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Prevalence, antimicrobial resistance, and genetic characteristics of *Staphylococcus aureus* isolates in frozen flour and rice products in Shanghai, China

Jiang Chang, Yi Zhang, Zengfeng Zhang, Bo Chen, Shoukui He, Zeqiang Zhan, Nan Zhong, Xiaorong Tian, Shimo Kang, Kannappan Arunachalam, Chunlei Shi *

MOST-USDA Joint Research Center for Food Safety, School of Agriculture and Biology, and State Key Laboratory of Microbial Metabolism, Shanghai Jiao Tong University, Shanghai, 200240, PR China

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ABSTRACT

Staphylococcus aureus is widely recognized as a highly hazardous pathogen that poses significant threats to food safety and public health. This study aimed to assess the prevalence, antimicrobial resistance, and genetic characteristics of *S. aureus* isolates recovered from 288 frozen flour and rice product samples in Shanghai, China, between September 2019 and May 2020. A total of 81 *S. aureus* isolates were obtained, representing 25 sequence types (STs), with ST7 being the most prevalent (17.28%, n = 14). The majority of *S. aureus* isolates (85.19%, n = 69) carried at least one enterotoxin gene, with the *seg* gene being the most frequently detected (51.85%, n = 42). Additionally, 12 isolates (14.81%) were identified as methicillin-resistant *S. aureus* (MRSA) through *mecA* gene detection. Notably, this study reported the presence of an ST398 MRSA isolate in frozen flour and rice products for the first time. All MRSA isolates displayed multidrug resistance, with the highest resistance observed against cefoxitin (100.00%), followed by penicillin (91.67%) and erythromycin (66.67%). Genomic analysis of the 12 MRSA isolates revealed the presence of twenty distinct acquired antimicrobial resistance genes (ARGs), eight chromosomal point mutations, and twenty-four unique virulence genes. Comparative genome analysis indicated close genetic relationships between these MRSA isolates and previously reported MRSA isolates from clinical infections, highlighting the potential transmission of MRSA through the food chain and its implications for public health. Significantly, the identification of three plasmids harboring ARGs, insertion sequences (ISs), the origin of transfer site (*oriT*), and the relaxase gene suggested the potential for horizontal transfer of ARGs via conjugative plasmids in *S. aureus*. In conclusion, this study revealed significant contamination of retail frozen flour and rice products with *S. aureus*, and provided essential data for ensuring food safety and protecting public health.

1. Introduction

Frozen flour and rice products have become highly popular for their convenience in consumers' daily lives. However, the production process of these products involves complex manual operations and the use of various ingredients (Wang et al., 2017), which increases the potential risk of contamination by *Staphylococcus aureus* (*S. aureus*), a significant foodborne pathogen (Fetsch and Johler, 2018). This contamination can significantly impact the quality of the products. Moreover, *S. aureus* can transmit through the food supply chain, posing a serious threat to consumer health, including severe infections, poisoning, or even death (Fetsch et al., 2018; Oniciuc et al., 2017). Therefore, it is essential to conduct comprehensive research on the characteristics of *S. aureus* in frozen flour and rice products, which could contribute to ensuring food safety and public health.

The primary mechanisms through which *S. aureus* poses a threat to human health are its ability to invade and cause infections in the human body while evading immune defenses, as well as its production of enterotoxins (Akineden et al., 2001; Papadopoulos et al., 2019). Enterotoxins, in particular, have been widely observed and even small amounts can cause serious food poisoning (Liu et al., 2022). Moreover, these enterotoxins have the capability to persist in various carriers for extended periods due to their resistance to harsh environments, further increasing the risk of *S. aureus*-related poisoning (Schelin et al., 2017).

* Corresponding author.
E-mail address: clshi@sjtu.edu.cn (C. Shi).

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Currently, over twenty types of staphylococcal enterotoxins have been reported, including the five classical staphylococcal enterotoxins (SEA, SEB, SEC, SEC, SED, and SEE), as well as several new staphylococcal enterotoxins (SEG, SEH, SEI, SEJ, etc.) (Chieffi et al., 2020). At the genetic level, genes such as *sea*, *seb*, *sec*, *sed*, and others are responsible for the production of these enterotoxins in *S. aureus* (Chieffi et al., 2020). In general, the presence and expression of enterotoxin genes indicate the ability of a given *S. aureus* isolate to produce enterotoxins (Cai et al., 2021), and this phenomenon was regarded as an important evidence of the production ability of enterotoxins in *S. aureus*, which can provide crucial insights into its enterotoxin-related pathogenic potential.

In recent years, the misuse of antimicrobial agents has led to the emergence of antimicrobial resistance in *S. aureus* isolates, posing a significant challenge for the treatment of *S. aureus* infections (Algammal et al., 2020). Methicillin-resistant *S. aureus* (MRSA) is a major global concern among antimicrobial-resistant *S. aureus*, as it confers resistance to most antimicrobial agents used in clinical settings, particularly β-lactam antimicrobial agents, significantly increasing the difficulty of clinical treatment (Algammal et al., 2020). Understanding the pathogenic characteristics and epidemiological patterns of drug-resistant bacteria is crucial for effectively preventing and controlling these significant threats. Whole-genome sequencing (WGS) is an exceptional technology that allows us to comprehensively understand the complete genetic determinants of foodborne pathogens, including antimicrobial resistance, virulence production, and pathogenesis. Moreover, WGS enables accurate identification of pathogens in food, facilitates source tracing of contamination, promotes data sharing within and between countries for comparative analysis, and supports the implementation of effective global control measures (Li et al., 2022). Given its importance, the characterization of MRSA using WGS can provide valuable insights into the mechanisms of antimicrobial resistance, transmission features, and potential risks to food safety and public health.

This comprehensive study aims to investigate the prevalence and characteristics of *S. aureus* in retail frozen flour and rice products in Shanghai, China. Initially, *S. aureus* was isolated from a certain number of retail frozen flour and rice products, and the sequence type profile, as well as enterotoxin genes of *S. aureus*, were identified and discussed. Additionally, the antimicrobial resistance patterns and genomic features of MRSA isolates were analyzed, providing new insights into the development of antimicrobial resistance in MRSA isolates. The obtained results will provide valuable data to support risk assessment related to frozen flour and rice products, ultimately contributing to improved food safety and public health measures.

2. Materials and methods

2.1. Sample collection and identification of isolates

During the period between September 2019 and May 2020, a total of 288 samples of frozen flour and rice products were collected from six local supermarkets across six districts of Shanghai, China. Specifically, on average, each supermarket was sampled twice, with an interval of approximately one month between two sampling events. In each round of sampling, about 25 samples were collected. The samples consisted of various frozen flour and rice products that were commercially available, such as frozen dumplings, frozen tangyuan, frozen buns, and others. Following collection, the samples were immediately stored at a temperature of 4 °C and transported to the laboratory within a maximum of 3 h. Subsequently, further processing was carried out. These samples were categorized into three groups based on their composition: meat-stuffed (38.54%, 111/288), vegetable-stuffed (34.38%, 99/288), and non-stuffed (27.08%, 78/288).

