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Investigation of a *Staphylococcus aureus* sequence type 72 food poisoning outbreak associated with food-handler contamination in Italy

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Abstract

On August 2019 a staphylococcal food poisoning outbreak occurred in an elderly home in Piedmont, Italy. The epidemiological investigation performed among the persons that consumed the meal identified chicken salad as the most likely source of the outbreak. *Staphylococcus aureus* was isolated from a total of seven samples, namely one vomit sample from a guest of the nursing home, two food samples (chicken salad with and without mayonnaise) and nasal swabs collected from a total of four persons working in the kitchen of the nursing home. The maximum likelihood tree obtained using single nucleotide polymorphisms analysis revealed that the isolates from the aforementioned samples clustered together. Multilocus sequence typing revealed that they belonged to Sequence Type 72. Fourier transform infrared spectroscopy (FTIR) was used in parallel to single nucleotide polymorphisms and whole genome sequencing for the determination of the degree of relatedness of the isolates. The results of the FTIR showed the same clustering obtained with single nucleotide polymorphisms and whole genome sequencing and revealed the source of infection. This study underlines the importance of both laboratory evidence and epidemiological data for outbreak investigation and further confirms that FTIR is a suitable support for the short-term epidemiological investigation on source attribution in case of a *S. aureus* infection.

KEYWORDS
foodborne outbreak, food safety, Fourier transform infrared spectroscopy, nasal swab, source tracking, *Staphylococcus aureus*

1 | INTRODUCTION

Staphylococcal food poisoning (SFP) is one of the most prevalent causes of foodborne intoxication worldwide. It is caused by the ingestion of preformed staphylococcal enterotoxins (SEs), thermostable proteins produced by enterotoxigenic strains of coagulase-positive staphylococci (CPS) mainly *S. aureus*. The pathology is typically self-limiting, presenting with severe vomiting,

diarrhoea and abdominal pain or nausea following a short incubation period.

Human food intoxication by *S. aureus* is mainly associated with inadequate handling of cooked or processed foods (Argudín et al., 2010) followed by favourable environmental conditions for its growth and enterotoxin(s) production during food storage and preparation (i.e. time and temperature). Food poisoning outbreaks associated with post-process contamination of foods with *S. aureus* are in part the responsibility of food handlers who carry enterotoxigenic staphylococci in their nares or on their skin (Angelillo et al., 2000; Portocarrero et al., 2002). Indeed, *S. aureus* represents a ubiquitous commensal that colonizes the anterior nares of healthy adults with percentages of the global population with 20% to 30% of intermittently and persistently infected respectively (Kluytmans & Wertheim, 2005; van Belkum et al., 2009). However, *S. aureus* can also cause serious infections, toxinoses and life-threatening diseases, including skin and soft tissue infections, toxic shock syndrome and septicaemia.

Subtyping of *S. aureus* is crucial to epidemiological investigations and phylogenetic studies (Johler et al., 2013; van Belkum et al., 2007) and common techniques used for subtyping of *S. aureus* are pulsed-field gel electrophoresis (PFGE), spa typing and multilocus sequence typing (MLST) (Aires-de-Sousa et al., 2006; Cookson et al., 2007; Strommenger et al., 2006). Ideally, a typing method needs to provide a reliable and accurate bacterial type, at the highest speed and lower cost possible (MacCannell, 2013).

The investigation of foodborne outbreaks studies is supported by many different culture-dependent or DNA-based, expensive, and/or time-consuming typing techniques such as next generation sequencing and pulsed-field gel electrophoresis, requiring at least several days until a complete characterization is available. Usually, investigations are limited to retrospective studies, nevertheless, to rapidly confine foodborne outbreaks, it is important to be able to identify and characterize the patient's pathogen and to link it with the food source, in order to take appropriate measures to prevent further spreading as quickly as possible. The development of easy, rapid and sensitive methods is, thus, still needed for implementing control measures in the case of outbreaks.

There is an increased interest in tracking, identifying and understanding the diversity of *S. aureus* in various settings. Fourier transform infrared (FTIR) spectroscopy is a phenotypic, rapid, non-destructive, simple, inexpensive, and high-throughput analytical tool, based on the differential vibrational modes of distinct chemical bonds when exposed to an infrared beam. Each bacterial cell exhibits a unique FTIR spectrum, corresponding to its specific fingerprint signature and correlating with genetic information (Helm et al., 1991; Naumann et al., 1991).

