," in *Aspergillus: Molecular Biology and Genomics*, eds M. Machida and K. Gomi (Norfolk, VA: Caister Academic Press), 1–17.

Bignell, E., Negrete-Urtasun, S., Calcagno, A. M., Haynes, K., Arst, H. N. Jr., and Rogers, T. (2005). The *Aspergillus* pH-responsive transcription factor PacC regulates virulence. *Mol. Microbiol.* 55, 1072–1084. doi: 10.1111/j.1365-2958.2004.04472.x

Bladt, T. T., Frisvad, J. C., Knudsen, P. B., and Larsen, T. O. (2013). Anticancer and antifungal compounds from *Aspergillus*, *Penicillium* and other filamentous fungi. *Molecules* 18, 11338–11376. doi: 10.3390/molecules180911338

Bok, J. W., Balajee, S. A., Marr, K. A., Andes, D., Nielsen, K. F., Frisvad, J. C., et al. (2005). LaeA, a regulator of morphogenetic fungal virulence factors. *Eukaryot. Cell* 4, 1574–1582. doi: 10.1128/EC.4.9.1574-1582.2005

Bok, J. W., and Keller, N. P. (2004). LaeA, a regulator of secondary metabolism in *Aspergillus* spp. *Eukaryot. Cell* 3, 527–535. doi: 10.1128/EC.3.2.527-535.2004

Bok, J. W., Noordermeer, D., Kale, S. P., and Keller, N. P. (2006). Secondary metabolic gene cluster silencing in *Aspergillus nidulans*. *Mol. Microbiol.* 61, 1636–1645. doi: 10.1111/j.1365-2958.2006.05330.x

Brakhage, A. A. (2013). Regulation of fungal secondary metabolism. *Nat. Rev. Microbiol.* 11, 21–32. doi: 10.1038/nrmicro2916

Bressac, B., Kew, M., Wands, J., and Ozturk, M. (1991). Selective G to T mutations of p53 gene in hepatocellular carcinoma from southern Africa. *Nature* 350, 429–431. doi: 10.1038/350429a0

Brown, D. W., Dyer, R. B., McCormick, S. P., Kendra, D. F., and Plattner, R. D. (2004). Functional demarcation of the *Fusarium* core trichothecene gene cluster. *Fungal Genet. Biol.* 41, 454–462. doi: 10.1016/j.fgb.2003.12.002

Brown, D. W., Yu, J. H., Kelkar, H. S., Fernandes, M., Nesbitt, T. C., Keller, N. P., et al. (1996). Twenty-five coregulated transcripts define a sterigmatocystin gene cluster in *Aspergillus nidulans*. *Proc. Natl. Acad. Sci. U.S.A.* 93, 1418–1422.

Caddick, M. X., Brownlee, A. G., and Arst, H. N. (1986). Regulation of gene expression by pH of the growth medium in *Aspergillus nidulans*. *Mol. Genet. Genom.* 203, 346–353. doi: 10.1007/BF00333978

Cardwell, K., and Miller, J. D. (1996). Mycotoxins in foods in Africa. *Nat. Toxins* 4, 103–107. doi: 10.1002/19960403NT1

Cardwell, K. F., Desjardins, A., Henry, H. S., Munkvold, G., and Robens, J. (2001). *Mycotoxins: The cost of achieving food security and food quality*. Saint Paul, MN: APSnet., APS.

Chang, P. K., Cary, J. W., Yu, J., Bhatnagar, D., and Cleveland, T. E. (1995). The *Aspergillus parasiticus* polyketide synthase genepksA, a homolog of *Aspergillus nidulans* wA, is required for aflatoxin B1 biosynthesis. *Mol. Gen. Genet.* 248, 270–277. doi: 10.1007/BF02191593

Cole, R. J., and Cox, R. H. (1981). *Handbook of toxic fungal metabolites, localization of stc gene products to subcellular sites*. New York, NY: Academic Press, 67–72.

