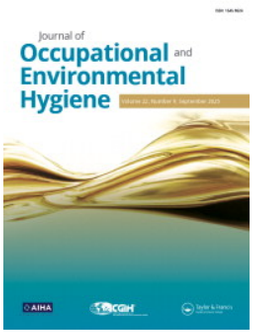

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REPORT

Microbial exposure and diversity in Norwegian shrimp processing plants

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ABSTRACT

Seafood processing workers have a high prevalence of respiratory symptoms and occupational asthma, primarily attributed to allergenic protein exposure. However, exposure to airborne microorganisms from raw materials can also contribute to allergic sensitization and other respiratory ailments. This study aimed to assess microbial exposure in shrimp processing plants and identify susceptible work tasks. Full-shift personal air samples were collected from two Norwegian shrimp processing plants across five distinct work processes: thawing, truck driving, cooking-peeling (technician), packing, and flour production. The samples were analyzed for the presence of endotoxin, Toll-Like Receptor (TLR) activation, bacterial and fungal DNA copies, and microbial composition. Endotoxin levels were generally low, with only one sample (98 EU/m^3) exceeding the recommended occupational exposure limit (OEL). A significant TLR2 activation was observed among thawers, indicating the presence of microbial ligands capable of triggering an immune response. The median bacterial (75×10^3 DNA copies/m^3) and fungal ($3{,}301 \times 10^3$ DNA copies/m^3) exposure were highest among the flour production workers, while the lowest bacterial and fungal exposure was among packers (1.5×10^3 DNA copies/m^3) and technicians (337 DNA copies/m^3), respectively. Several bacterial and fungal species were identified, including ten allergenic and sixteen pathogenic species. *Sporobolomyces roseus* and *Saccharomyces cerevisiae* were the two most frequently identified allergenic fungal species. Among the pathogenic bacterial species, *Prevotella nigrescens* and *Roseomonas gilardii* were the two most detected species. While the pathogenic species were identified mainly in the packing, truck driving, and flour production work processes, most of the allergenic species were found in all work processes. Altogether, work processes before the cooking of shrimp (thawing and truck driving) had higher endotoxin, bacterial load, and species richness than after cooking, suggesting that these work tasks are susceptible to bacterial exposure and that the cooking process significantly reduces bacterial exposure. By shedding light on microbial exposure and identifying high-exposure work tasks, this study enables the development of targeted interventions and implementation of measures for the prevention of occupational diseases.

KEYWORDS

Allergenic microbes; endotoxin; occupational exposure; seafood processing; shrimp industry; workplace microbiome

Introduction

The Norwegian economy relies greatly on the seafood industry, which involves the participation of numerous workers in seafood processing. Some workers within this industry have impaired lung function and have reported respiratory symptoms such as sneezing, a stuffy nose, and a runny nose (Bang et al. 2005; Jeebhay and Cartier 2010; Mason et al. 2020; Shiryaeva et al. 2010). Additionally, occupational asthma is common, affecting 2 to 36% of these workers (Jeebhay and Cartier 2010). Occupational exposures within this industry are complex, primarily because of the aerosolization of highly diverse bioaerosol components, including allergens, proteases, microbial particles, and toxins during various processing activities (Jeebhay 2011). There are minor differences in the processing procedures for shrimp (work processes) across various processing plants, whether the shrimp is farmed or caught in open waters, and the general steps typically involve washing, cooking, beheading, peeling, sorting, freezing, and packaging. Previous studies evaluated the microbial biota of

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shrimps during these processing steps and showed that washing, cooking, and UV treatment reduce microbial count and growth (Harrison and Lee 1969; Jeong et al. 2024). While shrimp microbial biota may generally not pose an immediate health risk to workers, studies have shown that workers who handle the raw material are at a greater risk of being exposed to endotoxins than those who handle the cooked shrimp (Thomassen et al. 2016). In contrast, cooking shrimp enhances its allergenicity due to the denaturation of the protein and the formation of new epitopes (Dong and Raghavan 2022).