Several studies using FTIR spectroscopy focused on foodborne and clinical pathogens, (Fetsch et al., 2014; Novais et al., 2019; Nyarko et al., 2014; Preisner et al., 2010), also in terms of outbreak investigation (Hu et al., 2021; Martak et al., 2019). Here we report a case of *S. aureus* foodborne intoxication where source tracking was performed.

Impacts

- In this study we report a *S. aureus* outbreak caused by the contamination of food from an asymptomatic food handler.
- *S. aureus* strain ST-72 identified as the cause of the foodborne outbreak is a common community acquired pathogen in South Korea.
- Fourier transform infrared spectroscopy allowed source tracking in a more rapid and less expensive way than WGS, thus it can be used for strain differentiation, identification and comparison for *S. aureus*.

2 | MATERIALS AND METHODS

On the evening of 7 August 2019, 11 persons manifested gastrointestinal symptoms, nausea and headache in a nursing home for the elderly. At 6.30 pm the doctor on duty of the Food Hygiene and Nutrition Service, Local Health Authority, Piedmont Region, Italy, received notification of a suspected food poisoning from the doctor of the nursing home for the elderly. An epidemiological investigation was carried out to determine the full extent of the outbreak and its probable source. Biological and food residual samples were collected, and microbiological analyses were performed.

Ethical approval was not required as this was a secondary data analysis and we report non-identifiable data.

2.1 | Epidemiological investigation

All the people that at lunch had consumed the meal were contacted, and structured interviews were performed by the local health authority to collect information on food exposure and illness symptoms. The guests self-reported what food exposures they had and whether or not they became ill following the exposure. Information about nature of symptoms and duration of illness was collected; the main symptoms and the time of onset were analysed to determine the possible causes of the outbreak and to draw the epidemic curve. The case definition included persons who developed specific symptoms (abdominal pain, nausea, vomit, and diarrhoea) with onset from the consumption of the common lunch until 12 h.

2.2 | Laboratory investigations

Only one emesis sample was collected and analysed for pathogenic bacteria and toxins potentially responsible for the reported symptoms. Analyses for the presence of *S. aureus*, *Bacillus cereus*, and Norovirus were performed. In addition, nasal swabs were collected from the personnel involved in food handling that were on duty on the day and the day before the outbreak (i.e. the cook and the

cook helpers) and analysed for the presence of *S. aureus*. From each positive sample, a maximum of three isolates were subjected to the analysis detailed below.

Food (each course of the consumed meal) samples were collected and sent to the Italian National Reference Laboratory for CPS (Turin), Istituto Zooprofilattico of Piemonte, Liguria and Valle d'Aosta for enumeration of CPS (ISO 6888-2:2021), *B. cereus* (ISO 7932:2004; ISO 7932:2004/AMD 1:2020), and Norovirus detection (IZSLER internal method). Food samples were tested for staphylococcal enterotoxins, according to ISO 19020:2017 (VIDAS) and *B. cereus* emetic toxin (Horwood et al., 2004).

All the *S. aureus* strains isolated from the different sources were identified by Matrix Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF, Vitek MS, Biomerieux) and the detection of genes encoding SEs was performed by multiplex PCR according to the European Union Reference Laboratory for Coagulase-Positive Staphylococci including *S. aureus* (EURL CPS) methods (De Buyser et al., 2009). The protocols included the detection of the genes from *sea* to *see* and *ser* for the first and from *seg* to *sej* and *ser* for the second PCR.