To isolate *S. aureus*, the collected samples were subjected to specific laboratory procedures (Ou et al., 2020). Firstly, the 7.5% (w/v) sodium chloride broth (Land Bridge Technology Ltd., Beijing, China) was incubated with the samples at 37 °C for 18 h. Subsequently, Baird-Parker (BP) agar plates supplemented with yolk potassium citrate potassium enrichment solution (Land Bridge Technology Ltd., Beijing, China) were used to selectively culture *S. aureus*. The presumptive colonies were then selected and subjected to genomic DNA extraction using the SPARKeasy Bacteria DNA Kit (Sparkjade Biotechnology Co., Ltd., Shandong, China). PCR amplification was performed using specific primers, namely nuc1F (5′-AGTATATAGTGCAACTTCAACTAA-3′) and nuc1R (5′-ATCAGCG TTGTCTTCGCTCCAAAT-3′), to detect the presence of *S. aureus*. Additionally, another PCR amplification was conducted using specific primers, mecAF (5′-ATCATAGCGTCATTATTCCAGG-3′) and mecAR (5′-TGTCTGCCAGTTTCTCCTTG-3′), to identify MRSA isolates by detecting the presence of *mecA* gene. *S. aureus* strain ATCC 25923 and MRSA strain ATCC 43300 were included as quality control measures.

2.2. Multilocus sequence typing (MLST)

The MLST analysis of *S. aureus* isolates was performed following established protocols (Goudarzi et al., 2017). The isolates were streaked onto BP agar plates and incubated at 37 °C. Genomic DNA was extracted using the SPARKeasy Bacteria DNA Kit (Sparkjade Biotechnology Co., Ltd., Shandong, China). PCR amplification was carried out for seven housekeeping genes specific to *S. aureus* (*arcC*, *aroE*, *glpF*, *gmk*, *pta*, *tpi*, and *yqiL*). The resulting PCR products were then sequenced bi-directionally (performed by Meiji Biology Ltd, Shanghai, China). These sequences were compared to the MLST database (https://pubmlst.org/organisms/staphylococcus-aureus) to determine the allelic profiles, represented by sequence types (STs) (Jolley et al., 2018). Finally, the phylogenetic relationship among the *S. aureus* isolates based on their STs was analyzed using the GrapeTree software (Tang et al., 2020).

2.3. Detection of staphylococcal enterotoxin genes

To detect the presence of staphylococcal enterotoxin genes in *S. aureus* isolates, the isolates were streaked onto BP agar plates and incubated at 37 °C. Genomic DNA was extracted using the SPARKeasy Bacteria DNA Kit (Sparkjade Biotechnology Co., Ltd., Shandong, China). The extracted DNA from *S. aureus* served as a template for PCR amplification using specific primers designed for 18 enterotoxin genes (*sea-see*, *seg-ser*, and *seu*) (Supplementary Table S1). These primers were synthesized at Sangon Ltd., (Shanghai, China). The PCR protocol consisted of an initial denaturation step of 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s. Agarose gel electrophoresis was then performed, and the presence of specific enterotoxin genes was determined based on the presence of corresponding bands on the gel. Positive controls were included using standard strains with known enterotoxin genes: strain ATCC 8095 for genes *sea*, *sed*, *sej*, *sek*, *seq*, and *ser*; strain ATCC 14458 for *seb* gene; strain ATCC 27664 for *see* gene; strain ATCC 27661 for genes *seg*, *sei*, *sem*, *sen*, and *seo*. Additionally, some *S. aureus* isolates previously detected and stored in our laboratory, known to carry specific enterotoxin genes, were also utilized as positive controls: strain I073 for genes sec and *sel*; strain C054 for *sep* gene; strain B044 for *seh* gene; strain C344 for *seu* gene (Song et al., 2015).

2.4. Antimicrobial susceptibility testing

The agar dilution method was used to perform the antimicrobial susceptibility assay (Wang et al., 2022). A selection of antimicrobial agents including oxacillin (OXA; 0.25–128 μg/mL), clindamycin (CLI; 0.03125–64 μg/mL), penicillin (PEN; 0.015625–16 μg/mL), gentamicin (GEN; 0.03125–128 μg/mL), rifampicin (RIF; 0.03125–32 μg/mL), ciprofloxacin (CIP; 0.125–64 μg/mL), erythromycin (ERY; 0.0625–128 μg/mL), cefoxitin (CFX; 0.5–512 μg/mL), vancomycin (VAN; 0.25–8 μg/mL), chloramphenicol (CHL; 0.5–256 μg/mL), linezolid (LZD; 0.25–8 μg/mL), tetracycline (TET; 0.0625–128 μg/mL), nitrofurantoin (NIT; 1–128 μg/mL), teicoplanin (TEC; 0.0625–8 μg/mL) and levofloxacin (LEV; 0.03125–64 μg/mL) were chosen for this assay. These

antimicrobial agents were obtained from Sigma Aldrich, Shanghai Trading Co. Ltd., China. The antimicrobial agents were added to Mueller Hinton (MH) agar plates (Land Bridge Technology Ltd., Beijing, China) at different concentrations. MRSA isolates were streaked onto BP agar plates and incubated at 37 °C. A single colony was selected and incubated with MH broth at 37 °C. The resulting suspension was diluted to a suitable concentration of $OD_{600} = 0.5$ (approximately 1×10^8 CFU/mL) and streaked onto the MH agar plates containing different antimicrobial agents. The plates were then incubated at 37 °C for 18 h. The growth of MRSA isolates on the plates was observed to determine their susceptibility to the antimicrobial agents. The breakpoints for each antimicrobial agent were set according to CLSI 2019: M100-S29 (CLSI, 2019) and EUCAST 2019: version 5.0 guidelines. *S. aureus* ATCC 29213 strain was used as a quality control in all the assays.

2.5. Whole-genome sequencing and sequence analysis

All MRSA isolates were streaked onto BP agar plates and incubated at 37 °C. A single colony was selected and incubated with Tryptone Soya broth (Land Bridge Technology Ltd., Beijing, China) at 37 °C. The resulting suspension was diluted to an appropriate concentration of $OD_{600} = 0.5$ (about 1×10^8 CFU/mL) and centrifuged at 8000 rpm for 5 min to remove the supernatant. Genomic DNA of the MRSA isolates was extracted from the bacterial block using the SPARKeasy Bacteria DNA Kit (Sparkjade Biotechnology Co., Ltd., Shandong, China). The concentration and purity of the DNA were assessed. Once the DNA sample met the quality standards for WGS, an Illumina NovaSeq platform was used to perform PE150 (paired-end) sequencing. The raw sequencing data needed to meet a minimum requirement of 100× genome coverage depth, and certain essential tests were conducted. Subsequently, the raw sequencing data was assembled using SOAPdenovo v2.04 software to ensure high quality. The assembled contigs were then analyzed using a BLAST search in the NCBI database to retrieve gene-related information (Tatusova et al., 2016). Plasmids were assembled using plasmidSPAdes based on the assembled contigs (Antipov et al., 2016). Assembled plasmids that exhibited at least 95.00% percent identity to any plasmid in the NCBI database through BLAST were included in the analysis.

Software from the Center for Genomic Epidemiology (http://www.genomicepidemiology.org/services/) was used to perform some necessary bioinformatic analyses. Specifically, spaTyper was used to predict the *spa* type of *S. aureus* (Bartels et al., 2020). ResFinder 4.1 was used to predict the acquired antimicrobial resistance genes, chromosomal point mutations, and acquired disinfectant resistance genes carried by *S. aureus* (Bortolaia et al., 2020; Zankari et al., 2017). The SCC*mec* element was identified using SCCmecFinder 1.2 (Li et al., 2009). VirulenceFinder 2.0 was used to identify virulence genes (Malberg Tetzschner Anna et al., 2020). PlasmidFinder 2.1 was used to identify the incompatibility group of the plasmids (Carattoli et al., 2014).

