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Surface microbiota and associated staphylococci of houseflies (*Musca domestica*) collected from different environmental sources

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ABSTRACT

Houseflies (*Musca domestica*) are important mechanical vectors for the transmission of pathogenic microorganisms. In this study, 129 houseflies (69 males and 60 females) were collected from 10 different environmental sources and a laboratory population was used. The surface microbiota of houseflies was identified by Next-Generation Sequencing. Staphylococci from the surfaces of houseflies were selectively isolated and their virulence genes, antibiotic susceptibilities, biofilm formation, and clonal relatedness were determined. Metagenomic analysis results demonstrated that *Staphylococcus, Bacillus,* and *Enterococcus* were mostly present on the surface of houseflies at the genus level. Additionally, the isolated 32 staphylococcal strains were identified as *Staphylococcus sciuri* (n = 11), *S. saprophyticus* (n = 9), *S. arlettae* (n = 6), *S. xylosus* (n = 4), *S. epidermidis* (n = 1) and *S. gallinarum* (n = 1). *tet*K, *tet*M, *tet*L, *erm*C, *msr*AB, and aad6 genes were found to carry by some of the staphylococcal strains. The strains were mostly resistant to oxacillin, penicillin, and erythromycin and three strains were multi-drug resistant. There was a statistical difference between housefly collection places and antibiotic resistance of isolated staphylococci to penicillin G, gentamicin, and erythromycin ($p < 0.05$). Biofilm test showed that 17 strains were strong biofilm formers, and it plays important role in the transmission of these bacteria on the surface of houseflies. Staphylococcal strains showed extracellular proteolytic and lipolytic activity in 31 and 12 strains, respectively. Closely related species were found in PFGE analysis from different environmental sources. By this study, surface microbiota and carriage of pathogenic staphylococci on the surfaces of houseflies and their virulence properties were elucidated.

1. Introduction

The housefly, *Musca domestica* L. (Diptera: Muscidae) is the common and widespread fly lives environment close to humans and animals. Their main breeding sources are high organic contents of manure and food wastes. Females are usually bigger than males and they can be distinguished by wider dorsal space between the eyes of female houseflies. Adults can survive a 15–30 days period [1].

The symbiotic relationship of bacterial populations present in the microbiota of houseflies form physiological development and adaptation to environmental conditions. The surface and gut microbiota help the development and survival stages of houseflies' life cycle. Especially Firmicutes were dominant at the early stages of development and larvae stage. Then Proteobacteria and Bacteroidetes become abundant at the phylum level when houseflies matured into adults [2].

Mechanical transmission of bacteria and viruses by houseflies has been shown in many studies [3–7]. They can carry human and animal pathogens on their legs, mouthparts, or regurgitated fluid after contacting contamination sources. De Jesús et al. [8] examined carriage of *Escherichia coli* ATCC 11775 by houseflies to different food and it was shown that 0.1 mg contaminated food was transferred per landing of houseflies. Carriage of *Salmonella* spp. [9], *Campylobacter jejuni* [10],

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and *Yersinia enterocolitica* [11] in farms and food processing environments have been previously reported. Recently, Cervelin et al. [12] isolated *E. coli* and *Salmonella* spp. strains from the surface of houseflies collected from swine farms with the abundance of *E. coli* from 10^4 to 10^6 CFU/20 flies and *Salmonella* spp. from 10^2 to 10^5 CFU/20 flies with various resistance to the antibiotics like ampicillin, cefalotin, ciprofloxacin, and norfloxacin. Additionally, houseflies (n = 94) collected from the dairy farms in Brazil were found to carry *E. coli* (n = 198) strains with >30% multi-drug resistance [13]. In a recent study, the carriage of the SARS-CoV-2 virus by *M. domestica* collected from the outdoor areas of two hospitals treating COVID-19 patients in Iran was reported. In that study, 156 adult houseflies were collected, and the outer surface and homogenized houseflies were examined to detect the presence of SARS-CoV-2 virions by TaqMan Real-Time PCR analysis. Their results showed that 75% of the outer surface samples and 37% of homogenized samples in other words inside the houseflies were positive for SARS-CoV-2. This was the first report to elucidate the transmission of SARS-CoV-2 by houseflies [14].

Next-Generation Sequencing (NGS) studies help to identify the microbiota of fermented foods, gut, feces, and various living materials. This DNA-based technique was use to describe both culturable and unculturable microorganisms. Whole bacterial and fungal communities that constitute microbiota could be characterized with a higher resolution than the classical microbiological techniques.

This study was aimed to evaluate an in-depth analysis of bacterial microbiota on the surface of each housefly collected from food and fish markets, waste treatment plants, and outdoor areas of the hospitals by amplicon sequencing of 16S rRNA gene using NGS. In this context, the important virulence factor genes, antibiotic susceptibility, biofilm-forming capacities, extracellular enzyme production, and clonal relationships of isolated staphylococci were examined.

2. Material and methods

2.1. Collection of houseflies

The houseflies were collected by an insect net from 10 different environmental sources between Aug. 20, 2018, and Oct. 05, 2018. An insecticide-susceptible World Health Organization (WHO) strain of *M. domestica* was used as the laboratory population. The collection places and numbers were shown in Table 1. The collected houseflies were freeze sacrificed at −20 °C for 5 min and they were phenotypically classified. After that houseflies separated based on their sex. Each housefly was transferred into a sterile 2 ml micro centrifuge tube and 1 ml of sterile 0.9% (w/v) NaCl was added. The tubes were mixed in a multi-rotator (Multi RS-60, Biosan, Latvia) for 1 h at room temperature to remove bacteria from external surfaces of the houseflies (Fig. 1). Then, 100 μl of suspension was transferred to 5 ml buffered peptone water (Oxoid CM0509B, UK), and bacteria were pre-enriched at 37 °C with shaking at 200 rpm for 24 h.

Table 1
Sampling locations and numbers of houseflies.