Cole, R. J., Schweikert, M. A., and Jarvis, B. B. (2003). *Handbook of secondary fungal metabolites*, Vol. 3. Houston, TX: Gulf Professional Publishing.

Coméra, C., André, K., Laffitte, J., Collet, X., Galtier, P., and Maridonneau-Parini, I. (2007). Gliotoxin from *Aspergillus fumigatus* affects phagocytosis and the organization of the actin cytoskeleton by distinct signalling pathways in human neutrophils. *Microbes Infect.* 9, 47–54. doi: 10.1016/j.micinf.2006.10.009

Delgado-Virgen, F., and Guzman-de-Peña, D. (2009). Mechanism of sterigmatocystin biosynthesis regulation by pH in *Aspergillus nidulans*. *Braz. J. Microbiol.* 40, 933–942. doi: 10.1590/S1517-838220090004000027

Díez, E., Álvaro, J., Espeso, E. A., Rainbow, L., Suárez, T., Tilburn, J., et al. (2002). Activation of the *Aspergillus* PacC zinc finger transcription factor requires two proteolytic steps. *EMBO J.* 21, 1350–1359. doi: 10.1093/emboj/21.6.1350

Domsch, K. H., Gams, W., and Anderson, T. H. (2007). *Compendium of soil fungi*, 2nd Edn. Eching: IHW-Verlag.

Feng, G. H., and Leonard, T. J. (1998). Culture conditions control expression of the genes for aflatoxin and sterigmatocystin biosynthesis in *Aspergillus parasiticus* and *A. nidulans*. *Appl. Environ. Microbiol.* 64, 2275–2277. doi: 10.1128/AEM.64.6.2275-2277.1998

Fente, C. A., Ordaz, J. J., Vazquez, B. I., Franco, C. M., and Cepeda, A. (2001). New additive for culture media for rapid identification of aflatoxin-producing *Aspergillus* strains. *Appl. Environ. Microbiol.* 67, 4858–4862. doi: 10.1128/AEM.67.10.4858-4862.2001

Fernandes, M., Keller, N. P., and Adams, T. H. (1998). Sequence-specific binding by *Aspergillus nidulans* AflR, a C6 zinc cluster protein regulating mycotoxin biosynthesis. *Mol. Microbiol.* 28, 1355–1365. doi: 10.1046/j.1365-2958.1998.00907.x

Garcia, M. V., Parussolo, G., Moro, C. B., Bernardi, A. O., and Copetti, M. V. (2018). Fungi in spices and mycotoxigenic potential of some *Aspergilli* isolated. *Food Microbiol.* 73, 93–98. doi: 10.1016/j.fm.2018.01.013

Gardiner, D. M., Cozijnsen, A. J., Wilson, L. M., Pedras, M. S., and Howlett, B. J. (2004). The sirodesmin biosynthetic gene cluster of the plant pathogenic fungus *Leptosphaeria maculans*. *Mol. Microbiol.* 53, 1307–1318. doi: 10.1111/j.1365-2958.2004.04215.x

Gardiner, D. M., and Howlett, B. J. (2005). Bioinformatic and expression analysis of the putative gliotoxin biosynthetic gene cluster of *Aspergillus fumigatus*. *FEMS Microbiol. Lett.* 248, 241–248. doi: 10.1016/j.femsle.2005.05.046

Guzman-de-Peña, D., Aguirre, J., and Ruiz-Herrrera, J. (1998). Corrrelation between the regulation of sterigmatocystin biosynthesis and asexual and sexual sporulation in *Emericella nidulans*. *Antonie Leeuwenhoek* 73, 199–205. doi: 10.1023/A:1000820221945

Hamzah, T. N. T., Lee, S. Y., Hidayat, A., Terhem, R., Faridah-Hanum, I., and Mohamed, R. (2018). Diversity and characterization of endophytic fungi isolated from the tropical mangrove species, *Rhizophora mucronata*, and identification of potential antagonists against the soil-borne fungus, *Fusarium solani*. *Front. Microbiol.* 9:1707. doi: 10.3389/fmicb.2018.01707