Although most of the respiratory symptoms and occupational asthma reported in this industry are associated with exposure to allergenic proteins, inhalation of airborne microorganisms, including bacteria, fungi, and viruses from raw shrimp under different working processes may also lead to allergenic sensitization (Abdurrahman et al. 2020; Edwards et al. 2012; Fukutomi and Taniguchi 2015; Jeebhay 2011; Kurup 2003; Levetin et al. 2016; Nordengrün et al. 2018). Studies have shown that exposure to endotoxins and other microbial components such as peptidoglycans from gram-positive bacteria may cause or exacerbate respiratory symptoms among workers mainly through an acute pro-inflammatory response (Bertelsen et al. 2016; Gilbert and Duchaine 2009; Poole and Romberger 2012). Moreover, exposure to certain microbial species such as *Staphylococcus aureus* and *Aspergillus fumigatus* is reported to cause IgE-mediated allergic reactions in addition to their well-known pathogenicity (Abdurrahman et al. 2020; Crameri et al. 2014; Kurup 2003; Levetin et al. 2016; Nordengrün et al. 2018; WHO 2022). Exposure to a mixture of endotoxin, bacterial species such as *Bacillus cereus,* and respirable dust from shrimp shell powder was reported to have caused symptoms of occupational asthma and organic dust toxic syndrome (Bertelsen et al. 2016).

These adverse health outcomes are triggered when immune or epithelial cells in the lung encounter pathogen-associated molecular patterns (PAMPs), allergens, or other substances such as bacterial or fungal components (Bianchi 2007). TLRs and C-type lectin receptors (CLRs) are among the key cell receptor types that play a crucial role in recognizing these agents and initiating immune responses (Kingeter and Lin 2012; Moresco et al. 2011). Studying the activation of these receptors could indicate potential immune responses to these bioaerosol exposures. Although more than 10 types of human TLRs have been identified, with many recognizing a wide range of patterns, some are known to be specific to a single PAMP. For example, TLR3 specifically binds with double-stranded RNA (dsRNA), while TLR7 is specific to single-stranded RNA (ssRNA) viral components (Moresco et al. 2011; Uematsu and Akira 2006). TLR2 and 4 can detect multiple ligands found in bacteria, fungi, viruses, protozoa, plants, and animals, but they are mainly known for detecting components of Gram-positive and Gram-negative bacteria, respectively (Akira and Takeda 2004; Kuzmich et al. 2017; Moresco et al. 2011; Zakeri and Russo 2018). The CLR member dectin-1a is an important receptor for β-glucans from the cell wall of fungi and carbohydrates from allergens (Angelina et al. 2023; Hardison and Brown 2012; Kingeter and Lin 2012). The activation of these receptors triggers a series of signaling pathways that lead to the activation of transcription factors, which promote the expression of genes involved in the immune responses. The activated immune cells release various signaling molecules, including inflammatory cytokines, chemokines, antimicrobial proteins, histamine, and leukotrienes (Larsen 2017; Larsen et al. 2015). If occurring acutely in the airways, activation is followed by the recruitment and proliferation of other cells such as neutrophils, mast cells, and macrophages, which can lead to classic acute respiratory symptoms like sneezing, itching, redness, or swelling. Over time, these symptoms could develop into chronic health problems such as asthma, rhinitis, and pneumonitis (Kurup 2003; Larsen 2017; Larsen et al. 2015).

Several studies in the seafood industry have primarily focused on examining particle fractions, total protein, and endotoxin concentrations (Heidelberg et al. 2021; Jeebhay and Cartier 2010; Laustsen et al. 2022; Lopata and Jeebhay 2013; Thomassen et al. 2016). Nevertheless, it is important to note that the aerosols in such a work environment comprise various other components, including microorganisms that need further characterization, particularly within the shrimp industry, where such studies are sparse. This study aimed to (1) evaluate the presence of airborne microbial ligands for TLRs, (2) analyze the diversity patterns of the microbial community in the shrimp processing plants, and (3) identify work processes with the highest risks of exposure.