Sample Code	Collection Date	Location	Coordinates	Number of collected houseflies	
				♂	♀
A	July 20, 2018	Laboratory population	37° 52′ 29.4″ N 32° 28′ 32.6″ E	4	6
B	July 20, 2018	Around Medical Faculty Hospital-Konya	37° 52′ 29.4″ N 32° 25′ 51.0″ E	5	3
C	July 20, 2018	Around Hospital-1 Konya	37° 51′ 32.2″ N 32° 26′ 47.7″ E	2	7
D	July 24, 2018	Rural area Ilgın-Konya	38° 17′ 24.1″ N 32° 00′ 29.6″ E	5	5
E	August 28, 2018	Fish market Konya	37° 51′ 49.4″ N 32° 29′ 19.3″ E	1	11
F	August 28, 2018	Food market-1 Konya	37° 53′ 33.2″ N 32° 29′ 37.1″ E	5	5
G	August 28, 2018	Food market-2 Konya	37° 52′ 05.5″ N 32° 30′ 04.4″ E	14	3
I	September 17, 2018	Dump site-1 (Waste storage area-Konya)	37° 44′ 1.75″ N 32° 36′ 23.85″ E	14	2
J	September 17, 2018	Dump site-2 Konya	37° 44′ 11.7″ N 32° 35′ 45.3″ E	4	6
K	October 5, 2018	Around Hospital-2 Konya	38° 01′ 10.6″ N 32° 30′ 39.2″ E	10	3
L	October 5, 2018	Water treatment plant-Konya	37° 57′ 33.1″ N 32° 37′ 26.7″ E	5	9
			Total	**69**	**60**

2.2. NGS and metagenomic analysis

Total bacterial DNA from pre-enriched bacterial cultures was extracted by the phenol/chloroform/isoamyl alcohol method [15] by adding 4 mg/ml lysozyme (Applichem, Germany) and 10 μg/ml lysostaphin (Sigma, Germany). Bacterial DNA was finally dissolved in 10 mM Tris pH 8.0 and it was quantified using AccuBlue™ NextGen dsDNA Quantitation kit (Biotium, Inc., USA) as described by manufacturer's instructions using a multimode plate reader (Mithras2 LB943, Berthold, Germany). A DNA library for amplicon sequencing was prepared according to the 16S metagenomic sequencing library preparation guide (Illumina, Inc., California, USA). The Nextera® XT index Kit v2 Set-A (Illumina) was used to attach dual indices to each sample. The library was prepared by equimolar proportions of the samples, and it was finally diluted to 35 pM containing 5% (v/v) PhiX control DNA. The sequencing was performed in the iSeq100 system (Illumina). NGS was carried out using pair-end read type and two reads of 151 bp read length. 16S Metagenomics, Version: 1.1.0 software (Illumina) was used to analyze the obtained sequencing raw data and the amplicons were clustered based on sequence identity (operational taxonomic unit (OTU) approach). Moreover, Shannon species diversity, the number of identified species, and bacterial taxonomic distributions of samples from the kingdom to the species level were identified by 16S Metagenomics software Version 1.1.0 (Illumina) using RefSeq RDP 16S v3 May 2018 DADA2 32 bp taxonomical interference and the Ribosomal Database Project (RDP) Classifier [16]. Principal Coordinates Components Analysis (PoCA) was also performed by Paleontological Statistics package for education and data analysis (PAST) program version 4.06b as described previously [17].

2.3. Identification of staphylococcal strains

Staphylococcal strains were isolated from the pre-enriched cultures using mannitol salt agar (CM0085B, Oxoid). The grown cultures at 37 °C were sub-cultured and purified colonies were biochemically identified

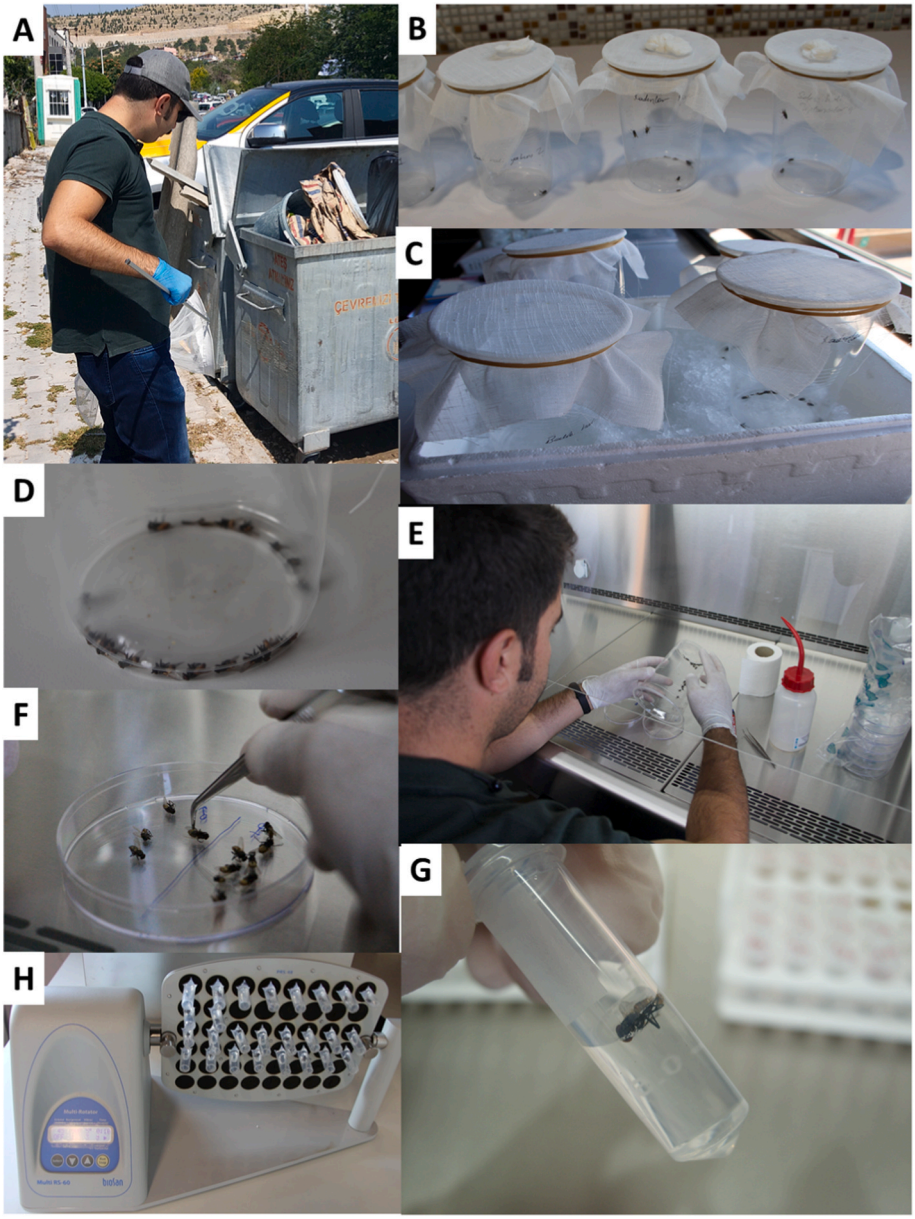

Fig. 1. Collection and removal of bacteria from the surface of houseflies. A, Collection of houseflies from an environment by an insect net; B, Sterile plastic storage containers of houseflies with a wet cotton; C and D, Freeze sacrificed houseflies; E and F, Selection of the houseflies based on their sex in the aseptic conditions; G, Transfer of each housefly into a micro centrifuge tube containing 0.9% NaCl; H, Removal of the surface bacteria by shaking in a rotator. (The photographs in this Figure were taken by M. Sudagidan for this study). (Color in online only).

based on Gram staining and catalase test. Bacterial genomic DNA of isolated bacteria was extracted as described previously [18]. 16S rRNA amplification of bacterial DNA by PCR and Sanger DNA sequencing of 16S rRNA PCR products were performed using the universal 16S rRNA primers [19]. The obtained gene sequences were analyzed and compared to GenBank sequences to identify staphylococci at the species level by Geneious Prime version 2020.2.4 software.