Han, X., Tang, X., Luo, X., Sun, C., Liu, K., Zhang, Y., et al. (2020). Isolation and identification of three new sterigmatocystin derivatives from the fungus *Aspergillus versicolor* guided by molecular networking approach. *Chem. Biodivers.* 17:2000208. doi: 10.1002/cbdv.202000208

Hicks, J. K., Yu, J. H., Keller, N. P., and Adams, T. H. (1997). *Aspergillus* sporulation and mycotoxin production both require inactivation of the FadA Gα protein-dependent signaling pathway. *EMBO J.* 16, 4916–4923. doi: 10.1093/emboj/16.16.4916

Hong, S. B., Kim, D. H., and Samson, R. A. (2015). *Aspergillus* associated with Meju, a fermented soybean starting material for traditional soy sauce and soybean paste in Korea. *Mycobiology* 43, 218–224. doi: 10.5941/MYCO.2015.43.3.218

Houbraken, J., and Samson, R. A. (2011). Phylogeny of *Penicillium* and the segregation of Trichocomaceae into three families. *Stud. Mycol.* 70, 1–51. doi: 10.3114/sim.2011.70.01

Hubka, V., Kubatova, A., Mallatova, N., Sedlacek, P., Melichar, J., Skorepova, M., et al. (2012). Rare and new etiological agents revealed among 178 clinical *Aspergillus* strains obtained from Czech patients and characterized by molecular sequencing. *Med. Mycol.* 50, 601–610. doi: 10.3109/13693786.2012.667578

Inglis, D. O., Binkley, J., Skrzypek, M. S., Arnaud, M. B., Cerqueira, G. C., Shah, P., et al. (2013). Comprehensive annotation of secondary metabolite biosynthetic genes and gene clusters of *Aspergillus nidulans*, *A. fumigatus*, *A. niger* and *A. oryzae*. *BMC Microbiol.* 13:91. doi: 10.1186/1471-2180-13-91

Jacxsens, L., and De Meulenaer, B. (2016). Risk assessment of mycotoxins and predictive mycology in Sri Lankan spices: Chilli and pepper. *Procedia Food Sci.* 6, 326–330. doi: 10.1016/j.profoo.2016.02.065

Javidnia, K., Dastgheib, L., Samani, S. M., and Nasiri, A. (2003). Anti-hirsutism activity of fennel (fruits of *Foeniculum vulgare*) extract–a double-blind placebo-controlled study. *Phytomedicine* 10, 455–458. doi: 10.1078/094471103322331386

Kang, Y. W., Baek, S. K., Choi, M., Lee, H. J., and Koo, Y. E. (2022). Occurrence and risk assessment of sterigmatocystin in agricultural products and processed foods in Korea. *Food Addit. Contam. A* 39, 373–381. doi: 10.1080/19440049.2021.1994156

Keller, N., Bok, J., Chung, D., Perrin, R. M., and Keats Shwab, E. (2006). LaeA, a global regulator of *Aspergillus* toxins. *Med. Mycol.* 44, S83–S85. doi: 10.1080/13693780600835773

Keller, N. P., and Hohn, T. M. (1997). Metabolic pathway gene clusters in filamentous fungi. *Fungal Genet. Biol.* 21, 17–29. doi: 10.1006/fgbi.1997.0970

Keller, N. P., Turner, G., and Bennett, J. W. (2005). Fungal secondary metabolism—from biochemistry to genomics. *Nat. Rev. Microbiol.* 3, 937–947. doi: 10.1038/nrmicro1286

Kimura, M., Tokai, T., O'Donnell, K., Ward, T. J., Fujimura, M., Hamamoto, H., et al. (2003). The trichothecene biosynthesis gene cluster of *Fusarium graminearum* F15 contains a limited number of essential pathway genes and expressed non-essential genes. *FEBS Lett.* 539, 105–110. doi: 10.1016/S0014-5793(03)00208-4