Methods

Study sites and sample collection

Sampling was conducted in two Norwegian shrimp processing plants, following ethical approval from the

Regional Committee for Medical and Health Research Ethics (384542).

These processing plants received frozen shrimp, and the processing steps included thawing, maturation, cooking, peeling, quick freezing, sorting, packing, and shell grinding. Out of 80 workers, 22 agreed to participate in the personal air sampling campaign. Sampling was conducted over two consecutive days, with 8 of 22 workers participating in sample collection on both days. Three different sample types were collected simultaneously from each participant, yielding a total of 90 samples. The sampling campaign included the following five different work processes: thawing (TH), truck driving (TR), cooking and peeling (referred to as technician (TE) hereafter), packing (PA), and flour production (FL), which entails grinding shells. The work process overview is shown in Supplementary Figure S1.

Each worker carried three personal air samplers connected to Casella Apex2 air sampling pumps (Casella UK, Wolseley Rd, Kempston, Bedford, UK). Airborne endotoxins were collected using a Personal Air Sampler (PAS-6) (Van der Wal 1983) equipped with a 25 mm glass microfiber filter (1.0 µm, GF/A Whatman, UK). Microbial DNA was collected using a Conical Inhalable Sampler (CIS) (Casella Solutions, Kempston, UK) with a 37 mm Polycarbonate track-etch (PCTE) (1.0 µm, GVS, ME, USA)), and *in vitro* assay samples were collected using total dust 3-pieces anti-static sampler equipped with a 37 mm polycarbonate membrane filter (0.8 µm, Merck Millipore KGaA, Germany). Before sampling, airflows were calibrated using a digital flow meter Defender 520 M (Defender; SKC Inc., Eighty-Four, PA, USA). Air flow rates were set at 3.5 L/min for the CIS and 2 L/min for the total dust anti-static sampler and PAS-6. The workers carried the samplers in a small backpack, placing the samplers in their personal breathing zone throughout an 8 to 9 hr work shift.

Endotoxin analysis

Endotoxins were extracted from glass microfiber filters using 0.05% Tween 20 in endotoxin-free water with shaking at 500 rpm for 1 hr, followed by centrifugation at 1,000 × *g*. Three blank filters were included and were treated the same way as the samples. Endotoxin levels were analyzed using Endozyme II Go (Biomerieux, Marcy-l'Etoile, France), a recombinant Factor C (rFC) kit with a Limit of Detection (LOD) of 0.005 EU/mL. In short, the extract was diluted 20 times and heat-treated at 75 °C for 15 min to inhibit potential protease interference, and endotoxin levels were analyzed according to the manufacturer's instructions. An internal spike control (endotoxin 0.5 EU/mL) was included for each sample to check for interferences. Two samples that failed the interference control were re-analyzed using the Limulus amebocyte lysate (LAL) assay (Lonza, Basel, Switzerland), following the manufacturer's instructions. All samples were analyzed in quadruplicate: two with a known endotoxin spike (0.5 EU/mL) and two without. Endotoxin concentrations were calculated using a four-parameter regression fit model based on a five-point standard curve (0.005–50 EU/mL).

TLR2, TLR3, TLR4, TLR7, and dectin-1a activation assay

To investigate the presence of microbial ligands with potential health effects upon exposure in workers, Human Embryonic Kidney (HEK) 293 reporter cells TLR2, TLR3, TLR4, TLR7, and dectin-1a (Invivogen, France) were exposed to the air sample extract. Activation of each of these receptors results in the induction of NF-κB, leading to the release of measurable secreted embryonic alkaline phosphatases.

The polycarbonate membrane filters from the total dust anti-static sampler were extracted by immersing in 5 mL 0.1% Bovine Serum Albumin in Phosphate Buffer Saline (0.1% BSA-PBS) using a clean tweezer and sonicated for 5 min in a water bath sonicator, followed by shaking for 60 min (500 rpm) at room temperature. The filter was removed carefully, and the eluate was aliquoted in sterile tubes and stored at −80 °C until the *in vitro* assay experiment.