2.3.1. Detection of virulence genes

The presence of antibiotic resistance genes (*mec*A (1235 bp) [20], *tet*K (1153 bp) [21], *tet*M (405 bp), *tet*S (589 bp), *tet*L (739 bp) [22], *erm*A (421 bp), *erm*B (359 bp), *erm*C (572 bp), *msr*A (940 bp), *msr*B (595 bp) [23], aad6 (978 bp), mefA (345 bp), and cat (384 bp) [22]), staphylococcal enterotoxin genes (*sea* (560 bp) [24], *seb* (477 bp), *sec* (257 bp), *sed* (318 bp), and *see* (169 bp) [25]), and Panton-Valentine Leukocidin (PVL) gene (*lukSF*, 433 bp) [26] were searched by PCR. Methicillin-resistant *S. aureus* ATCC 43300, *S. aureus* ATCC 25923, and methicillin-susceptible *S. aureus* M1-AAG42B (PVL+) strains were used as the positive controls in the PCR experiments. The amplicons were resolved in 1.5% (w/v) agarose gels containing 5% (v/v) fluorescent DNA dye (SafeView Classic, Applied Biological Materials Inc., Canada) and the gels were visualized by the ChemiDoc gel imaging system (Bio-Rad, Hercules, California, USA).

2.3.2. Antibiotic susceptibility testing

Antibiotic susceptibilities of the isolated staphylococcal strains were examined based on CLSI (2021) [27] using the agar disk diffusion assay. The strains were tested against penicillin G (10U, Oxoid CT0043B), cefoxitin (30 μg, Oxoid CT0119B), oxacillin (1 μg, Oxoid CT0159B), gentamicin (10 μg, Oxoid CT0024B), erythromycin (15 μg, Oxoid CT0020B), tetracycline (30 μg, Oxoid CT0054B), ciprofloxacin (5 μg, Oxoid CT0425B), clindamycin (2 μg, Oxoid CT0064B), sulphamethoxazole/trimethoprim (1.25/23.75 μg, Oxoid CT0052B), chloramphenicol (30 μg, Oxoid CT0013B), rifampicin (5 μg, Oxoid CT0207B), linezolid (30 μg, Oxoid CT1650B), and mupirocin (200 μg, Oxoid CT0523B). The minimum inhibitory concentration (MIC) values of the resistant strains against penicillin G, oxacillin, erythromycin, tetracycline, gentamicin, chloramphenicol, and vancomycin were determined using the MICE test (Oxoid) or the E-test (BioMerieux, France) on Mueller-Hinton agar plates (Oxoid CM0337B). The recorded breakpoint values were

evaluated on the basis of CLSI MIC breakpoints [27]. Inducible clindamycin resistance was also performed using clindamycin (2 μg) and erythromycin (15 μg) disks and D-shaped zone formation was evaluated according to CLSI (2021) [27]. *S. aureus* ATCC 25923 was used as a control strain in antibiotic susceptibility testing.

2.3.3. Biofilm tests

The biofilm-forming properties of staphylococci were determined using a microplate test in the 96-well tissue culture plates (F-bottom, 3599 Corning Costar, USA) in Tryptone soya broth (TSB, Oxoid CM0129) supplemented with 1% (w/v) sucrose and Brain Heart Infusion (BHI, Oxoid CM1135) broth. In the microplate test, the triplicate wells were used. In each well, 200 μl of each media was inoculated with 20 μl bacterial strains adjusted to McFarland 0.5 in 0.9% (w/v) NaCl using a densitometer (Den-1B, Biosan, Latvia). The plates were incubated at 37 °C for 24 h and the biofilm formation was detected after staining with crystal violet (Merck, Germany) [28]. The absorbance values were measured at 595 nm using a microplate reader (Epoch2, Bio-Tek, USA). The test was repeated two times and *S. epidermidis* YT-169a was used as a biofilm-forming positive control strain [29].

2.3.4. Extracellular enzyme production

Staphylococcal strains were grown in TSB for 24 h at 37 °C with shaking at 200 rpm. Then, 1 ml grown culture was centrifuged at 10,000 rpm for 5 min and the supernatant was removed. The pellet was washed with TSB and centrifuged again. The pellet was resuspended in 200 μl TSB. After that, 10 μl of bacterial culture were spotted on a substrate containing media. For detection of lipase production tributyrin agar (Liofilchem 402520, Italy), for protease production casein agar, skim milk, and milk agar and for amylase production starch agar were used [30]. After 72 h incubation at 35 °C, zone formation around spotted bacteria was examined visually in tributyrin agar, skim milk, and milk agar plates. Protease activity was determined after the addition of 5% (v/v) trichloroacetic acid (TCA, Sigma) on casein agar and amylase was examined by the addition of Gram's iodine solution (1 g iodine and 2 g KI in 300 ml deionized water) on starch agar plates.

2.3.5. Pulsed field gel electrophoresis

PFGE was performed as described previously [31]. 30 U *Sma*I restriction enzyme (1085AH, Takara, Japan) and 1% (w/v) PFGE gel in 0.5x TBE buffer were used. PFGE gel was run at 6 V/cm for 22 h, 5–40 s pulse time at 14 °C in the CHEF DR-II electrophoresis system (Bio-Rad). PFGE band patterns were evaluated using BioNumerics Version 7.6 (AppliedMaths, Belgium) with Dice similarity coefficient with 0.5% optimization and 1% band tolerance. In the analysis of band patterns, Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering method with 80% degeneracy cut-off value was applied.