In addition, ISfinder was used to identify the insertion sequence (IS) in the plasmid (Siguier et al., 2006). The conjugative ability of the plasmids was predicted using oriTfinder (Li et al., 2018). For phylogenetic analysis based on the maximum-likelihood (ML) method, the KSNP3 software was utilized (Gardner et al., 2015). Finally, the composition of the phylogenetic tree was refined using MEGA X and iTOL (Kumar et al., 2018; Letunic and Bork, 2021).

2.6. Nucleotide sequence accession numbers

The complete genomes were deposited in GenBank under the accession numbers JAHXFZ000000000 (SJTUF21415), JAHXGB000000000 (SJTUF21434), JAHXGC000000000 (SJTUF21454), JAHXGD000000000 (SJTUF21455), JAHXGE000000000 (SJTUF21457), JAHXGF000000000 (SJTUF21470), JAHXGG000000000 (SJTUF21473), JAHXGH000000000 (SJTUF21491), JAHXGI000000000 (SJTUF21492), JAHXGJ000000000 (SJTUF21501), JADRJJ000000000 (SJTUF21502) and JAHXGK000000000 (SJTUF21506).

3. Results

3.1. The prevalence and sequence type profiles of S. aureus

A total of 81 *S. aureus* isolates were identified in 81 retailed frozen flour and rice samples, resulting in an overall contamination rate of 28.13% (81/288). Among these samples, meat-stuffing samples accounted for 44 isolates (39.64%, 44/111), vegetable-stuffing samples accounted for 23 isolates (23.23%, 23/99), and non-stuffing samples accounted for 14 isolates (17.95%, 14/78) (Supplementary Table S2). Notably, meat-stuffing samples exhibited the highest detection rate. Furthermore, among the isolated *S. aureus* strains, 12 were identified as MRSA, resulting in an overall contamination rate of 4.17% (12/288) (Supplementary Table S2).

In order to determine the genetic relatedness among *S. aureus* isolates, all 81 strains were subtyped using MLST. The results, as depicted in Fig. 1 and Supplementary Table S2, revealed a total of twenty-five different STs among the 81 *S. aureus* isolates. Among them, ST7 was the most prevalent, accounting for 17.28% (14/81) of the isolates. Following ST7, ST1 and ST5 were the next most commonly represented STs, each accounting for 8.64% (7/81) of the isolates.

3.2. Carriage of enterotoxin genes by S. aureus

The presence of 18 enterotoxin genes (*sea* ~ *see*, *seg* ~ *ser*, and *seu*) was examined in the 81 *S. aureus* isolates, and the results are presented in Fig. 2. Among all the isolates, 85.19% (69/81) were found to carry at least one enterotoxin gene. Notably, five isolates (6.17%, 5/81) were found to harbor ten different enterotoxin genes. The most commonly detected enterotoxin gene among the isolates was *seg* (51.85%, 42/81), followed by *sei* (48.15%, 39/81) and *sem* (40.74%, 33/81). Additionally, 37.04% (30/81) of the isolates carried at least one classical enterotoxin

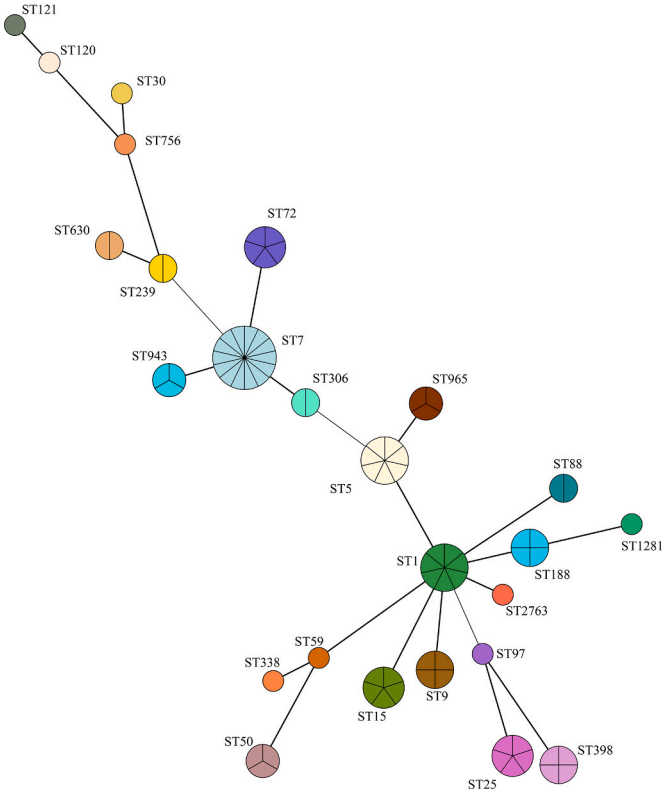


Fig. 1. Minimum spanning tree analysis of 81 *S. aureus* isolates. Each circle represents one ST, and the area of the circle corresponds to the number of isolates.

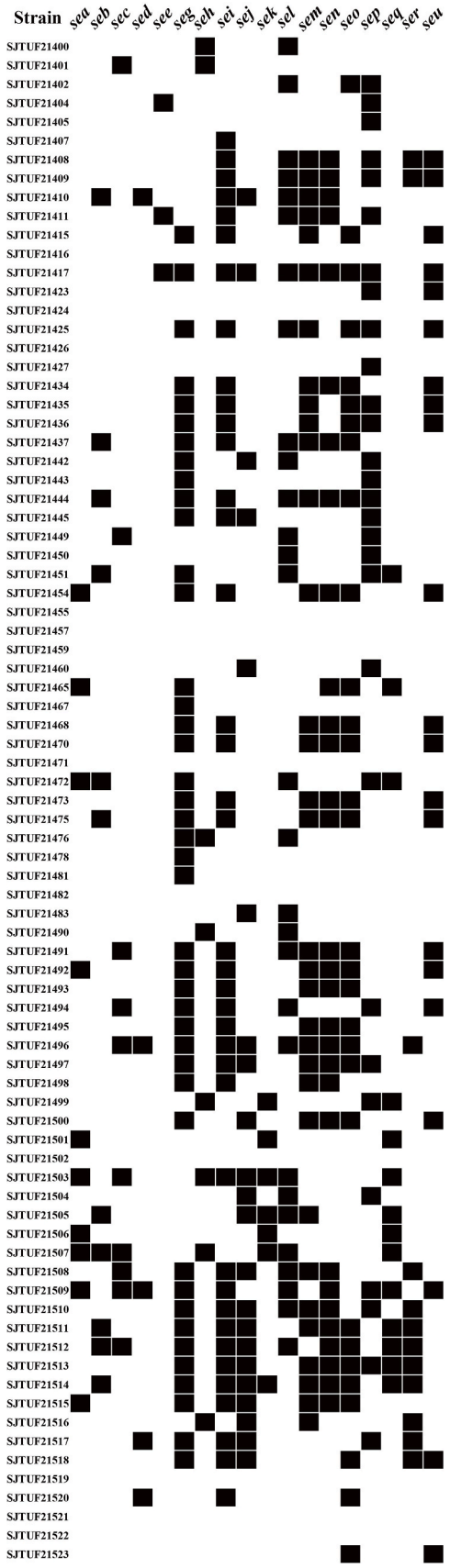


Fig. 2. Distribution of 18 enterotoxin genes (*sea* ~ *see*, *seg* ~ *ser*, *seu*) in 81 *S. aureus* isolates.

gene (*sea* ~ *see*). The most frequently observed classical enterotoxin gene was *seb* (13.58%, 11/81), followed by *sea* (12.35%, 10/81) and *sec* (12.35%, 10/81). Supplementary Table S3 provides further details on the different profiles of classical enterotoxin genes identified among these 30 isolates. The most predominant genotype was *seb* (23.33%, 7/30), followed by *sea* (20.00%, 6/30). The maximum number of multi-enterotoxin gene profiles observed was three, represented by combinations such as *sea-seb-sec* or *sea-sec-sed*.