For the exposure experiments, cells were seeded in 96-well plates at a density of 2.8×10^5 cells/mL in 180 µL fresh culture media prepared with HEK Blue selection media according to the manufacturer's recommendation and incubated overnight in a humidified atmosphere at 37 °C with 5% CO_2. Cells were then exposed to 20 µL of air sample extract for 24 hr, whereafter 20 µL of cell suspension was transferred to a new 96-well Nunc plate, and 180 µL Quanti-Blue (Invivogen, San Diego, USA) was added. The plate was incubated for 180 min, and the color development was measured at 649 nm by a BioTek Synergy Neo2 Hybrid Multimode Reader (Agilent Technologies, Winooski, VT, USA). All samples were run in triplicate, and each experiment was repeated three times. The experiment included a blank unexposed filter as a negative control and positive controls for TLR2 (ultrapure LTA, lipoteichoic acid), TLR3 (Poly (I: C)

LMW), TLR4 (ultrapure LPS, lipopolysaccharide), TLR7 (CL264), and dectin-1a (Zymosan); all were purchased from InvivoGen. The data were reported as a fold change against the blank sample.

Extraction of microbial DNA from PCTE filter

When sampling was completed, the exposed and unexposed (blank) samples were immediately subjected to extraction by immersing the filter in 5 mL phosphate-buffered saline with 0.1% Tween 20 (PBST) with 400 rpm shaking for 3 min, followed by 3 min sonication. The extraction was repeated with another 5 mL PBST buffer. The two extractions were combined and centrifuged at 3,000 rpm for 30 min. The supernatant was discarded, and the pellet was stored at −80 °C until DNA isolation.

DNA was isolated from the samples using CTBA-lysis buffer (PanReac AppliChem, Barcelona, Spain), bead beating, and different temperature treatments as previously described (Straumfors et al. 2019). Genomic DNA extraction was finalized using a Qiagen isolation kit (DNeasy Plant Mini Kit, Qiagen GmbH, Hilden, Germany). DNA concentration was quantified using a Qubit 1X ds DNA HS assay kit from Invitrogen (Thermo Fisher Scientific, Waltham, MA, USA).

Digital droplet PCR (ddPCR)

A master mix was made using EvaGreen supermix (Bio-Rad, Hercules, CA) and a primer set P11P and P13P (Widjojoatmodjo et al. 1995) for bacterial DNA quantification. Primer sequences are shown in Supplementary Table S1. Droplets were generated using a QX200 droplet generator (Bio-Rad, Hercules, CA). The droplets underwent thermocycling using the following program: 95 °C for 5 min, 94 °C for 30 sec, annealing at 60 °C for 1 min, 4 °C for 5 min, and extension at 90 °C for 5 min, for a total of 40 cycles.

The same procedure was repeated for fungal DNA quantification using primer duo FF390/FR1 (Chemidlin Prévost-Bouré et al. 2011) and a slightly different thermocycling program as follows: 95 °C for 5 min, 95 °C for 30 sec, 50 °C for 30 sec, 60 °C for 1 min and 4 °C for 5 min, and 90 °C for 5 min for 40 cycles. The number of DNA copies was quantified using a QX200 droplet reader (Bio-Rad, Hercules, CA, USA) and QX Manager software version 2 (Bio-Rad). To avoid uncertainty, the threshold for recognizing positive droplets was set at 10,000 for bacteria and 9,000 for fungi. Airborne bacterial and fungal DNA concentrations are presented as DNA copies/m^3.