Kwon-Chung, K. J., and Sugui, J. A. (2009). What do we know about the role of gliotoxin in the pathobiology of *Aspergillus fumigatus*? *Med. Mycol.* 47, S97–S103. doi: 10.1080/13693780802056012

Lauer, J. M., Duggan, C. P., Ausman, L. M., Griffiths, J. K., Webb, P., Wang, J. S., et al. (2019). Maternal aflatoxin exposure during pregnancy and adverse birth outcomes in Uganda. *Matern. Child Nutr.* 15:12701. doi: 10.1111/mcn.12701

Lee, B. N., and Adams, T. H. (1994). The *Aspergillus nidulans fluG* gene is required for production of an extracellular developmental signal. *Genes Dev.* 8, 641–651. doi: 10.1101/gad.8.6.641

Leema, G., Chou, D. S., Jesudasan, C. A. N., Geraldine, P., and Thomas, P. A. (2011). Expression of genes of the aflatoxin biosynthetic pathway in *Aspergillus flavus* isolates from keratitis. *Mol. Vis.* 17:2889.

Liew, W. P. P., and Mohd-Redzwan, S. (2018). Mycotoxin: Its impact on gut health and microbiota. *Front. Cell. Infect. Microbiol.* 8:60. doi: 10.3389/fcimb.2018.00060

Lind, A. L., Wisecaver, J. H., Smith, T. D., Feng, X., Calvo, A. M., and Rokas, A. (2015). Examining the evolution of the regulatory circuit controlling secondary metabolism and development in the fungal genus *Aspergillus*. *PLoS Genet.* 11:1005096. doi: 10.1371/journal.pgen.1005096

López-Berges, M. S., Hera, C., Sulyok, M., Schäfer, K., Capilla, J., Guarro, J., et al. (2013). The velvet complex governs mycotoxin production and virulence of *Fusarium oxysporum* on plant and mammalian hosts. *Mol. Microbiol.* 87, 49–65. doi: 10.1111/mmi.12082

Mahata, P. K., Dass, R. S., Pan, A., and Muthusamy, B. (2022). Substantive morphological descriptions, phylogenetic analysis and single-nucleotide polymorphisms of *Aspergillus* species from *Foeniculum vulgare*. *Front. Microbiol.* 13:832320. doi: 10.3389/fmicb.2022.832320

Malini, T., Vanithakumari, G., Megala, N., Anusya, S., Devi, K., and Elango, V. (1985). Effect of *Foeniculum vulgare* Mill seed extract on the genital organs of male and female rats. *Indian J. Physiol. Pharmacol.* 29, 21–26.

Marley, E., Brown, P., Mackie, J., Donnelly, C., Wilcox, J., Pietri, A., et al. (2015). Analysis of sterigmatocystin in cereals, animal feed, seeds, beer and cheese by immunoaffinity column clean-up and HPLC and LC-MS/MS quantification. *Food Addit. Contam. A* 32, 2131–2137. doi: 10.1080/19440049.2015.1100331

Moloney, C. (2019). *First post*. Available online at: https://www.firstpost.com/business/indias-major-agricultural-produce-losses-estimated-at-rs-92000-cr-2949002.html (accessed February 16, 2021).

Motloung, L., De Saeger, S., De Boevre, M., Detavernier, C., Audenaert, K., Adebo, O. A., et al. (2018). Study on mycotoxin contamination in South African food spices. *World Mycotoxin J.* 11, 401–409. doi: 10.3920/WMJ2017.2191

Navale, V., Vamkudoth, K. R., Ajmera, S., and Dhuri, V. (2021). *Aspergillus* derived mycotoxins in food and the environment: Prevalence, detection, and toxicity. *Toxicol. Rep.* 8, 1008–1030. doi: 10.1016/j.toxrep.2021.04.013

Osman, N. N., Backer, W. S., and Al-Ahmadi, A. M. (2017). The role of senna and fennel in ameliorating cardiovascular disease in diabetic rats. *Am. J. Pharm. Health Res.* 5, 48–59. doi: 10.21786/bbrc/15.1.32