2.4. Statistical analysis

The One-Way Analysis of Variance (ANOVA) test was applied to determine the significance of the differences between tested antibiotics and staphylococci isolated from different collection places of the houseflies as described in Table 1 (Table S3). In addition, the differences between staphylococci and antibiotic resistance were also evaluated (Table S4). In statistical analysis, $p < 0.05$ was used as the criterion for statistical significance. Statistics were performed using IBM SPSS statistical program v.22.0 (2020).

3. Results and discussion

The results of this study provide a culture-independent description of the surface microbiota of houseflies collected from different environments. A total number of 129 houseflies (69 male and 60 female in sex) were used to isolate bacteria from the external surface of houseflies. In total, NGS resulted in 8,974,153 reads for 91 housefly samples. In 31 samples, amplification of 16S rRNA gene could not be achieved in amplicon PCR and 7 samples from the laboratory population did not include the results due to all showing the same microbial diversity on their surface. Metagenomic analysis results of 91 housefly samples demonstrated that Firmicutes were dominant at the phylum level. However, Proteobacteria were also observed in a high read numbers in 20 houseflies, and in 3 houseflies Actinobacteria were recorded with high abundance (Fig. S1). The top three family level taxonomic distribution of the surface microbiota included *Staphylococcaceae*, *Enterococcaceae,* and *Bacillaceae* (Fig. S2). Furthermore, *Staphylococcus*, *Bacillus,* and *Enterococcus* were dominated at the genus level (Fig. S3).

In the examination of housefly microbiota based on collection environment, *Enterococcus* were dominant in the laboratory population (A9D, A10D, and A1E) at the genus level (Fig. 2A). The fermentation of the growth medium of the laboratory population could lead to the overgrowth of enterococci. This medium contains skim milk, sugar, bran, etc. The houseflies collected around the Medical Faculty Hospital in Konya (B8D, B1E–B5E) contained mainly *Enterococcus*, *Enterobacter,* and *Vagococcus* (Fig. 2B). *Enterococcus*, *Acinetobacter,* and *Bacillus* were determined the surface microbiota of houseflies (C3D-C9D, C1E, and C2E) collected around the Hospital-1 area (Fig. 2C). The houseflies from a rural area (Ilgın-Konya) (Fig. 2D) showed diverse bacterial populations. Moreover, in the samples collected from a fish market, *Staphylococcus*, *Bacillus*, and *Enterococcus* were found to be dominant (Fig. 2E). Similarly, *Staphylococcus* was dominant in the surface microbiota of houseflies from the food market-1 (Fig. 2F) and the food market-2 (Fig. 2G). In fact, the human effect and handling of food products and fish by food vendors may cause the dominance of staphylococci. In dump site-1 and dump site-2, similar bacterial populations were dominant with different ratios. *Enterococcus*, *Staphylococcus,* and *Bacillus* were found to be dominant bacteria at the genus level (Fig. 2I and J). In out of the hospital environment, *Staphylococcus*, *Bacillus*, and in three houseflies' surface microbiota *Brachybacterium* were dominant (Fig. 2K). The houseflies collected from a water treatment plant showed that *Bacillus*, *Enterococcus,* and *Escherichia/Shigella* were dominant at the genus level (Fig. 2L). Due to wastewater and human-induced wastes, *Enterococcus,* and *Escherichia/Shigella* were found to be dominant in this environment.

The surface microbiota of houseflies was a good indicator of the environmental habitat where they were collected and lived. Zhao et al. [32] indicated the importance of the food source of housefly larvae and how it affects gut microbiota and helps to degrade polysaccharides present in feeding materials. When larvae feed on wheat bran, their results showed that Proteobacteria and Firmicutes were dominant in houseflies' larvae, and in control wheat bran samples Proteobacteria were abundant at the phylum level.

Culture-dependent and culture-independent methods were used to identify bacterial communities present in the gut microbiota of 65 adult *M. domestica* collected from public places in India. The mainly identified bacteria belong to *Klebsiella*, *Aeromonas*, *Shigella*, *Morganella*, *Providencia*, and *Staphylococcus* genera. The results of the culture-independent method (16S rRNA gene clone library) were consistent of the culture-dependent taxonomic results [33].

In addition to pathogenic bacteria, lactic acid bacteria, *Lactococcus*, *Enterococcus*, and *Lactobacillus* were also present on the external surface of houseflies. The diet and feeding materials or food sources of houseflies can shape their microbiota. In fact, collection environments (manure, farm, home, inside or outdoors of hospitals or wastewater treatment plant) play an important role to establish the microbiota of houseflies and they reflect their habitat. Bahrndorff et al. [34] identified microbiota of 90 houseflies collected within and between ten dairy farms in Denmark by 16S rRNA amplicon sequencing. Their results indicated that *Corynebacterium*, *Lactobacillus*, *Staphylococcus*, *Vagococcus*, *Weissella*, *Lactococcus*, and *Aerococcus* were dominant at the genus level. The average Shannon diversity index was calculated from 3.4 to 5.4. In our study, the highest Shannon species diversity value