In addition, bacterial genomic DNA was extracted using the EXTRACTME Genomic DNA isolation kit (Blirt) and Whole Genome Sequencing (WGS) was performed on the MiSeq platform (Illumina) using paired-end libraries which were prepared following the Illumina™ DNA Library Prep Kit (Illumina), with 150-bp read length. The reads were first subjected to the Galaxy tool 'FastQC Read Quality reports', accessed via the Galaxy public server at https://usegalaxy.org, (Afgan et al., 2016) to provide the quality control checks on raw sequence data. Raw reads were trimmed using the Galaxy tool Trimmomatic 0.38 (Bolger et al., 2014) by removing Nextera adaptors and other Illumina-specific sequences ("Illuminaclip" set to value "Nextera (paired-ended)"), removing low-quality residues at the start and end of the reads ("leading:10" and "trailing:10"), clipping reads when average Q-scores dropped below 20 over a sliding window of four residues ("slidingwin-dow:4:20"), and dropping reads shorter than 40 bases after processing ("minlen:40").and finally the reads were assembled to genomes by means of Unicycler (ver. 0.4.1.1) via Galaxy (Wick et al., 2017) using for the bridging mode moderate contig size and misassembly rate ("Bridging mode" set to value "Normal") and contigs below 200 bp in length were excluded ("Exclude contigs from the FASTA file which are shorter than this length (bp)" set to value "200"). The assembled genomes were processed to determine the multilocus sequence typing (MLST) in silico with MLST 1.8 (accessed via https://cge.food.dtu.dk/services/MLST//) (Larsen et al., 2012) selecting "5x" for minimum depth for an allele. The antimicrobial resistance genes were identified using ResFinder 4.1 (accessed via https://cge.cbs.dtu.dk/services/ResFinder/; Cosentino et al., 2013). Finally, genomes were analysed for virulence gene detection with VirulenceFinder 2.0 (accessed via https://cge.food.dtu.dk/services/VirulenceFinder/) selecting 90% as threshold for identification and 60% for minimum length (Joensen et al., 2014). The fastq files of paired reads were processed with CSI Phylogeny 1.4 (accessed via https://cge.cbs.dtu.dk/services/CSIPhylogeny/) to call and filter single nucleotide polymorphisms (SNPs) and infer phylogeny based on the concatenated alignment of the high-quality SNPs (Kaas et al., 2014). SNP analysis was performed with the following parameters: 10× minimum depth at SNP position, 10% minimum relative depth at SNP position, 100 bp minimum distance between SNPs, 30 for minimum SNP quality, 25 for minimum read mapping quality, 1.96 minimum Z-score for each SNP, and including *S. aureus* NCTC 8325 (GenBank accession number: NC_007795.1) as reference.

The evolutionary history was inferred by using the maximum likelihood method and Tamura–Nei model (Tamura & Nei, 1993). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura–Nei model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved nine nucleotide sequences and there were a total of 3.978 positions in the final dataset. Evolutionary analyses were conducted in MEGA11 (Tamura et al., 2021). Finally, FTIR was investigated in parallel for a preliminary determination of the degree of relatedness of the isolates, using the IR Biotyper® system (IRBT—Bruker Daltonics GmbH & Co. KG). Sample preparation was performed accordingly to manufacturer's instruction. Briefly, bacterial isolates were cultured on CBA (Columbia Blood Agar – Becton Dickinson) for 24 h at 37°C and a 1 µL loop full of bacterial cells was suspended in 50 mL of 70% v/v ethanol solution. Suspensions were mixed before addition of 50 mL of deionized water. The suspensions were homogenized by vortexing, and 15 µL of each suspension were spotted in five technical replicates on the IR Biotyper silicon sample plate (Bruker Daltonics GmbH & Co. KG) and dried for 25 min at 37°C. Infrared absorption spectra were acquired in transmission mode by the OPUS software (Bruker Daltonics), in the spectral range 4000–500 cm^{-1}. Spectra processing and visualization was performed with the IR BIOTYPER Client software V3.1 (Bruker Daltonics), using default settings as recommended by the manufacturer. After spectra smoothing using the Savitzky–Golay algorithm, the second derivative of the spectra was calculated by the software. After vector normalization, spectra relation within a wavenumber range from 1300 to 800 cm^{-1} was analysed. IRTS 1 and IRTS 2 (Infrared Test Standard) were measured as quality control prior to sample spectra acquisition, in each run. All spectra were acquired intercalating a background spectrum between each sample/control measurement. Each sample spectra was evaluated by the software considering four quality parameters: absorption (range: 0.4–2 cm^{-1}), noise (value: <300 cm^{-1}), water vapour (value: <300 cm^{-1}) and fringes (value: <100 cm^{-1}). For each strain at least three good quality spectra were considered for further analysis. Exploratory analysis was performed applying principal components analysis (PCA) and linear discriminant analysis (LDA). Hierarchical cluster analysis (HCA) was done using Euclidean metric and average linkage algorithm. Results were displayed as scatter plot (PCA/LDA) and dendrogram (HCA).