Next generation sequencing (NGS) (16S and 18S)

The DNA was amplified for NGS analysis using Phusion high fidelity PCR Master Mix with 5x HF PCR buffer, 40 mM dNTPs, and 1 µM of forward and reverse primers with a 25 µL final PCR reaction volume using PCR program setting: 98 °C for 30 sec, 98 °C for 10 sec, annealing temperature at 52 °C for bacteria and at 60 °C for fungi for 30 sec, 72 °C for 30 sec, and 72 °C for 4.5 min for a total of 30 cycles. Amplification was done using primer duo 515FB/926R targeting the 16S rRNA V4–V5 region (Parada et al. 2016; Walters et al. 2016) and fungal-specific primer set ITS86/ITS4 (Op De Beeck et al. 2014). The DNA concentrations obtained were lower than the criteria (>1 ng/µL) set by the sequencing company. Therefore, PCR products were re-amplified using their respective primers and the same PCR program but with a reduced number of cycles (25 cycles). All samples were run in four replicates, and the replicates were combined into one sample before PCR purification using a Norgen PCR purification kit (Norgen Bioptek Corporation, Thorold, ON, Canada). A mock community of fungi and bacteria was included as a positive control for amplification and sequencing, and extracts of blank filters were included as a negative control.

Bioinformatic analysis

Sequencing and bioinformatic analysis were performed by the Integrated Microbiome Resource (IMR) at Dalhousie University, Canada. The samples were sequenced in NextSeq2000, which can provide up to 4× depth and a Q-score of 30, read accuracy of 90%. The bioinformatics analysis was performed using the QIIME2-based Microbiome Helper pipeline (https://github.com/LangilleLab/microbiome_helper/wiki/Amplicon-SOP-v2-(qiime2-2022.11)). In short, the primers were removed using cutadapt after reading quality inspection using FASTQC and MultiQC to make sure the samples were of high quality with per-base quality >30 across most of the reads and no outliers. Forward and reverse reads were joined with VSEARCH, and low-quality reads were filtered out. Additional filtering was performed with a mean error rate set ≤0.5% and acceptable trim length to remove low-quality reads and ambiguous bases. This step was followed by deblurring, which generates an Amplicon Sequence Variant (ASV). Finally, rare ASVs with a

frequency <0.1% of the mean depth and contaminant ASVs, such as sequencing data from mitochondria and chloroplasts, were removed, and taxonomic assignment was done using reference databases SILVA and UNITE.

In the refined dataset, 835 OTUs for bacteria and 288 OTUs for fungi were retained, excluding negative control and mock community samples. While the blank (background) samples showed a limited number of species, the mock community samples demonstrated the anticipated species, thus meeting expectations. The final dataset contained a total of 5,447,715 bacterial reads and 9,578,418 fungal reads across the 30 samples collected in the five work processes.

To identify allergenic species among the species identified in the air samples, a search was carried out on the Allergome platform (Allergome 2024). Additionally, to evaluate the risk group of the species identified, searches were conducted in the GESTIS Biological Agents Database (https://gestis-database.dguv.de) and ePATHogen Risk Group Database (https://health.canada.ca/en/epathogen) (Government of Canada 2024; IFA 2024), and species that belong to risk group 2 in either database are reported. Risk Group 2 is defined as biological agents that can cause human disease and may pose a risk to workers but are unlikely to spread to the community. Generally, effective prophylaxis or treatment is available.

Data treatment and statistical analysis

Exposure concentrations of endotoxin (EU/m^3), bacteria, and fungi as DNA copies/m^3 are reported as min, max, median, arithmetic mean, and standard deviation. Values below the LOD were treated as zero. The relationship between bacterial DNA copies and endotoxin levels was examined using the Spearman correlation coefficient. Alpha diversity analysis, which includes parameters species richness (the number of species), evenness (distribution of individuals among species), and Shannon index (which accounts for both species abundance and evenness), was conducted using the vegan package (2.6.4) in R Studio. Further, different work processes were compared using either One-way ANOVA and Turkey's HSD post hoc analysis or the Kruskal-Wallis test, followed by Dunn's test, depending on the normality of the Shapiro test result. Before and after cooking, comparisons were made using a t-test or Mann-Whitney test, with a two-sided level of significance of 0.05. Log transformation was used when necessary. Data transformations and analysis types are specified in the figure legends.