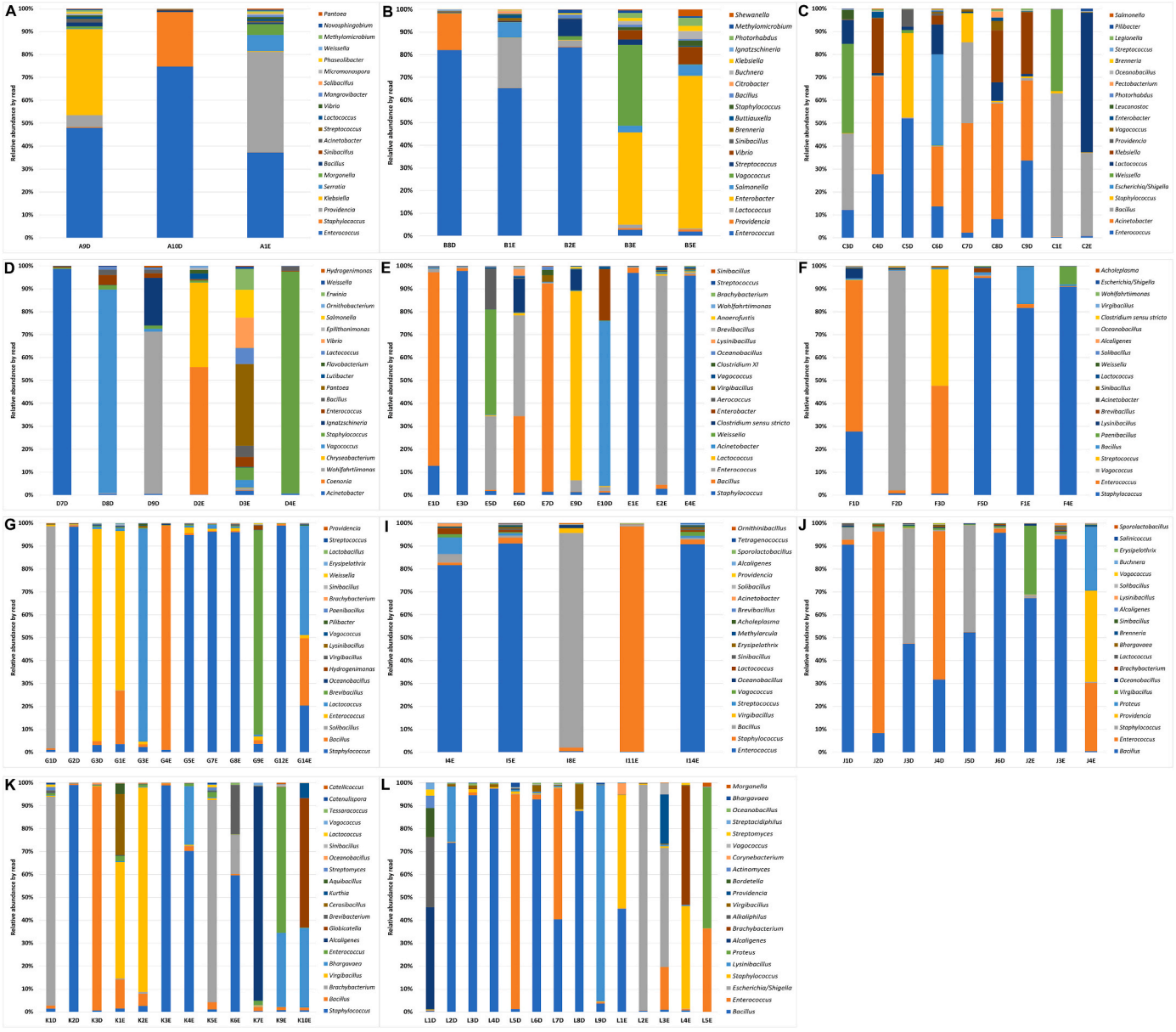

Fig. 2. The genus level taxonomic distributions of the bacterial communities on the surface microbiota of houseflies. Each graph represents a collection place of the housefly samples coded as A, Laboratory population; B, Around Medical Faculty Hospital; C, Around Hospital-1; D, Rural area; E, Fish market; F, Food market-1; G, Food market-2; I, Dump site-1 (Waste storage area); J, Dump site-2; K, Around Hospital-2; and L, Water treatment plant. (Color in online only).

(1.677) was recorded in the J4E sample obtained from a male housefly in the dump site-2 and the lowest Shannon diversity value was 0.123 and it was recorded in the I11E samples obtained from a male housefly collected from the dump site-1 (Waste storage area) (Table S1). Moreover, the highest number of species (298) were identified in the L1E sample collected from the water treatment plant and the lowest number of species (104) were identified in G7E collected from the food market-2 (Table S1). Our results indicated that the most variation in species richness and diversity were found between individuals of examined male and female houseflies, but not locations where houseflies were collected.

Recently, Park et al. [35] examined 400 houseflies from Belgium and Rwanda to identify the external and internal bacterial and fungal microbiota. The study showed that the external microbiota of houseflies varies in geographic location, and isolation habitat, but internal microbiota is limited by their physiology. The internal and external microbiota of houseflies from the hospital, homes, and environments were mainly dominated by *Staphylococcus*, *Weissella,* and *Acinetobacter*. Paulson et al. [36] also studied external and internal (gut) bacterial populations in 100 randomly selected houseflies from the fish markets, butcheries, vegetable markets, and residential areas by microbiological techniques. Their results showed that *Pseudomonas* spp. and *E. coli* were dominant both the external and internal microbiota, moreover *Staphylococcus* spp. were also isolated from both the external and internal parts of houseflies. In another study, Sukontason et al. [37] collected 130 *M. domestica* from five fresh-food markets in Chiang Mai Province and coagulase-negative staphylococci (n = 57) were the most commonly isolated bacteria followed by *E. coli* (n = 10) and Viridans streptococci (n = 10). Vazirianzadeh et al. [38] also determined bacteria present on the surface of 230 houseflies and *E. coli* was dominant with 36.5% and *S. aureus* with 26.9%.

In our study, PoCA analysis of houseflies at the genus level grouped the sample locations into 4 major gatherings although the groups were not distinctly separated from each other. Group 1 contained mostly

dump areas and the water treatment plant (L, J, and I), Group 2 contained most of the locations from the hospitals (K and B), fish market (E) and a rural area (D), food market-2 (G) and the water treatment plant (L), Group 3 contained the water treatment plant (L), food market-1&2 (F and G), fish market (E), a rural area (D), and hospital (K), while Group 4 contained samples from the fish market (E), hospitals (B and C) and the lab population (A). These results can be interpreted as evidence for distinct genus members for water treatment and dump areas, fish markets with significant overlap with the hospitals and some dump locations, a food market with overlap with some hospital locations (Fig. 3).

Staphylococci were selectively isolated and applied partial 16S rRNA Sanger sequencing revealed that 32 staphylococcal strains were identified as *S. sciuri* (n = 11), *S. saprophyticus* (n = 9), *S. arlettae* (n = 6), *S. xylosus* (n = 4), *S. epidermidis* (n = 1), and *S. gallinarum* (n = 1). *S. aureus* is the most virulent staphylococcal species, however, none of the collected houseflies were found to carry *S. aureus*. All isolated staphylococcal species were found to be coagulase-negative. In another study, 1349 *M. domestica* were collected and 439 of them were found to carry bacteria on their surface in Thailand. Coagulase-negative staphylococci were identified as dominant bacteria and they were followed by *Streptococcus* group D non-enterococci and *Bacillus* spp [39].

Virulence gene contents of staphylococci were searched by PCR and the results revealed that all strains were found to be negative for *mec*A, *tet*S, *erm*A, *erm*B, *cat*, *mef*A, *seb*, *sec*, *sed*, *see*, and *lukSF* PVL genes. *tet*K was detected in 2 strains (*S. saprophyticus* F4E/S1 and *S. sciuri* I11E/S1-Y), *tet*M in only *S. xylosus* G7E/S1, *tet*L in only *S. saprophyticus* C7D/S1, and *erm*C in 2 strains (*S. saprophyticus* F4E/S1 and *S. arlettae* K9E/S3). The eight strains (*S. arlettae* K2E/S1, K4E/S1, K6E/S1, K9E/S3, L1E/S1, L4E/S1, *S. epidermidis* J5D/S2, and *S. saprophyticus* K3E/S1) were found to contain *msr*A and *msr*B genes. In the PCR experiments, only *S. xylosus* D4E/S2 was positive for aad6 gene.