3 | RESULTS

3.1 | Epidemiological and clinical characteristics of cases

From the performed interviews resulted that an overall of 69 people (60 guests, 5 beneficiaries of home assistance, 2 healthcare professionals, 1 canteen cook and 1 cook helper) consumed the meal. The menu provided pasta with pesto and fresh tomatoes, pasta with oil, chicken salad with or without mayonnaise and cooked vegetables. The meal was prepared by the internal canteen. The interviewed personnel referred that out of the 33 people who consumed the chicken salad, 11 (9 guests and 2 healthcare professionals) were symptomatic, while 22 were asymptomatic (15 guests, 2 food handlers and 5 beneficiaries of home assistance; Table 1). Since the remaining 36 people did not consume the chicken salad, it resulted as the most likely source of the outbreak.

The 11 cases reported the same following clinical symptoms: in particular, nine guests and two healthcare professionals manifested gastrointestinal symptoms, nausea, vomiting and headache about 3 h after the food consumption. The clinical picture faded within about 10 h and no subject presented symptoms such as to require hospitalization.

3.2 | Laboratory investigations

Emesis sample resulted positive for *S. aureus* but negative for *B. cereus*, and for the presence of Norovirus. Nasal swab culture revealed the presence of *S. aureus* in all of the swabs collected from the nares of the four food handlers as reported in Table 2. Among the food tested, all samples tested negative for Norovirus, *B. cereus* and its emetic toxin and, whereas high levels of CPS (>100,000 CFU g^{-1}) were isolated from the chicken salad (served both with and without mayonnaise), no staphylococcal enterotoxins were detected.

Overall *S. aureus* was isolated from a total of seven samples, namely one vomit, two food (chicken salad with and without mayonnaise) samples and nasal swabs collected from a total of four persons working in the nursing home for the elderly.

The WGS run had the following overall statistics: cluster passing filter of 98.01%, quality score ≥30 of 97.54% with a total yield of 10.30 Gbp achieved. The analysed *S. aureus* isolates obtained from the same matrix shared the same anti-microbial resistance genes, enterotoxin genes and ST, hence only one strain has been reported in Table 2. Only for operator 3 (O3), the two detected isolates belonged to two strains differing from one another for the presence/absence of the enterotoxin genes. No staphylococcal enterotoxins were identified in food, but WGS analysis revealed the presence of the *sei* and *seg* enterotoxin genes in every sample, while *sec* was identified in

TABLE 1 Summary of the subjects involved in the outbreak.

Role	Chicken salad consumer	Symptomatic	Asymptomatic
Guests	24	9	15
Home assisted	5	0	5
Healthcare professionals	2	2	0
Canteen cook	1	0	1
Cook helper	1	0	1

TABLE 2 Summary of the sampled matrices that tested positive for *Staphylococcus aureus*.

Sample ID	Matrix	Role	Multilocus sequence typing	SEs toxins genes	Anti-microbial resistance genes
F1	Chicken salad with mayonnaise (one strain)	Food	ST-72	*sei*, *seg*	*blaZ*, *norA*
F2	Chicken salad without mayonnaise (one strain)	Food	ST-72	*sei*, *seg*	*blaZ*, *norA*
P1	Emesis (one strain)	Patient	ST-72	*sei*, *seg*	*blaZ*, *norA*
O1	Nasal swab (one strain)	Food handler #1	ST-72	*sei*, *seg*	*blaZ*, *norA*
O2	Nasal swab (one strain)	Food handler #2	ST-1162	*sei*, *seg*, *seh*, *sec*	*blaZ*, *norA*, *ermC*
O3-1	Nasal swab (two strains)	Food handler #3	ST-22	n.d.	*blaZ*, *norA*
O3-2			ST-22	*sei*, *seg*	*blaZ*, *norA*
O4	Nasal swab (one strain)	Canteen cook	ST-45	*sei*, *seg*, *sec*	*blaZ*, *norA*

Note: Results of multilocus sequence typing, the presence of toxins genes and anti-microbial resistance genes demonstrated by whole genome sequencing.