Partial least squares discriminant analysis (PLS-DA) was employed to visualize and assess workgroup patterns, relationships, and differences while enhancing group separation.

Statistical analysis and data visualization were performed in R Studio (V 4.3.1), Graph pad Prism version 9, and jvenn (Bardou et al. 2014).

Results

Microbial DNA and ligands in the airborne dust

Exposure to endotoxin, bacteria, and fungi

Personal exposure to endotoxin was generally low and detectable only among thawers, truck drivers, and technicians, with a concentration ranging from <0.005 EU/mL (LOD) to 98 EU/m^3 (Table 1). Workgroup comparison showed that the endotoxin exposure among thawers was significantly higher than that of the technicians ($p = 0.02$). The median bacterial exposure was 14×10^3 DNA copies/m^3, with the highest median exposure levels among flour production workers (75×10^3 DNA copies/m^3) and the lowest levels among the packers (1.5×10^3 DNA copies/m^3), with concentrations below the detection limit in a few samples (Table 1). The bacterial exposure was significantly higher in the thawing than in the packing ($p = 0.03$) (Table 1). Furthermore, the comparison between workgroups before cooking the shrimp (thawers and truck drivers) and after cooking the shrimp (technician, packers, and flour production workers) showed a significant reduction in endotoxin exposure ($p < 0.01$)and bacterial exposure ($p = 0.04$) (Supplementary Figure S2). Moreover, the bacterial DNA copies were weakly correlated to the endotoxin levels ($r = 0.39$, $p = 0.04$).

The highest fungal exposure (6.5×10^6 DNA copies/m^3) was measured in flour production, which was significantly higher than all other work processes ($p < 0.01$). The lowest fungal exposure was among the packers (below the limit of detection), with an overall median exposure concentration of 337 DNA copies/m^3 (Table 1). The fungal exposure comparison between work processes before and after cooking the shrimp showed a significant increase in exposure after the cooking ($p = 0.04$) (Supplementary Figure S2). However, this increase is primarily attributed to flour production, as indicated in Table 1.

Microbial ligands by TLR activation

Significant activation of TLR2 was detected only in the thawing work processes (20% of the air samples), with a two-fold increase in receptor activation

Table 1. Exposure levels of endotoxin, bacteria, and fungi in personal air samples stratified by workgroups.

		Before cooking		After cooking		
	All samples	Thawers (TH)	Truck drivers (TR)	Technicians (TE)	Packers (PA)	Flour production workers (FL)
Endotoxin (EU/m^3)						
N	29	5	4	7	11	2
Median (Min-Max)[1]	<LOD (<LOD–98)	35 (7–49)^{a}	3 (<LOD–98)^{ab}	<LOD (<LOD–28)^{b}	<LOD	<LOD
Mean ± SD	11 ± 22	33 ± 16	26 ± 48	7 ± 12	<LOD	<LOD
Bacterial DNA Copies × 10^3/m^3						
N	30	6	4	7	11	2
Median (Min-Max)	14 (<LOD–130)	60 (4–130)	23 (8–89)	12 (0.1–83)	1.5 (<LOD–34)	75 (21–129)
Mean ± SD[2]	30 ± 38	58 ± 46^{a}	36 ± 37^{ab}	25 ± 30^{ab}	7 ± 11^{b}	75 ± 76^{ab}
Fungal DNA Copies × 10^3/m^3						
N	30	6	4	7	11	2
Median (Min-Max)	0.3 (<LOD–6,546)	0.6 (0.1–3.9)	1.1 (0.6–6.7)	0.2 (0.03–0.6)	0.3 (<LOD–1.2)	3,301 (55–6,546)
Mean ± SD[2]	221 ± 1,195	1.1 ± 1.4^{a}	2.4 ± 2.9^{a}	0.2 ± 0.2^{a}	0.4 ± 0.4^{a}	3,301 ± 4,590^{b}