Antibiotic susceptibility testing showed that 29/32 (90.63%) and 18/32 (56.25%) staphylococcal strains were resistant to oxacillin and penicillin G, respectively. In addition, erythromycin resistance was found in 9/32 (28.13%) strains. Resistance to clindamycin (n = 5), tetracycline (n = 3), ciprofloxacin (n = 2), gentamicin (n = 1), and chloramphenicol (n = 1) were found in staphylococcal strains. Three strains *S. saprophyticus* C7D/S1 and F4E/S1 as well as *S. arlettae* K9E/S3 were multi-drug resistant with resistance to more than three antibiotics. The agar disk diffusion assay results of resistance profiles to antibiotics were also confirmed with MIC breakpoints values. Resistance of multi-drug resistant strains to vancomycin was tested with E-test and the obtained breakpoints showed that all multi-drug resistant strains were susceptible to vancomycin with 1–2 μg/ml breakpoints. None of the strains were found to be resistant to cefoxitin, mupirocin, linezolid, sulphamethoxazole/trimethoprim, and rifampicin. There was a statistical difference between housefly collection places and antibiotic resistance to penicillin G, gentamicin, and erythromycin ($p < 0.05$), but there was no difference with other antibiotics (Table S3). In the evaluation of staphylococci with tested antibiotics, there were statistical differences between isolated staphylococcal strains and penicillin G, oxacillin, cefoxitin, gentamicin, linezolid, erythromycin, sulphamethoxazole/trimethoprim, and rifampicin with $p < 0.05$ value (Table S4). D-test results demonstrated that two *S. saprophyticus* strains F1E/S1 and K2D/S1 showed inducible clindamycin resistance (Fig. S4). In fact, the strains that were found to have inducible clindamycin resistance were negative for *erm*ABC genes. However, both clindamycin and erythromycin-resistant *S. saprophyticus* F4E/S1 and *S. arlettae* K9E/S3 were positive for *erm*C gene.

Recent studies showed that Gram-positive and Gram-negative bacteria isolated from houseflies were resistant to antibiotics. Bouamama et al. [40] isolated 21 coagulase-negative staphylococci and 17 *S. aureus* strains from the external surface of 600 *M. domestica* collected in Morocco and their antibiotic susceptibility testing showed that the staphylococcal strains were resistant to penicillin (65.2%), oxacillin (26.1%), erythromycin (8.7%), and clindamycin (8.7%). All staphylococci showed 100% susceptibility to vancomycin, linezolid, daptomycin, gentamicin, levofloxacin, and co-trimoxazole. In another study, Nazari et al. [41] isolated 394 bacterial strains (*Bacillus* spp., *Staphylococcus* spp., and *E. coli*) from the surface of 275 houseflies in Iran by the microbiological methods. In that study, coagulase-negative staphylococci from houseflies in the non-hospital environments and *S. aureus* strains from the hospital environments were mostly resistant to ciprofloxacin and cefotaxime. Hemmatinezhad et al. [42] isolated *Pseudomonas aeruginosa* in 8.8% (53/600) from the houseflies collected from farms, hospitals, and slaughterhouses. All isolates were found to be

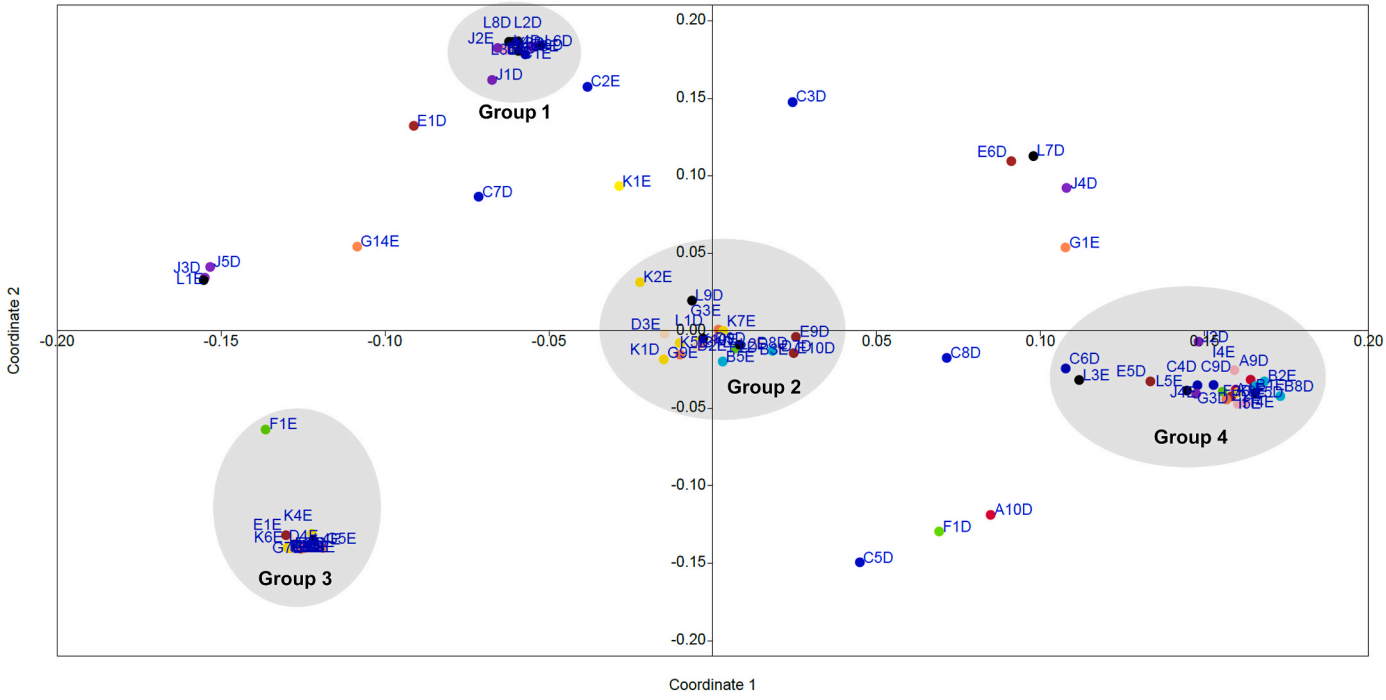

Fig. 3. PoCA analysis results of the houseflies' surface microbiota at the genus level taxonomic distribution. (Color in online only).

resistant to ampicillin, cefalexin, ceftriaxone, cefotaxime, and kanamycin. Furthermore, Davari et al. [43] examined 908 houseflies and 366 bacterial strains were isolated mostly *Klebsiella pneumoniae* (43.3%), *P. aeruginosa* (37%), *Proteus mirabilis* (29.1%), and *Citrobacter freundii* (28.4%). The strains were found to be highly resistant to cephalexin and chloramphenicol. Fukuda et al. [44] also showed that the inoculated houseflies with an antimicrobial-resistant *E. coli* strain persisted during their life cycle. Moreover, the colistin resistance genes (mcr-1, 2, and 3) were searched in 252 *M. domestica* flies, and 4.8% (9/189) bacteria isolated from houseflies were found to carry only mcr-1 gene [45].