Patananan, A. N., Palmer, J. M., Garvey, G. S., Keller, N. P., and Clarke, S. G. (2013). A novel automethylation reaction in the Aspergillus nidulans LaeA protein generates S-methylmethionine. *J. Biol. Chem.* 288, 14032–14045. doi: 10.1074/jbc.M113.465765

Payne, G. A., and Brown, M. P. (1998). Genetics and physiology of aflatoxin biosynthesis. *Annu. Rev. Phytopathol.* 36, 329–362. doi: 10.1146/annurev.phyto.36.1.329

Pitt, J. I., and Hocking, A. D. (eds). (2009). "Aspergillus and related teleomorphs," in *Fungi and food spoilage*, (Boston, MA: Springer). doi: 10.1007/978-0-387-92207-2_8

Polizeli, M. L., Vici, A. C., Scarcella, A. S., Cereia, M., and Pereira, M. G. (2016). "Enzyme system from *Aspergillus* in current industrial uses and future applications in the production of second-generation ethanol," in *New and future developments in microbial biotechnology and bioengineering*, ed. V. K. Gupta (Amsterdam: Elsevier), 127–140. doi: 10.1016/B978-0-444-63505-1.00009-9

Proctor, R. H., Brown, D. W., Plattner, R. D., and Desjardins, A. E. (2003). Coexpression of 15 contiguous genes delineates a fumonisin biosynthetic gene cluster in *Gibberella moniliformis*. *Fungal Genet. Biol.* 38, 237–249. doi: 10.1016/S1087-1845(02)00525-X

Pusztahelyi, T., Holb, I. J., and Pócsi, I. (2015). Secondary metabolites in fungus-plant interactions. *Front. Plant Sci.* 6:573. doi: 10.3389/fpls.2015.00573

Ramamoorthy, V., Dhingra, S., Kincaid, A., Shantappa, S., Feng, X., and Calvo, A. M. (2013). The putative C2H2 transcription factor MtfA is a novel regulator of secondary metabolism and morphogenesis in *Aspergillus nidulans*. *PLoS One* 8:74122. doi: 10.1371/journal.pone.0074122

Rather, M. A., Dar, B. A., Sofi, S. N., Bhat, B. A., and Qurishi, M. A. (2016). *Foeniculum vulgare*: A comprehensive review of its traditional use, phytochemistry, pharmacology, and safety. *Arab. J. Chem.* 9, S1574–S1583. doi: 10.1016/j.arabjc.2012.04.011

Refai, M., El-Yazid, H. A., and Hassan, A. (2014). "Monograph on *Aspergillus* and aspergillosis in man, animals and birds," in *A guide for classification and identification of Aspergilli, diseases caused by them, diagnosis and treatment*, eds M. Refai, H. A. El-Yazid, and A. Hassan (Cambridge, MA: Academia Press).

Reinholds, I., Pugajeva, I., Bavrins, K., Kuckovska, G., and Bartkevics, V. (2017). Mycotoxins, pesticides and toxic metals in commercial spices and herbs. *Food Addit. Contam. B* 10, 5–14. doi: 10.1080/19393210.2016.1210244

Samadi-Noshahr, Z., Hadjzadeh, M. A., Moradi-Marjaneh, R., and Khajavi-Rad, A. (2021). The hepatoprotective effects of fennel seeds extract and trans-anethole in streptozotocin-induced liver injury in rats. *Food Sci. Nutr.* 9, 1121–1131. doi: 10.1002/fsn3.2090

Sarikaya Bayram, Ö., Bayram, Ö., Valerius, O., Park, H. S., Irniger, S., Gerke, J., et al. (2010). LaeA control of velvet family regulatory proteins for light-dependent development and fungal cell-type specificity. *PLoS Genet.* 6:1001226. doi: 10.1371/journal.pgen.1001226

Segal, B. H. (2009). "Invasive *Aspergillosis* in chronic granulomatous disease," in *Aspergillosis: From diagnosis to prevention*, ed. A. C. Pasqualotto (Dordrecht: Springer), 527–543. doi: 10.1007/978-90-481-2408-4_31