SD – Standard Deviation, LOD – Limit of detection.
[1]Group comparisons were made using the Kruskal-Wallis test and Dunn's test for endotoxin measurement.
[2]One-way ANOVA and Tukey's HSD post hoc test were used for bacterial and log-transformed fungal data sets. Workgroups that do not share the same letter (^{ab}) are significantly different ($p < 0.03$).

compared to a blank (unexposed) sample ($p < 0.01$) (Supplementary Figure S3a). No TLR3, TLR4, TLR7, and dectin-1a activation was observed (Supplementary Figures S3 and S4).

Diversity patterns and microbial community composition

Bacterial diversity

The two most common phyla identified were Bacteroidota (29%) and Firmicutes (24%) in the overall samples. Bacteroidota was dominant in samples among truck drivers (29%) and technicians (34%). Bacteroidota was equally abundant with Firmicutes, each comprising 24% of the bacterial community among the packers (Figure 1a). In flour production and thawing, Proteobacteria (47%) and Firmicutes (40%) were dominant (Figure 1a). The alpha diversity analysis showed that bacterial species richness was significantly higher among thawers than technicians and packers ($p = 0.01$) (Supplementary Figure S5b). In addition, samples among truck drivers exhibited a higher species richness than samples from technicians and packers, with a $p < 0.01$ (Supplementary Figure S5b). Furthermore, a significant reduction in species richness was observed in processes after cooking ($p < 0.01$) (Supplementary Figure S5e). The Shannon index and evenness parameters did not reflect these differences, indicating similar biodiversity and uneven species abundance between work processes (Supplementary Figure S5). A score plot from Partial Least Square Discriminant Analysis (PLS-DA) with a 95% confidence interval showed a clear separation between processes before and after cooking and truck drivers and thawers (Figure 1b). Although there is some separation between the packers and technicians, the clusters exhibited partial overlap. The samples from flour production are situated within the same cluster as the thawing, suggesting similarity (Figure 1b).

The three most abundant genera identified are *Deinococcus*, *Brevibacterium*, and *Vagococcus* (Supplementary Figure S7a). However, most species identified belong to the genera *Prevotella* (5.2%) and *Deinococcus* (4.9%). In total, 117 bacterial species were identified. *Deinococcus radiodurans* (18%), *Prevotella nigrescens* (17.2%), and *Deinococcus murrayi* (17.1%) were the three most abundant species identified. While *Deinococcus radiodurans* and *Prevotella nigrescens* were common among the technician samples, *Deinococcus murrayi* was frequently identified among truck drivers (Figure 1c). From the total number of species identified, nine were shared among the five workgroups (Figure 1d). Distinct bacterial species profiles were observed for each workgroup, with the highest number of unique species found among packers (21 species), followed by thawers (11 species), an equal number of species among truck drivers and technicians (6 species), and flour production workers (2 species) (Figure 1d).

Fungal diversity

Basidiomycota was the most frequently identified phylum in the overall samples, accounting for 72%. In the