3.3. The antimicrobial resistance phenotype of MRSA

All 12 MRSA isolates exhibited 100.00% resistance to CFX, which aligns with the typical MRSA phenotype. Specifically, 9 out of 12 (75.00%) MRSA isolates were susceptible to OXA, classifying them as OXA-susceptible MRSA. Among the 12 MRSA isolates, PEN resistance was the second most prevalent, detected in 91.67% (11/12) of the isolates, followed by ERY in 66.67% (8/12) and TET in 58.33% (7/12) (Fig. 3). No resistance to VAN, LZD, NIT, and TEC was observed. All 12 MRSA isolates displayed resistance to a minimum of three antimicrobial agents, with one isolate demonstrating resistance to nine antimicrobial agents (Supplementary Table S4).

3.4. Characterization of the genomic features of MRSA

WGS was conducted on all 12 MRSA isolates, and the results are summarized in Table 1. The analysis revealed the presence of eight prevalent STs among the MRSA isolates, which include ST9, ST72, ST965, ST239, ST338, ST630, ST5, and ST398, consistent with the ST identification results in section 3.1 (Supplementary Table S2). Moreover, seven distinct *spa* types were identified in the 12 MRSA isolates, namely t002, t030, t062, t2970, t437, t4549, and t899. Notably, two isolates could not be classified using *spa* typing. All MRSA isolates harbored a characteristic SCC*mec* element. Specifically, four isolates possessed SCC*mec* type IV, three isolates had SCC*mec* type V, two isolates carried SCC*mec* type III, two isolates contained SCC*mec* type XII, and one isolate featured SCC*mec* type II.

A total of 20 antimicrobial resistance genes (ARGs) were identified in all MRSA isolates (Fig. 4A). These ARGs primarily confer resistance against aminoglycosides, folate pathway antagonists, phenicols, beta-lactams, streptogramin A/pleuromutilin/lincosamide, tetracycline, lincosamides, streptogramin B/macrolide/lincosamide, and steroid antibacterial agents. Each of the 12 isolates carried at least three different ARGs, with 41.67% (5/12) of them harboring at least six ARGs. Notably,

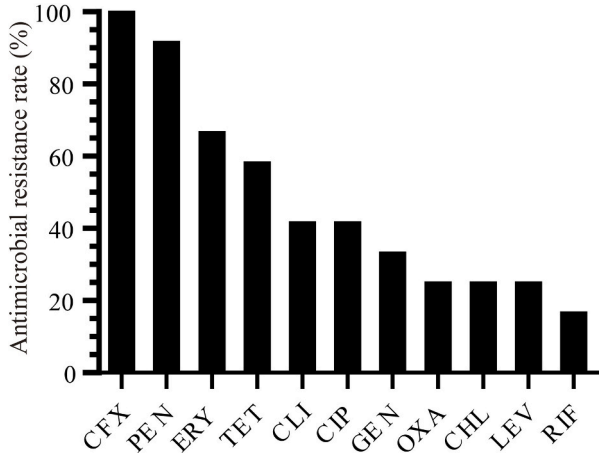


Fig. 3. Antimicrobial resistance rates of 12 MRSA isolates to 15 antimicrobials. OXA: oxacillin, CLI: clindamycin, PEN: penicillin, GEN: gentamicin, RIF: rifampicin, CIP: ciprofloxacin, ERY: erythromycin, CFX: cefoxitin, VAN: vancomycin, CHL: chloramphenicol, LZD: linezolid, TET: tetracycline, NIT: nitrofurantoin, TEC: teicoplanin, LEV: levofloxacin.

Table 1
The genetic characterization of MRSA isolates.

Strain	ST	*spa*	SCC*mec*
SJTUF21415	9	t899	SCC*mec*_type_XII(9C2)
SJTUF21434	9	t899	SCC*mec*_type_XII(9C2)
SJTUF21454	965	t062	SCC*mec*_type_IVc(2B)
SJTUF21455	338	t437	SCC*mec*_type_Vb(5C2&5)
SJTUF21457	630	t4549	SCC*mec*_type_V(5C2&5)
SJTUF21470	72	–	SCC*mec*_type_IVc(2B)
SJTUF21473	72	–	SCC*mec*_type_IVc(2B)
SJTUF21491	5	t002	SCC*mec*_type_II(2A)
SJTUF21492	965	t062	SCC*mec*_type_IVc(2B)
SJTUF21501	239	t030	SCC*mec*_type_III(3A)
SJTUF21502	398	t2970	SCC*mec*_type_V(5C2)
SJTUF21506	239	t030	SCC*mec*_type_III(3A)

the SJTUF21434 isolate was found to carry nine different ARGs, indicating a high potential for multidrug resistance. The presence of the *mecA* gene was confirmed in all isolates, consistent with the findings from section 3.1. The *blaZ* gene was the second most prevalent, detected in 83.33% (10/12) of the isolates, followed by *erm(C)* in 50.00% (6/12) of the isolates. Furthermore, eight chromosomal point mutations were observed across all MRSA isolates, contributing to resistance against steroid antibacterials, fluoroquinolones, and rifamycins (Fig. 4A). Specifically, eight isolates (66.67%, 8/12) exhibited chromosomal point mutations, while no such mutations were detected in SJTUF21455, SJTUF21457, SJTUF21470, and SJTUF21473. Notably, the fusion of *fusA*:p.L461K, associated with steroid antibacterial resistance, was only found in the SJTUF21491 isolate. Additionally, the *qacA* gene, responsible for resistance to quaternary ammonium compounds, was detected in the SJTUF21491 isolate (Fig. 4A).