Seo, J. A., Guan, Y., and Yu, J. H. (2006). FluG-dependent asexual development in *Aspergillus nidulans* occurs via derepression. *Genetics* 172, 1535–1544. doi: 10.1534/genetics.105.052258

Seyedmousavi, S., Guillot, J., Arné, P., De Hoog, G. S., Mouton, J. W., Melchers, W. J., et al. (2015). *Aspergillus* and aspergilloses in wild and domestic animals: A global health concern with parallels to human disease. *Med. Mycol.* 53, 765–797. doi: 10.1093/mmy/myv067

Sharma, D., Capoor, M., Ramesh, V., Gupta, S., Shivaprakash, M., and Chakrabarti, A. (2015). A rare case of onychomycosis caused by *Emericella quadrilineata* (*Aspergillus tetrazonus*). *Indian J. Med. Microbiol.* 33, 314–316. doi: 10.4103/0255-0857.153561

Siruguri, V., and Bhat, R. V. (2015). Assessing intake of spices by pattern of spice use, frequency of consumption and portion size of spices consumed from routinely prepared dishes in southern India. *Nutr. J.* 14:7. doi: 10.1186/1475-2891-14-7

Slot, J. C., and Rokas, A. (2011). Horizontal transfer of a large and highly toxic secondary metabolic gene cluster between fungi. *Curr. Biol.* 21, 134–139. doi: 10.1016/j.cub.2010.12.020

Sugui, J. A., Pardo, J., Chang, Y. C., Müllbacher, A., Zarember, K. A., Galvez, E. M., et al. (2007). Role of *laeA* in the regulation of *alb1*, *gliP*, conidial morphology, and virulence in *Aspergillus fumigatus*. *Eukaryot. Cell* 6, 1552–1561. doi: 10.1128/EC.00140-07

Tabata, S. (2011). "Yeasts and moldsâĂŞmycotoxins: Aflatoxins and related compounds," in *Encyclopedia of dairy sciences*, 2nd Edn, ed. J. W. Fuquay (Cambridge, MA: Academic Press), 801–811. doi: 10.1016/B978-0-12-374407-4.00369-1

Tam, E. W., Chen, J. H., Lau, E. C., Ngan, A. H., Fung, K. S., Lee, K. C., et al. (2014). Misidentification of *A. nomius* and *Aspergillus tamarii* as *Aspergillus flavus*: Characterization by internal transcribed spacer, β-tubulin, and calmodulin gene sequencing, metabolic fingerprinting, and matrix-assisted laser desorption ionization–time of flight mass spectrometry. *J. Clin. Microbiol.* 52, 1153–1160. doi: 10.1128/JCM.03258-13

Thom, C., and Raper, K. B. (1945). *A manual of the Aspergilli*, Vol. 60. Philadelphia: LWW, 333.

Tilburn, J., Sarkar, S., Widdick, D. A., Espeso, E. A., Orejas, M., Mungroo, J., et al. (1995). The *Aspergillus* PacC zinc finger transcription factor mediates regulation of both acid-and alkaline-expressed genes by ambient pH. *EMBO J.* 14, 779–790. doi: 10.1002/j.1460-2075.1995.tb07056.x

Uhrin, G. B., Jensen, R. H., Korup, E., Grønlund, J., Hjort, U., Moser, C., et al. (2015). Recurrent prosthetic valve endocarditis caused by *Aspergillus delacroxii* (formerly *Aspergillus nidulans* var. *echinulatus*). *Med. Mycol. Case Rep.* 10, 21–23. doi: 10.1016/j.mmcr.2015.12.005

Varga, J., and Samson, R. A. (2008). *Aspergillus* in the genomic era. Wageningen: Academic Publishers.