Biofilm formation is an important virulence factor of staphylococci. Especially *S. epidermidis* and *S. aureus* strains form an intense biofilm layer on biomaterial, medical devices as well as food contact surfaces [46–48]. In this study, the biofilm-forming capacity of staphylococci was examined in two media (TSB+1% sucrose and BHI) using the microplate test. The results showed that 17 strains formed biofilm in both media (Fig. 4). Whereas 15 strains were non-biofilm formers. *S. sciuri* C9D/S1, F1D/S1, G1D/S1, G7E/S2, and *S. saprophyticus* F1E/S1, G8E/S2, and G12E/S1 form a dense biofilm layer in both TSB and BHI media on the microplate surfaces (Fig. 4). Although, the biofilm formers *S. sciuri* C7D/S1, C9D/S1, and *S. saprophyticus* K3E/S1 were isolated from the houseflies collected from around the hospitals, the biofilm-forming strains (*S. sciuri* F1D/S1, G1D/S1, G7E/S2, and *S. saprophyticus* F1E/S1, G8E/S2, and G12E/S1) were isolated from the food markets. Most of the strains (coded C and K) isolated from the surface of houseflies collected around the hospitals did not form biofilm. This study also indicated the importance of biofilm-forming strains in the mechanical transport of pathogenic bacteria from surface-to-surface.

The extracellular enzyme production of staphylococci was determined using the substrate containing media and the zone formation in the plates were evaluated visually (Fig. S5). The results demonstrated that 12 strains showed lipolytic activity in tributyrin agar. These were mostly *S. saprophyticus* (n = 7), *S. xylosus* (n = 4), and *S. epidermidis* (n = 1) strains (Table S2). In casein agar plates, 10 strains had proteolytic activity after TCA treatment with halo formation around colonies and all of them were *S. sciuri* strains. Moreover, 27 strains in skim milk agar plates and 31 strains in milk agar plates showed proteolytic activity (Table S2). None of the strains had amylase activity in starch agar. Contamination of food by extracellular enzyme-producing bacteria can cause adverse effects in especially in the food processing. The necessary hygiene precautions should be taken in the food processing and production environments.

Clonal relationships among the same species of staphylococci were determined by PFGE analysis. The constructed dendrograms based on band patterns that were created by macrorestriction of the whole bacterial genome showed that three groups of *S. sciuri* (G7D/S2 and G1D/S1, C9D/S1, and C4D/S1, A9D/S1, and A10D/S1) were identical with indistinguishable band patterns (Fig. 5A). The *S. sciuri* strains A9D/S1 and A10D/S1 isolated from *M. domestica* lab population were separated from all other *S. sciuri* strains with 30% homology. Indeed, the growth medium of the lab population shapes the surface microbiota. Moreover, *S. sciuri* I11E/S1-Y strain isolated from the dump site-1 showed distinct band patterns and it was separated from the others with 44.4% homology (Fig. 5A). None of the *S. saprophyticus* strains were identical. Two *S. saprophyticus* G8E/S2 and F4E/S1 isolated from the food market-2 and the food market-1 respectively were most closely related with 92.3% homology (Fig. 5B). *S. saprophyticus* E5D/S1A from the fish market showed distinct band patterns and it was separated from all other *S. saprophyticus* strains with 26.3% homology. Furthermore, *S. arlettae* strains from the water treatment plant and around the hospital-2 showed 100% homology (Fig. 5C). *S. arlettae* K6E/S1 and K2E/S1 were separated from the main group with 87.5% and 53.5% homology, respectively. Similar to *S. saprophyticus* strains, each *S. xylosus* strains showed different band patterns, and two main groups were separated with 47.1% homology (Fig. 5D). The PFGE analysis results demonstrated that the environmental conditions and the habitat of houseflies also characterize staphylococci.