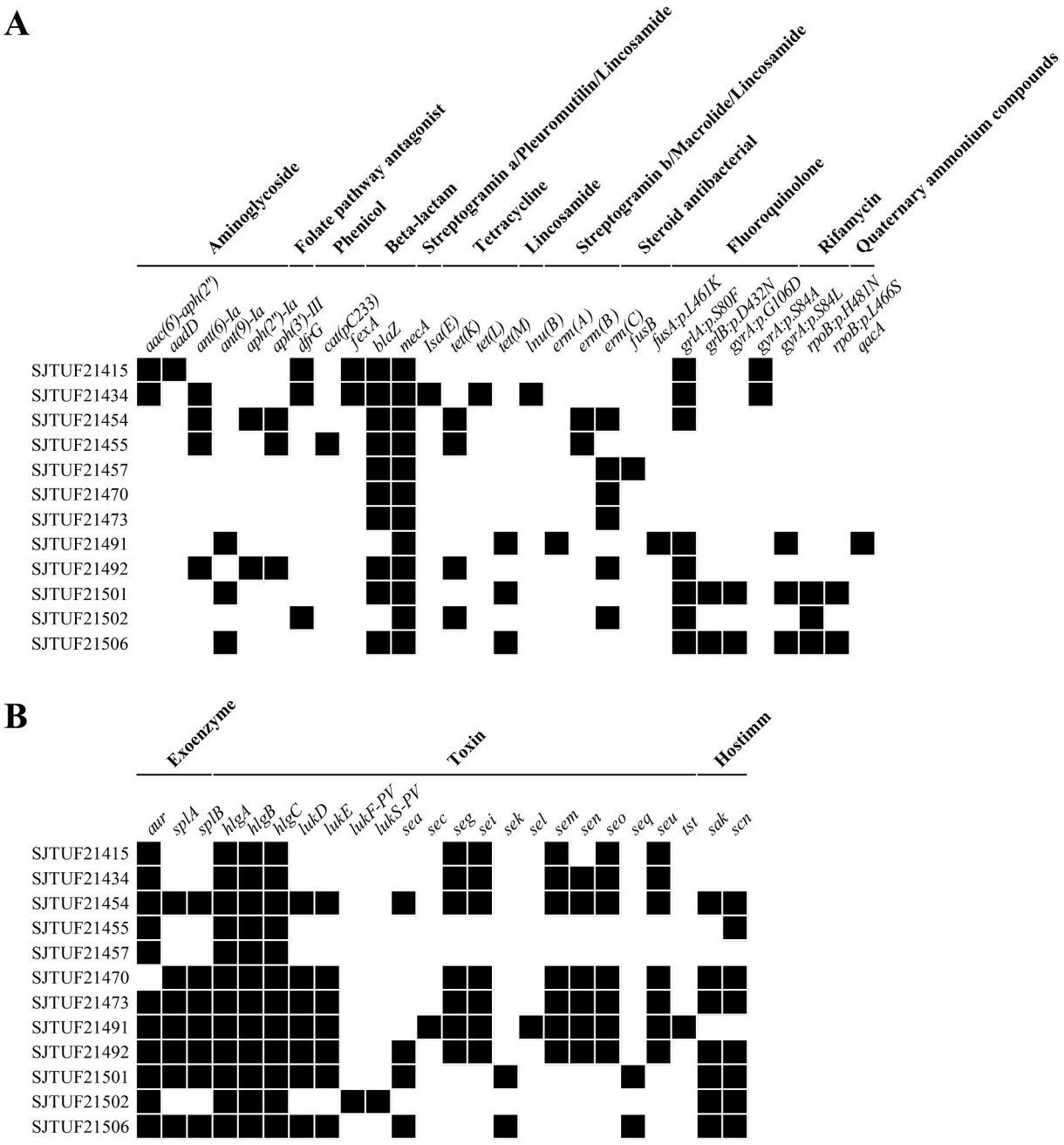


Fig. 4. Prediction results for (A) acquired antimicrobial resistance genes, chromosomal point mutations, acquired disinfectant resistance genes, and (B) virulence genes in 12 MRSA isolates.

A total of 24 virulence genes were identified in all MRSA isolates (Fig. 4B), primarily contributing to various virulence factors such as exoenzymes, toxins, and host immune-modulating proteins. Each of the 12 isolates carried at least four different virulence genes, with an average of 12 virulence genes per isolate. Notably, both the SJTUF21491 and SJTUF21492 isolates were found to harbor 17 different virulence genes. Three virulence genes, *hlgA*, *hlgB*, and *hlgC*, exhibited the highest detection rate of 100.00% (12/12), followed by *aur*, with a detection rate of 91.67% (11/12). The predicted results for enterotoxin genes aligned with the PCR amplification results from section 3.2. Particularly, the SJTUF21502 isolate exhibited the presence of *lukF-PV* and *lukS-PV* genes, while the SJTUF21491 isolate demonstrated the presence of the *tst* gene.

A total of 14 plasmids were identified and classified into six different incompatibility groups in this study (Table 2, Supplementary Material 1). These groups include rep10, rep16-rep19, rep20, rep21, rep7a, and rep7a-rep19. In particular, plasmids sharing identical replicon types exhibited 100.00% sequence identity in their genomes (Data not shown). The rep10 plasmid was universally found to carry the *ermC* gene, while the rep7a plasmid contained the *tet(K)* gene (Table 2). Moreover, the rep7a-rep19 plasmid harbored both the *tet(K)* and *blaZ* genes (Fig. 5B). It is worth noting that the rep16-rep19 plasmid carried the *erm(B)*, *aph(2″)-Ia*, *dfrE*, and *blaZ* genes (Fig. 5A). No virulence genes were detected in any of the plasmids. Additionally, the presence of the origin of transfer site (*oriT*) and relaxase gene was observed in the rep16-rep19 and rep7a-rep19 plasmids (Table 2, Fig. 5), suggesting their potential for conjugative transfer.

3.5. Comparative genome analysis of MRSA

In order to conduct a comprehensive phylogenetic analysis, we compared the genome sequences of our 12 MRSA isolates with previously reported MRSA isolates from various sources and countries. These additional isolates were selected from the third-generation complete genome sequences in the GenBank database and were confirmed to carry the *mecA* gene through genome sequence analysis. The inclusion of MRSA isolates from different countries and isolation sources enhances the comprehensiveness of genome sequence database. A ML method was employed to construct a phylogenetic tree based on SNP analysis, as depicted in Fig. 6. While certain MRSA isolates obtained in this study can be grouped into closely related branches, such as SJTUF21454 and SJTUF21492, indicating potential transmission of these MRSA isolates within similar food types, the overall findings reveal substantial diversity in the evolutionary relationships among the MRSA isolates investigated. This suggests that contamination of frozen flour and rice products by these strains may occur via distinct pathways, emphasizing the diverse and complex nature of the contamination status associated with frozen flour and rice products. Furthermore, the tree revealed that isolates with different STs, hosts, sources, locations, and collection dates could be clustered together, and conversely, isolates from the same region could be found in different lineages. The MRSA isolates exhibited a wide range of phylogenetic relationships, and they clustered with other highly pathogenic MRSA isolates from diverse sources and countries. For instance, the SJTUF21491 isolate displayed a close relationship with two isolates (SR153 and ZJ5499) obtained from human blood samples in Zhejiang, China. Similarly, the isolates SJTUF21470 and SJTUF21473 exhibited proximity to the E16SA093 isolate originating from a human blood sample in South Korea. Likewise, the isolate SJTUF21455 demonstrated a close relationship with other isolates, including ZY05 (human blood infection sample in Zhejiang, China), VGC1 (human blood infection sample in Taiwan, China), SA957 (human blood infection sample in Taiwan, China), and SA268 (human sputum infection sample in Zhejiang, China).

4. Discussion

Frozen flour and rice products have gained popularity in our fast-paced lifestyle due to their convenience, making them an essential component of the food supply chain. However, the presence of *S. aureus* in these frozen products poses a hidden risk to food safety and public health. Unfortunately, there is a lack of comprehensive studies focusing on *S. aureus* in these products, and the available information is insufficient. To bridge this knowledge gap, the current study investigated the prevalence, virulence factors, antimicrobial resistance, and genomic characteristics of *S. aureus* in frozen flour and rice products in Shanghai, China. The findings from this study greatly enhance our understanding of the characteristics of *S. aureus* strains isolated from frozen flour and rice products, providing vital information for ensuring food safety and public health.