Abbreviations: F, food; O, operator; P, patient; SE, staphylococcal enterotoxin.

the strains of operators 3 and 4 (O2 and O4) and *seh* was additionally identified in the strain identified from O2 (Table 2). All identified *S. aureus* strains isolated from the different sources showed the *bla*Z and *nor*A anti-microbial resistance genes, encoding beta-lactam and fluoroquinolones resistance respectively, while *S. aureus* strain isolated from O2 showed *erm*C anti-microbial resistance gene, encoding macrolide resistance. The *S. aureus* isolates were successfully typed by MLST and classified in four sequence types (STs), namely STs 72, 45, 22 and 1162. Four ST-72 isolates originated from vomit, chicken salad with and without mayonnaise, and nasal swab of one food handler operating during the day before the outbreak during the meal preparation of chicken salad and the other foods.

The maximum likelihood tree obtained using SNP analysis with CSI Phylogeny 1.2 revealed that the strains isolated from the nasal swab of O1, food samples (F1 and F2) and one emesis sample (P1) clustered together, while isolates from the three other operators (O2, O3 and O4) were located on another branch of the tree (Figure 1).

IRBT acquired at least four spectra of good quality for each isolate. Regarding hierarchical cluster analysis, the clustering cut-off value, automatically calculated by the software, revealed overall five clusters, one including samples F1, F2, O1 and P1, the other four clusters including one sample each (Figure 2).

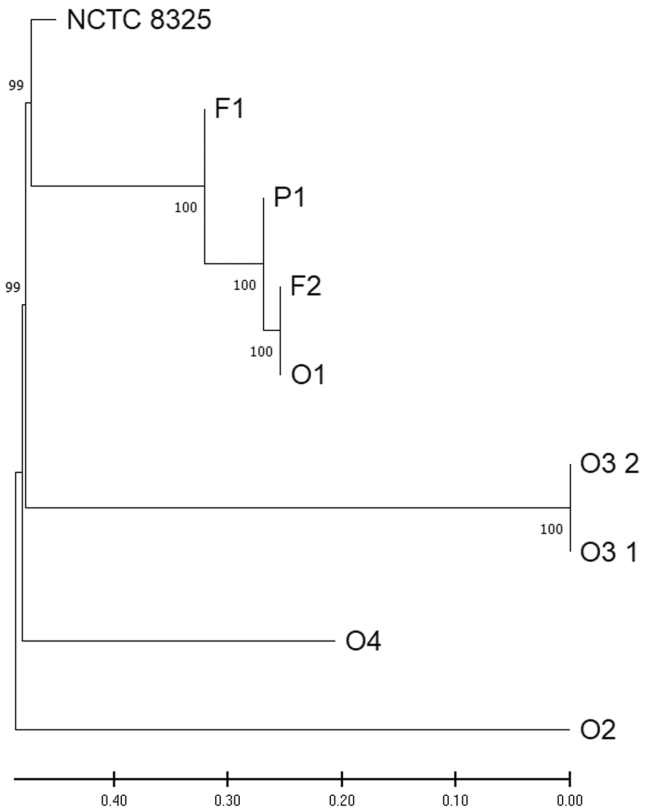

FIGURE 1 Maximum likelihood tree obtained using SNP analysis with CSI Phylogeny 1.2 highlights the genomic correlation between the strains isolated from the nasal swabs of the caterer (O1), the vomitus sample of a patient (P1) and the food samples from chicken salad with (F1) and without mayonnaise (F2) and that cluster differ from the other isolates of operators (O2, O3 and O4).

Based on these findings we hypothesize that this outbreak has been provoked by O1 that contaminated with *S. aureus* the chicken salad by failure to follow good hygienic practices. The results of the FTIR confirmed the clusterization previously described (Figures 2 and 3).