Variane, A. C., Santos, F. C., Castro, F. F., Barbosa-Tessmann, I. P., Santos, G. T., and Pozza, M. S. (2018). The occurrence of aflatoxigenic *Aspergillus* spp. in dairy cattle feed in Southern Brazil. *Braz. J. Microbiol.* 49, 919–928. doi: 10.1016/j.bjm.2018.05.005

Veršilovskis, A., De Saeger, S., and Mikelsone, V. (2008). Determination of sterigmatocystin in beer by high performance liquid chromatography with ultraviolet detection. *World Mycotoxin J.* 1, 161–166. doi: 10.3920/WMJ2008.x012

Watanabe, C. M., and Townsend, C. A. (2002). Initial characterization of a type I fatty acid synthase and polyketide synthase multienzyme complex NorS in the biosynthesis of aflatoxin B1. *Chem. Biol.* 9, 981–988. doi: 10.1016/S1074-5521(02)00213-2

Watanabe, C. M., Wilson, D., Linz, J. E., and Townsend, C. A. (1996). Demonstration of the catalytic roles and evidence for the physical association of type I fatty acid synthases and a polyketide synthase in the biosynthesis of aflatoxin B1. *Chem. Biol.* 3, 463–469. doi: 10.1016/S1074-5521(96)90094-0

Wiemann, P., Brown, D. W., Kleigrewe, K., Bok, J. W., Keller, N. P., Humpf, H. U., et al. (2010). FfVel1 and *FfLae1*, components of a velvet-like complex in *Fusarium fujikuroi*, affect differentiation, secondary metabolism, and virulence. *Mol. Microbiol.* 77, 972–994. doi: 10.1111/j.1365-2958.2010.07263.x

Wieser, J., Lee, B. N., Fondon, J. W., and Adams, T. H. (1994). Genetic requirements for initiating asexual development in *Aspergillus nidulans*. *Curr. Genet.* 27, 62–69. doi: 10.1007/BF00326580

Xie, G. F., Li, W. J., Lu, J., Cao, Y., Fang, H., Zou, H. J., et al. (2007). Isolation and identification of representative fungi from Shaoxing rice wine wheat Qu using a

polyphasic approach of culture-based and molecular-based methods. *J. Inst. Brew.* 113, 272–279. doi: 10.1002/j.2050-0416.2007.tb00287.x

Young, C. A., Felitti, S., Shields, K., Spangenberg, G., Johnson, R. D., Bryan, G. T., et al. (2006). A complex gene cluster for indole-diterpene biosynthesis in the grass endophyte *Neotyphodium lolii*. *Fungal Genet. Biol.* 43, 679–693. doi: 10.1016/j.fgb.2006.04.004

Yu, J., Chang, P. K., Ehrlich, K. C., Cary, J. W., Bhatnagar, D., Cleveland, T. E., et al. (2004). Clustered pathway genes in aflatoxin biosynthesis. *Appl. Environ. Microbiol.* 70, 1253–1262. doi: 10.1128/AEM.70.3.1253-1262.2004

Yu, J., Mu, X., and Li, R. (2013). Invasive pulmonary aspergillosis due to *Emericella nidulans* var. *echinulata*, successfully cured by voriconazole and micafungin. *J. Clin. Microbiol.* 51, 1327–1329. doi: 10.1128/JCM.02487-12

Yu, J. H., and Keller, N. (2005). Regulation of secondary metabolism in filamentous fungi. *Annu. Rev. Phytopathol.* 43, 437–458. doi: 10.1146/annurev.phyto.43.040204.140214

Yu, J. H., Butchko, R. A., Fernandes, M., Keller, N. P., Leonard, T. J., and Adams, T. H. (1996b). Conservation of structure and function of the aflatoxin regulatory gene *aflR* from *Aspergillus nidulans* and *A. flavus*. *Curr. Genet.* 29, 549–555. doi: 10.1007/BF02426959

Yu, J. H., Wieser, J., and Adams, T. H. (1996a). The *Aspergillus FlbA* RGS domain protein antagonizes G-protein signaling to block proliferation and allow development. *EMBO J.* 15, 5184–5190. doi: 10.1002/j.1460-2075.1996.tb00903.x

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