A total of 81 strains of *S. aureus* were isolated, exhibiting 25 distinct STs. The wide range of ST patterns observed indicates that contamination of retailed frozen flour and rice products with *S. aureus* could originate from diverse sources, underscoring the complexity of the issue and the formidable challenge of preventing *S. aureus* contamination. In addition, compared to other food types such as milk (Naushad et al., 2020), ready-to-eat foods (Islam et al., 2019), and pork (Li et al., 2021), frozen flour and rice products exhibited a more diverse composition of ST types in *S. aureus* in this study. This may be related to the complex processing ingredients and multi-step processing procedures of frozen flour and rice products, which contributes to diverse potential sources for *S. aureus* contamination. Additionally, it is noteworthy that the diverse composition of ST types in *S. aureus* is often observed in the analysis of clinical isolates (Li et al., 2019; Gu et al., 2020). This indicates the complexity and difficulty in the prevention of *S. aureus* infections from clinical sources. Furthermore, frozen flour and rice products may play an important role in the process of *S. aureus* clinical

Table 2
The genetic characterization of predicted plasmids in MRSA isolates.

Plasmid	Strain	Replicon type	Antimicrobial resistance gene	Virulence gene	Insertion sequence	*oriT*	Relaxase
plasmid21454-1	SJTUF21454	rep16-rep19	*erm(B), aph(2″)-la, dfrE, blaZ*	-	IS*6*	+	+
plasmid21454-2	SJTUF21454	rep7a	*tet(K)*	-	-	-	-
plasmid21454-3	SJTUF21454	rep10	*erm(C)*	-	-	-	-
plasmid21455	SJTUF21455	rep7a-rep19	*tet(K), blaZ*	-	IS*6*	+	+
plasmid21457	SJTUF21457	rep10	*erm(C)*	-	-	-	-
plasmid21470-1	SJTUF21470	rep21	-	-	-	-	-
plasmid21470-2	SJTUF21470	rep10	*erm(C)*	-	-	-	-
plasmid21473-1	SJTUF21473	rep21	-	-	-	-	-
plasmid21473-2	SJTUF21473	rep10	*erm(C)*	-	-	-	-
plasmid21491-1	SJTUF21491	rep20	-	-	-	-	-
plasmid21492-1	SJTUF21492	rep16-rep19	*erm(B), aph(2″)-la, dfrE, blaZ*	-	IS*6*	+	+
plasmid21492-2	SJTUF21492	rep7a	*tet(K)*	-	-	-	-
plasmid21492-3	SJTUF21492	rep10	*erm(C)*	-	-	-	-
plasmid21502-2	SJTUF21502	rep10	*erm(C)*	-	-	-	-

'+': detection positive; '-': detection negative.

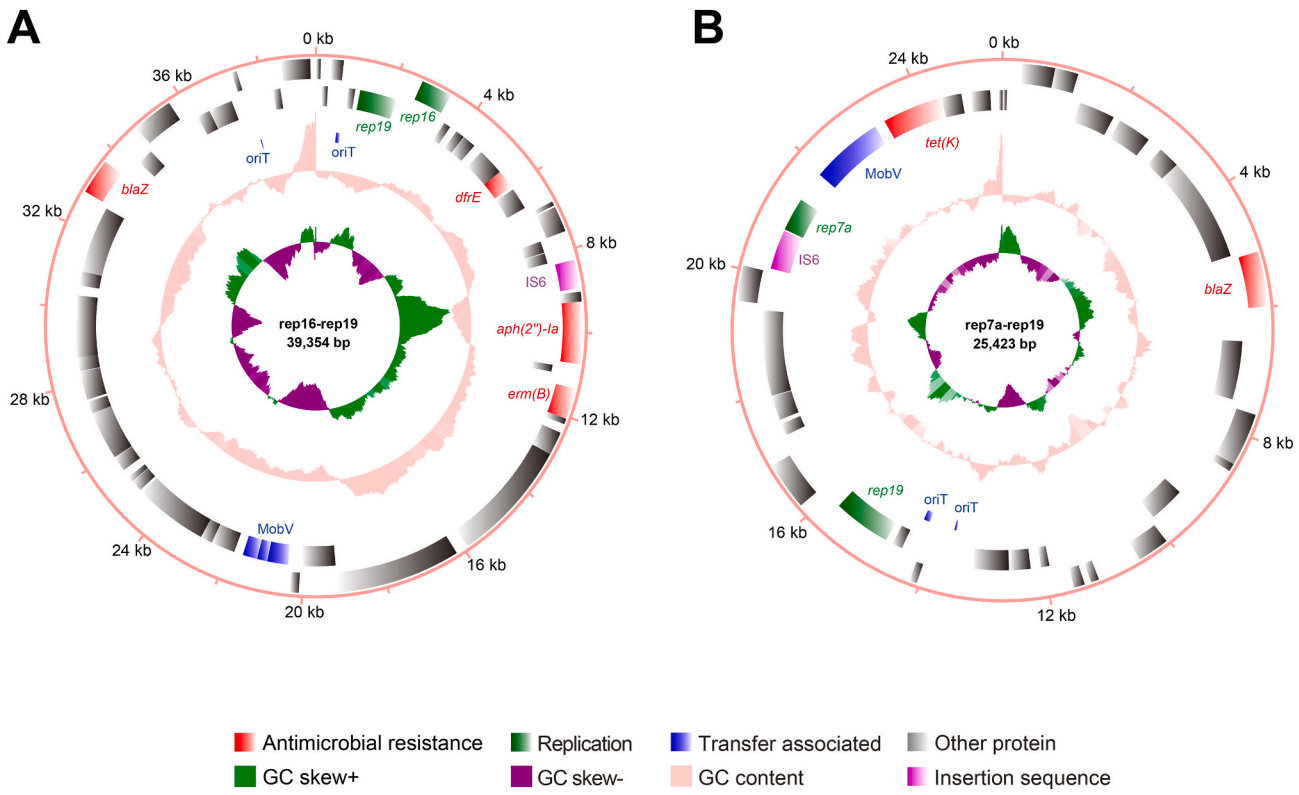


Fig. 5. Representation of the completed genome sequence of predicted plasmids in MRSA isolates. (A) Complete genome sequence map of the rep16-rep19 type plasmid. (B) Complete genome sequence map of the rep7a-rep19 type plasmid.