4 | DISCUSSION

Here, we describe a confirmed food poisoning outbreak due to CPS that can be considered an outbreak with strong microbiological and epidemiological evidence according to the European Food Safety Authority (EFSA) nomenclature (EFSA manual, 2013). The strong microbiological evidence includes the identification of an indistinguishable causative agent in a human case and in a food vehicle, which is unlikely to have been contaminated coincidentally or after the event (EFSA, 2014).

The conclusive diagnostic criterium is mainly based on at least one of the following: (1) the recovery of $N10^5$ *S. aureus* g^{-1} from food remnants, (2) the detection of SEs in food remnants and (3) the isolation of identical *S. aureus* clones from both patients and food remnants (Bryan et al., 1997). During the investigation, we met criteria 1 and 3 both in food samples and patient.

Taken all together, the investigated outbreak can be considered as a typical Staphylococcal intoxication in which food handlers carrying enterotoxin-producing *S. aureus* in their noses or on their hands are regarded as the main source of food contamination (Argudín et al., 2010; Bencardino et al., 2021), involving a high protein content that favours growth of CPS (Gumbo et al., 2015; Wieneke et al., 1993) in which the organism was able to multiply.

Different STs among different food handlers namely STs -22, -45, -1162 and -72, were isolated, none showing resistance to methicillin, in line with previous findings reporting that food handlers are rarely carriers of MRSA isolates (de Jonge et al., 2010; Udo et al., 2009).

The ST-72 strain identified to be the cause of the outbreak is the most commonly community-associated methicillin-resistant or -sensitive *S. aureus* in South Korea (Joo et al., 2012). Indeed, recent findings have demonstrated that these isolates have been developing resistance to desiccation and adaptation to hypotonic solutions, promoting their survival in a hospital setting (Joo et al., 2017). In addition, within food ST-72 has been identified in Korea in the meat and milk production chain (Kim et al., 2015; Lim et al., 2013), and in Uruguay in food premises (Machado et al., 2020) while interestingly, the first identification of the isolate in Europe has been recently described in Italy in buffalo milk products (Normanno et al., 2020). Here we report the first foodborne intoxication due to *S. aureus* ST-72 in Italy and in Europe.

For operator 3, two isolates belonging to two strains differing for the presence/absence of the *seg* and *sei* enterotoxin genes were detected. Much of the variation between *S. aureus* strains appears to be attributable to mobile genetic elements, such as plasmids,