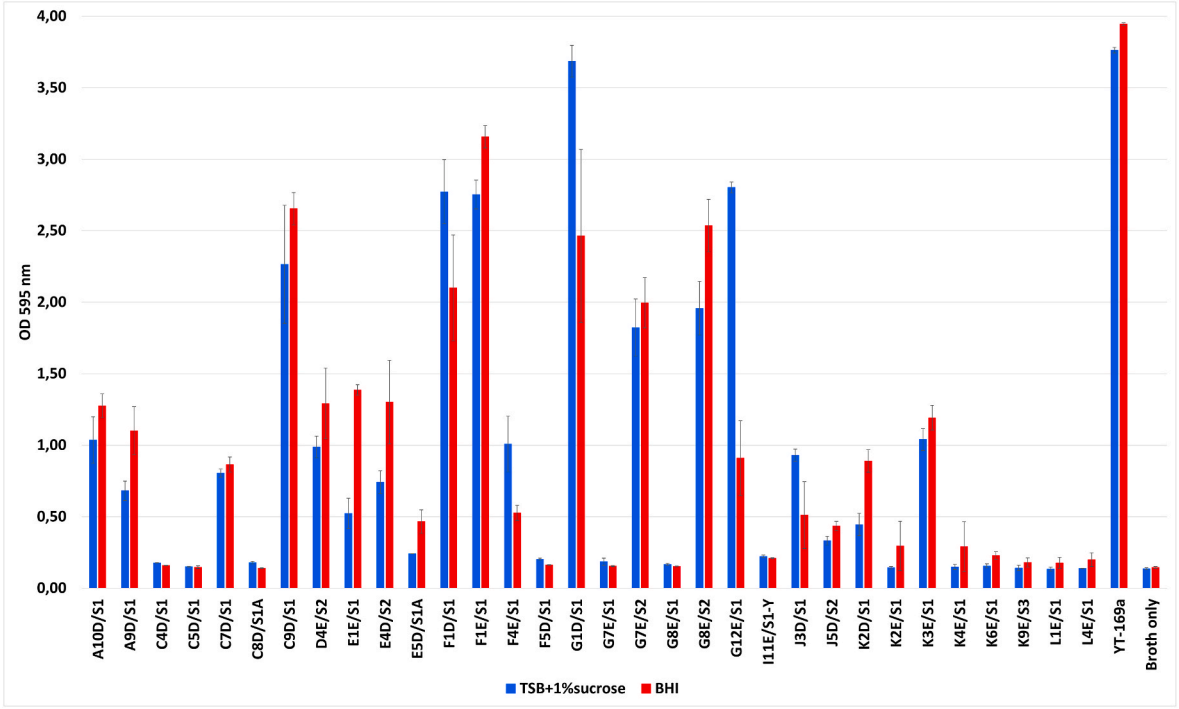

Fig. 4. The microplate test results show the biofilm formation of staphylococci isolated from the houseflies' external surfaces in TSB supplemented with 1% sucrose and BHI media. *S. epidermidis* YT-169a was used as a biofilm forming positive control strain. The results were shown mean and standard deviations of two measurements with triplicate wells for each bacterial strain. (Color in online only).

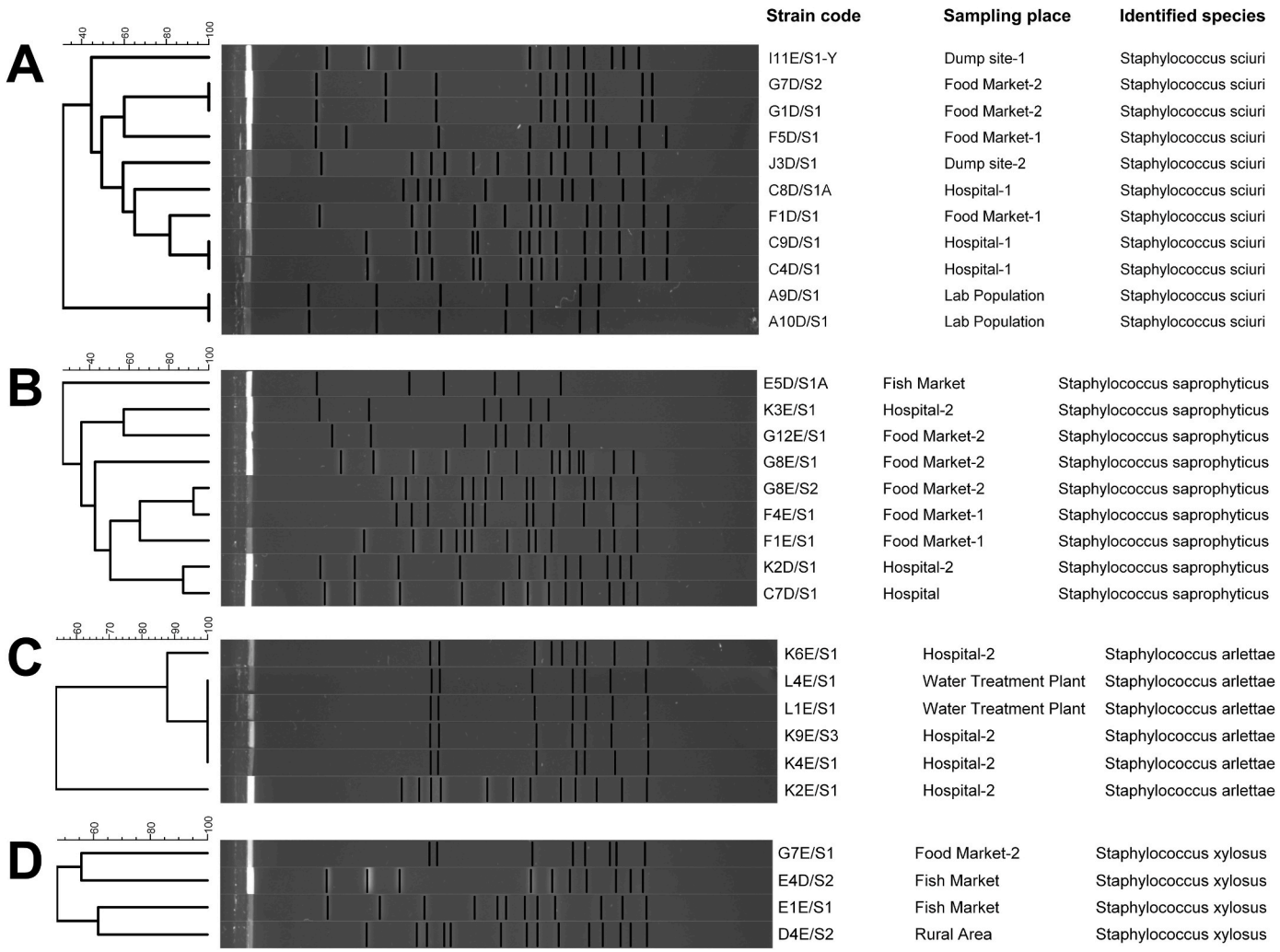

Fig. 5. Dendrograms of PFGE analysis show the clonal relationships of the isolated staphylococcal strains at the species level. The codes of strains and sampling places were indicated. (A) *S. sciuri* strains (n = 11), (B) *S. saprophyticus* strains (n = 9), (C) *S. arlettae* strains (n = 6), and (D) *S. xylosus* strains (n = 4).

4. Conclusion

Houseflies contain a large and highly diverse bacterial microbiota on their external surfaces. In fact, this diverse microbiota of houseflies is likely to reflect their habitat where they live, breed, and decay organic matters. Especially the environmental effects in other words feeding sources like manure, food, and human wastes which are rich in different kind of microorganisms shape the external microbiota of houseflies. Flying long distances is one of the reasons for the distribution of microorganisms to other houseflies, to contaminate foods sold in the open market areas, and finally reach to humans. In this way, pathogenic, antimicrobial-resistant, and biofilm-forming microorganisms could be easily spread to other environments and among humans and animals. Hospitals are also suitable environments for the houseflies to carry vector-borne pathogens between patients and from hospital to other environments. Our culture-independent metagenomics and culture-dependent analysis results indicated the carriage of antibiotic-resistant, biofilm-forming, and virulent staphylococci on the external surfaces of houseflies. The necessary hygiene precautions should be taken to protect against contamination of food and the spread of pathogens by houseflies.

Data availability

The metagenomic amplicon sequencing NGS data have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA749668.

Declaration of interests

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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2022.105439.

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