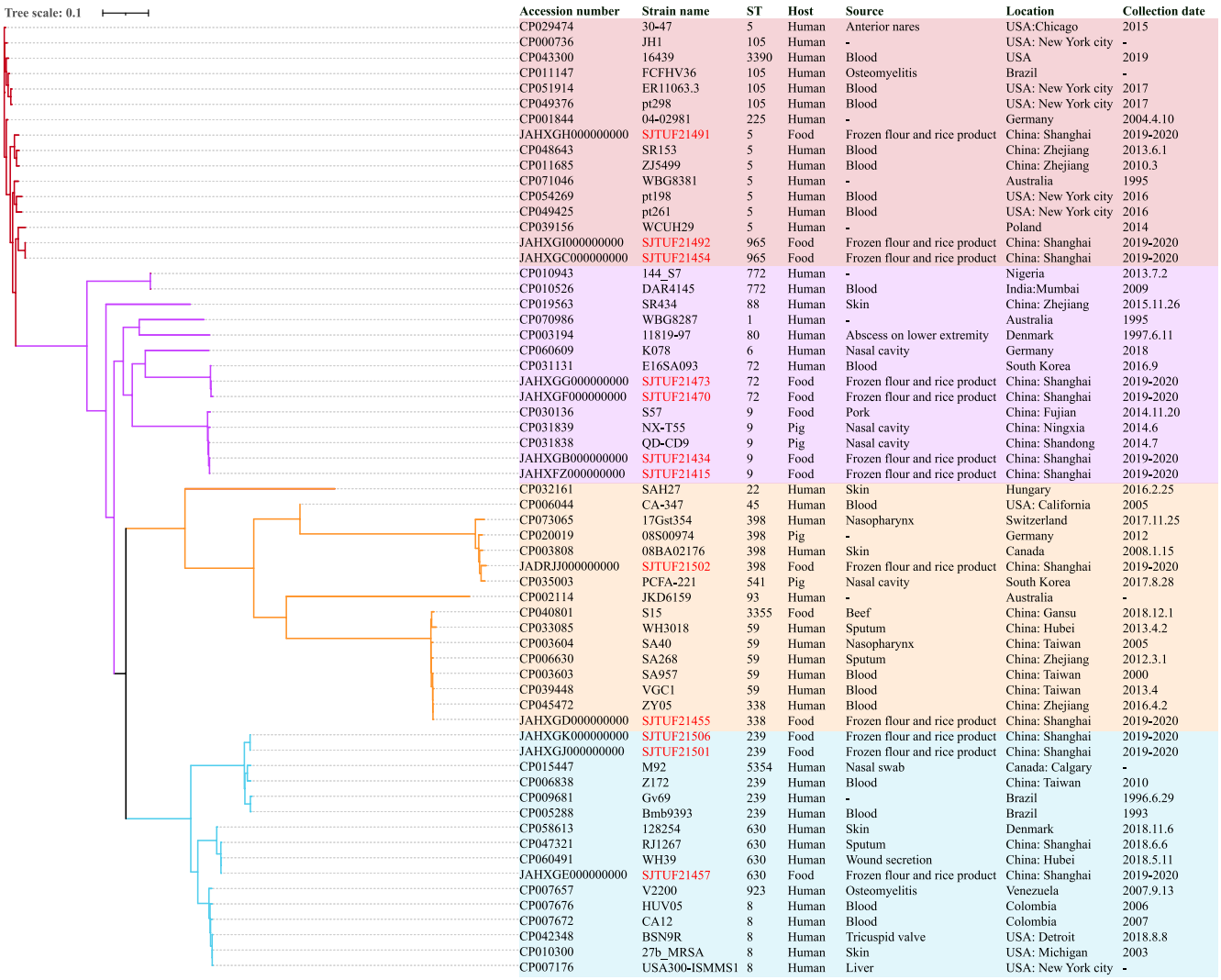
infections through the food chain. This is due to the potential of frozen flour and rice products to serve as carriers for the transmission of multiple ST types of *S. aureus*, providing a possibility for human exposure of various ST types of *S. aureus*. Notably, this study identified several prevalent STs, specifically ST7, ST5, and ST72, which have been linked to clinical infections (Gu et al., 2023; Jian et al., 2021; Yang et al., 2021). All in all, this finding suggests that *S. aureus* present in retailed frozen flour and rice products may serve as potential sources for clinical *S. aureus* infections, highlighting the critical role of frozen flour and rice products in the transmission of *S. aureus*.

In our study, we observed that 85.19% of the isolated strains of *S. aureus* carried enterotoxin genes, and 37.04% of these strains harbored at least one classical enterotoxin gene (*sea* ~ *see*). This finding underscores the widespread capacity of *S. aureus* in frozen flour and rice products to produce enterotoxins. Notably, the detection rate of novel enterotoxin genes surpassed that of classical enterotoxin genes, indicating an emerging characteristic of enterotoxin gene profiles. While it has been commonly reported that five classical enterotoxins are responsible for 95% of *S. aureus* food poisoning (Argudín et al., 2010), recent evidence has revealed the involvement of novel enterotoxin genes, such as *seg* and *sei* genes (Yan et al., 2012), as well as *seg*, *sei*, *sem*, *sen*, *seo*, and *seu* genes (Umeda et al., 2017), in various foodborne outbreaks worldwide. The presence of these novel enterotoxins in *S. aureus* clearly poses a significant threat to human health. Therefore, it is imperative to enhance the significance of detecting novel enterotoxin genes and their corresponding enterotoxins in routine food safety monitoring.

A variety of virulence genes were identified in the MRSA isolates examined in this study. These virulence determinants are associated with the invasion of host tissues and the development of infections, which are distinct from enterotoxins. Specifically, the SJTUF21491 isolate was predicted to carry the *tst* gene, while both the *lukF-PV* and *lukS-PV* genes were predicted in the SJTUF21502 isolate. The *tst* gene encodes toxic shock syndrome toxin-1 (TSST-1), a superantigen produced by *S. aureus* and considered the primary etiological agent of Staphylococcal toxic shock syndrome (Sharma et al., 2019). The *lukF-PV* and *lukS-PV* genes have the potential to encode Panton-Valentine leukocidin, a cytotoxin capable of inducing cell death in various immune cells, including neutrophils, macrophages, and lymphocytes, while also triggering inflammatory responses (Tran et al., 2020). These virulence factors are widely recognized as posing a significant threat to human health, indicating that *S. aureus* prevalent in frozen flour and rice products exhibits diverse and high-risk pathogenicity. Therefore, it is crucial to consider *S. aureus* prevalent in frozen flour and rice products as significant risk factors when monitoring food safety and public health.

ST398 MRSA, a lineage of livestock-associated MRSA, has increasingly been recognized as a human pathogen in clinical settings, exhibiting a growing propensity for causing infections (Ballhausen et al., 2017). Previous studies have documented the isolation of ST398 MRSA from food animals and bulk tank milk (Cui et al., 2020; Schnitt et al., 2020; Tomao et al., 2020). Significantly, our study identified an ST398 MRSA isolate (SJTUF21502) in frozen flour and rice products, which has not been reported previously. These notable findings serve as a reminder that frozen flour and rice products may potentially serve as reservoirs for ST398 MRSA, thereby enhancing our understanding of the evolution and transmission dynamics of this strain.

Through the utilization of WGS and bioinformatics analysis, we have identified a diverse range of ARGs carried by MRSA isolates in frozen flour and rice products. This finding has greatly contributed to the understanding that MRSA isolates present in these products act as a significant reservoir for ARGs. It has also shed light on the important role played by retail frozen flour and rice products in the transmission and evolution of *S. aureus*-related ARGs. Furthermore, studies have reported that exposure to cold stress can upregulate ARGs and induce gene mutations in *S. aureus*, leading to increased resistance against antimicrobial agents (Qiao et al., 2020). Therefore, considering the specific storage conditions of frozen flour and rice products, cold stress may exacerbate the risk posed by MRSA isolates in these products. This could be a