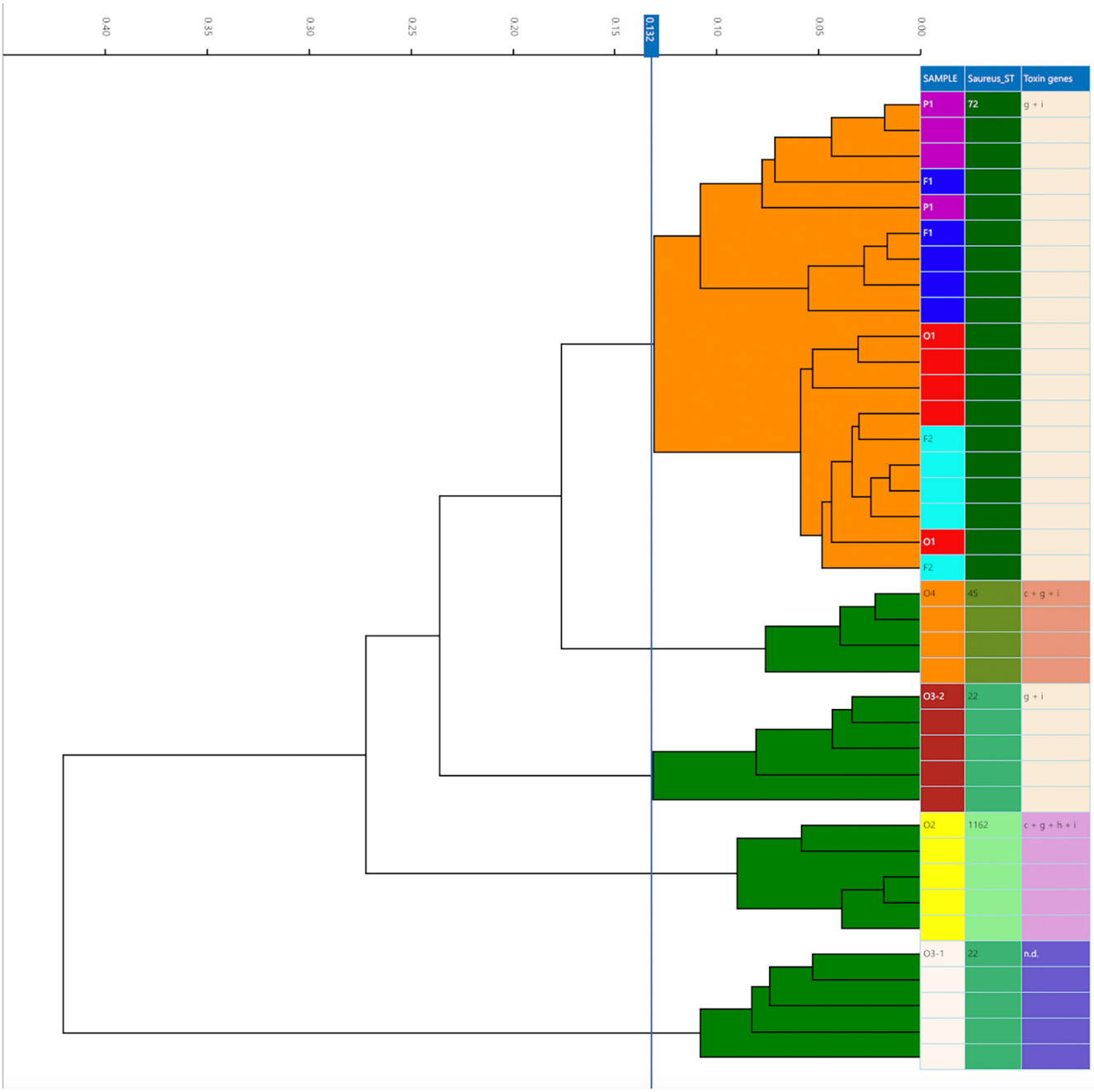

FIGURE 2 Dendrogram delivered by IRBT clustering analysis (WN: 1300-800/cm; Dim. reduction: PCA (7PCs/95.2% variance); Averaging: None; Metric/linkage: Euclidean/Average; Cophenetic correlation coefficient: 0.949; Calculated cut-off: 0.119). On the right side of the figure, the columns show the samples spectra (each line represents a technical replicate). The upper axis shows the cut-off distance that is calculated by the algorithm: Simpson's Diversity Index (SDI)×mean Coherence (mC).

bacteriophages, transposons and insertion sequences (Sumby & Waldor, 2003). The *seg* and *sei* genes are present in *S. aureus* in a tandem orientation belonging to the same operon (Jarraud et al., 2001) and as reported by Sumby and Waldor (2003) *seg* is a protophage-borne gene that occasionally could lead to excision and consequently the loss of SE genes.

The analyses performed with FTIR and WGS gave comparable results in terms of their ability to link different isolates, but, while whole genome sequencing provides more information, Fourier transform infrared spectroscopy is a rapid and inexpensive method that can be applied as a real-time surveillance method.

In addition, with FTIR two ST-22 isolates that differed for the absence/presence of enterotoxin genes (*seg* toxin and *sei* toxin) were discriminated from one another. Even though there is no indication that exactly this difference is responsible for the different clustering, similar results have been described in a previous study (Meyers, 2000), where spectra have been suggested to serve as "fingerprint" usable in taxonomic discrimination.

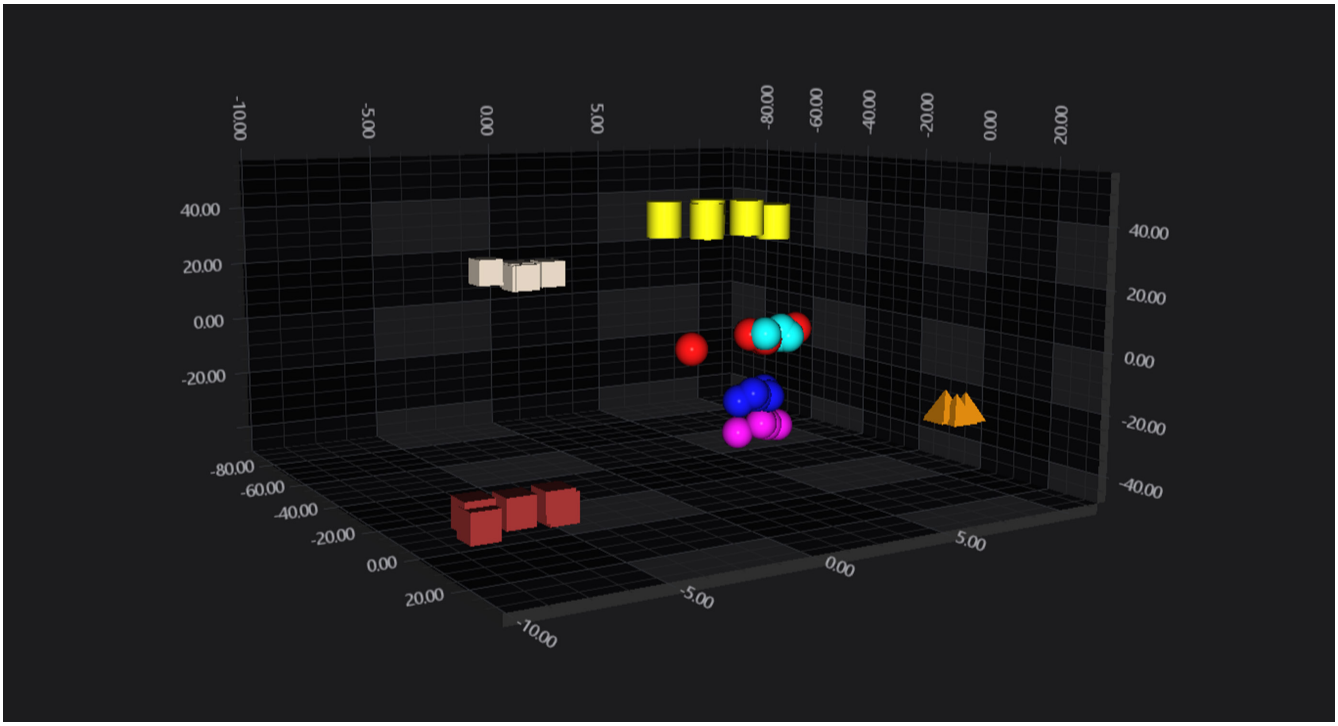

FIGURE 3 Results of LDA analysis depicted as 3D scatterplot. Each geometric form represents one spectrum, while colours and shapes are attributed to identify isolates and multilocus sequence typings respectively.

Fourier transform infrared spectroscopy has already been described as a promising tool for rapid discrimination among strains in outbreak investigations (Johler et al., 2013) as well as in source attribution studies (Harmsen et al., 2003), and recently, the performance of the identification technique was compared to conventional MLST for typing of pathogens collected during outbreaks (Martak et al., 2019).

Fourier transform infrared spectroscopy does not need highly trained staff to perform and couples rapidity, cost- and time-effectiveness to the requirement for little biomass, as well as the necessity for little or no sample pre-treatment. In this light the routinary use of the instrument from the official control laboratories would constitute an important advantage for public health from an epidemiological perspective. Indeed, continuous collection of FTIR data would support the passive surveillance enabling on one hand to speed up source identification during an ongoing outbreak, and on the other hand to reveal unidentified outbreaks by linking spectra from isolates of human and animal origin. Early identification of the source of food contamination during a foodborne outbreak is of crucial importance for robust contact tracing, cohorting and other infection control practices.

From our results it is possible to further confirm that this technique can easily and successfully be applied to the source tracking of *S. aureus*, as previously demonstrated by (Piva et al., 2021) during a zoonotic transmission from goat to veterinarians, as well as during nosocomial and community acquired infections (Vogt et al., 2019).

This is particularly important in case of *S. aureus* outbreaks since some humans are only intermittently infected in the nasal cavity (van Belkum et al., 2009). Staphylococcal intoxications are usually self-limiting and without severe consequences, but in the case of an outbreak within a home for the elderly the rapid identification of the source of contamination can be crucial.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

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

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