Accession number	Strain name	ST	Host	Source	Location	Collection date
CP029474	30-47	5	Human	Anterior nares	USA:Chicago	2015
CP000736	JH1	105	Human	-	USA: New York city	-
CP043300	16439	3390	Human	Blood	USA	2019
CP011147	FCFHV36	105	Human	Osteomyelitis	Brazil	-
CP051914	ER11063.3	105	Human	Blood	USA: New York city	2017
CP049376	pt298	105	Human	Blood	USA: New York city	2017
CP001844	04-02981	225	Human	-	Germany	2004.4.10
JAHXGH000000000	SJTUF21491	5	Food	Frozen flour and rice product	China: Shanghai	2019-2020
CP048643	SR153	5	Human	Blood	China: Zhejiang	2013.6.1
CP011685	ZJ5499	5	Human	Blood	China: Zhejiang	2010.3
CP071046	WBG8381	5	Human	-	Australia	1995
CP054269	pt198	5	Human	Blood	USA: New York city	2016
CP049425	pt261	5	Human	Blood	USA: New York city	2016
CP039156	WCUH29	5	Human	-	Poland	2014
JAHXGI000000000	SJTUF21492	965	Food	Frozen flour and rice product	China: Shanghai	2019-2020
JAHXGC000000000	SJTUF21454	965	Food	Frozen flour and rice product	China: Shanghai	2019-2020
CP010943	144_S7	772	Human	-	Nigeria	2013.7.2
CP010526	DAR4145	772	Human	Blood	India:Mumbai	2009
CP019563	SR434	88	Human	Skin	China: Zhejiang	2015.11.26
CP070986	WBG8287	1	Human	-	Australia	1995
CP003194	11819-97	80	Human	Abscess on lower extremity	Denmark	1997.6.11
CP060609	K078	6	Human	Nasal cavity	Germany	2018
CP031131	E16SA093	72	Human	Blood	South Korea	2016.9
JAHXGG000000000	SJTUF21473	72	Food	Frozen flour and rice product	China: Shanghai	2019-2020
JAHXGF000000000	SJTUF21470	72	Food	Frozen flour and rice product	China: Shanghai	2019-2020
CP030136	S57	9	Food	Pork	China: Fujian	2014.11.20
CP031839	NX-T55	9	Pig	Nasal cavity	China: Ningxia	2014.6
CP031838	QD-CD9	9	Pig	Nasal cavity	China: Shandong	2014.7
JAHXGB000000000	SJTUF21434	9	Food	Frozen flour and rice product	China: Shanghai	2019-2020
JAHXFZ000000000	SJTUF21415	9	Food	Frozen flour and rice product	China: Shanghai	2019-2020
CP032161	SAH27	22	Human	Skin	Hungary	2016.2.25
CP006044	CA-347	45	Human	Blood	USA: California	2005
CP073065	17Gst354	398	Human	Nasopharynx	Switzerland	2017.11.25
CP020019	08S00974	398	Pig	-	Germany	2012
CP003808	08BA02176	398	Human	Skin	Canada	2008.1.15
JADRJJ000000000	SJTUF21502	398	Food	Frozen flour and rice product	China: Shanghai	2019-2020
CP035003	PCFA-221	541	Pig	Nasal cavity	South Korea	2017.8.28
CP002114	JKD6159	93	Human	-	Australia	-
CP040801	S15	3355	Food	Beef	China: Gansu	2018.12.1
CP033085	WH3018	59	Human	Sputum	China: Hubei	2013.4.2
CP003604	SA40	59	Human	Nasopharynx	China: Taiwan	2005
CP006630	SA268	59	Human	Sputum	China: Zhejiang	2012.3.1
CP003603	SA957	59	Human	Blood	China: Taiwan	2000
CP039448	VGC1	59	Human	Blood	China: Taiwan	2013.4
CP045472	ZY05	338	Human	Blood	China: Zhejiang	2016.4.2
JAHXGD000000000	SJTUF21455	338	Food	Frozen flour and rice product	China: Shanghai	2019-2020
JAHXGK000000000	SJTUF21506	239	Food	Frozen flour and rice product	China: Shanghai	2019-2020
JAHXGJ000000000	SJTUF21501	239	Food	Frozen flour and rice product	China: Shanghai	2019-2020
CP015447	M92	5354	Human	Nasal swab	Canada: Calgary	-
CP006838	Z172	239	Human	Blood	China: Taiwan	2010
CP009681	Gv69	239	Human	-	Brazil	1996.6.29
CP005288	Bmb9393	239	Human	Blood	Brazil	1993
CP058613	128254	630	Human	Skin	Denmark	2018.11.6
CP047321	RJ1267	630	Human	Sputum	China: Shanghai	2018.6.6
CP060491	WH39	630	Human	Wound secretion	China: Hubei	2018.5.11
JAHXGE000000000	SJTUF21457	630	Food	Frozen flour and rice product	China: Shanghai	2019-2020
CP007657	V2200	923	Human	Osteomyelitis	Venezuela	2007.9.13
CP007676	HUV05	8	Human	Blood	Colombia	2006
CP007672	CA12	8	Human	Blood	Colombia	2007
CP042348	BSN9R	8	Human	Tricuspid valve	USA: Detroit	2018.8.8
CP010300	27b_MRSA	8	Human	Skin	USA: Michigan	2003
CP007176	USA300-ISMMS1	8	Human	Liver	USA: New York city	-

Fig. 6. Phylogenetic tree of MRSA isolates with complete genome sequences. Fifty isolates were obtained from the GenBank database, and 12 isolates were from this study. The strain names marked in red indicated 12 MRSA isolates in this study. The sequences were aligned for phylogenetic analysis based on the maximum-likelihood method. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

contributing factor to the observed serious multidrug resistance in MRSA isolates from frozen flour and rice products. While plasmids typically play a significant role in the transmission of ARGs, the specific contribution of plasmids to ARG transmission in *S. aureus* remains largely unclear (Castañeda-Barba et al., 2023). The results of this study demonstrate that three identified plasmids carry ARGs and have the potential for conjugative transfer. Additionally, the conjugative plasmids in this study carried a greater number of ARGs compared to the non-conjugative plasmids. These findings emphasize the significance of conjugative plasmids as reservoirs and vectors of ARGs in *S. aureus*, which facilitated a notable transfer of ARGs. These results contribute to our understanding of ARG transmission patterns in *S. aureus*.

Through comparative genomic analysis, the MRSA strains identified in this study were found to be closely related to highly pathogenic MRSA isolates previously reported, suggesting that frozen flour and rice products may serve as a source of MRSA infections in clinical settings. This highlights the possibility of *S. aureus*-related diseases resulting from exposure to or consumption of frozen flour and rice products. Moreover, the observed serious multidrug resistance in the MRSA isolates underscores the significant challenges in clinical treatment posed by *S. aureus* prevalent in these products. Overall, the role of frozen flour and rice products as a potential source of MRSA infections in clinical settings should be emphasized in sustainable "One Health" surveillance efforts for the preservation of public health.

5. Conclusions

Our study has revealed a high contamination rate of *S. aureus* in retailed frozen flour and rice products, with a diverse range of STs observed. The detection of abundant enterotoxin genes suggests a strong ability of these *S. aureus* isolates to produce enterotoxins. Furthermore, the MRSA isolates, including the identification of ST398 MRSA for the first time in frozen flour and rice products, exhibited serious multidrug resistance, as well as a high prevalence of ARGs and virulence genes. The plasmids harbored by these MRSA isolates were found to carry ARGs and ISs, and possessed the ability to undergo conjugative transfer, highlighting the role of plasmids in facilitating the development of antimicrobial resistance in *S. aureus*. Lastly, comparative genomic analysis suggests that frozen flour and rice products could serve as a potential source of MRSA infections in clinical settings. This comprehensive report provides essential data for ensuring food safety and protecting public health in relation to *S. aureus* contamination in retailed frozen flour and rice products.

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CRediT authorship contribution statement

Jiang Chang: Conceptualization, Methodology, Validation, Investigation, Data curation, Writing – original draft. **Yi Zhang:** Methodology, Writing – original draft. **Zengfeng Zhang:** Writing – review & editing. **Bo Chen:** Investigation. **Shoukui He:** Writing – review & editing. **Zeqiang Zhan:** Writing – review & editing. **Nan Zhong:** Data curation. **Xiaorong Tian:** Investigation, Data curation. **Shimo Kang:** Writing – review & editing. **Kannappan Arunachalam:** Writing – review & editing. **Chunlei Shi:** Supervision, Project administration, Funding acquisition.

Declaration of competing interest

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

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2023.100631.

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