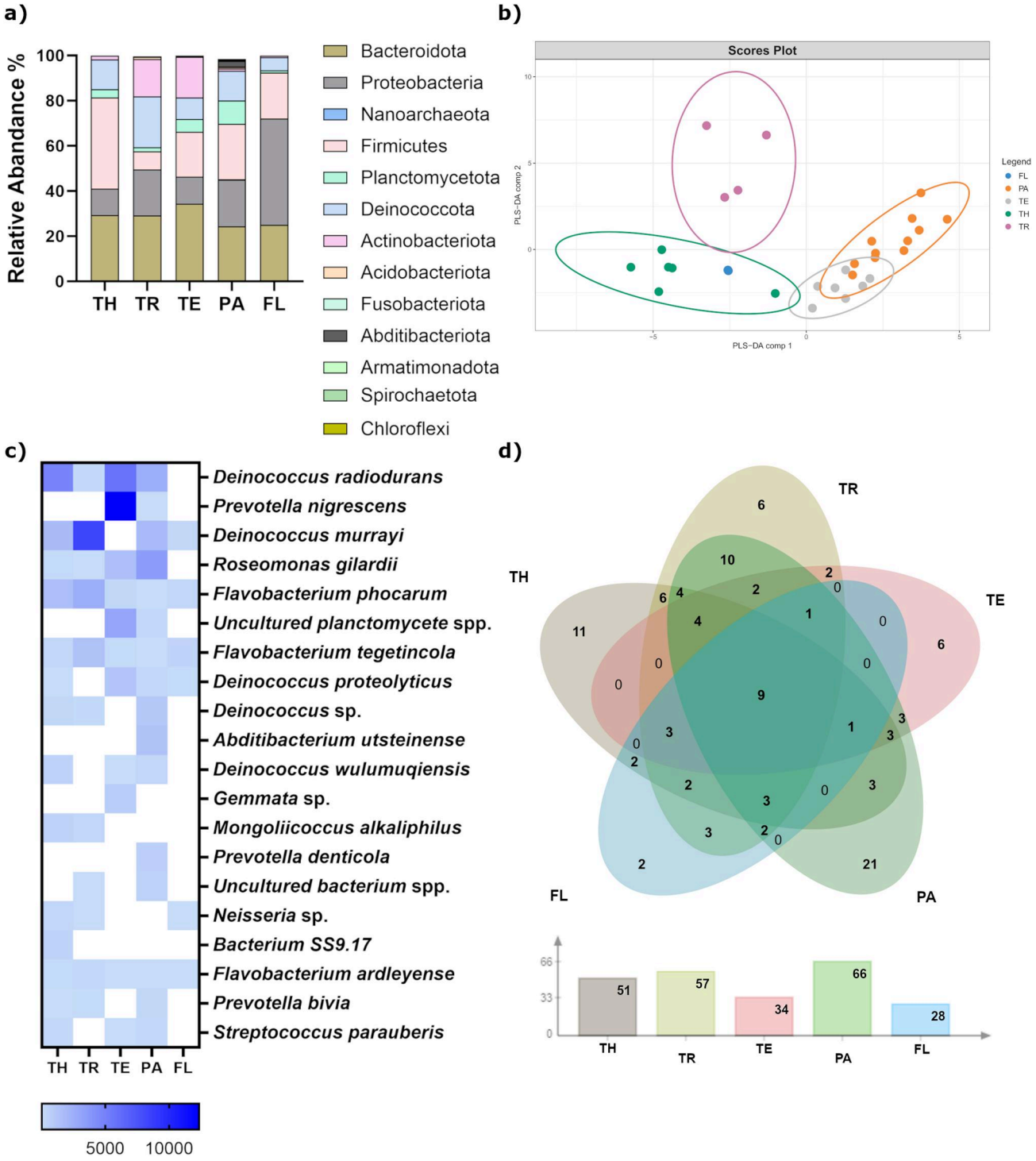

Figure 1. Bacterial abundance and distribution stratified by workgroups (a) relative abundance of bacterial phylum. (b) Partial least square discriminant analysis (PLS-DA) score plot of bacterial communities showing group separation within the 95% confidence interval (circled area). Each point represents a community profile, with cluster proximity indicating compositional differentiation. (c) Heatmap showing the 20 most abundant bacterial species. (d) Venn diagram showing unique and shared bacterial species between workgroups. TH – thawers ($n = 6$), TR – truck drivers ($n = 4$), TE – technicians ($n = 7$), PA – packers ($n = 11$), and FL – flour production workers ($n = 2$)). Full shift (8–9 hr) samples taken from the worker's breathing zone.

different workgroups, Basidiomycota ranged from 53 to 99%. The second most abundant phylum was Ascomycota, representing 20% of the overall samples, 30% among the packers, and 27% among the technicians (Figure 2a). Although there was a slight variation in the Shannon index, richness, and evenness between the different workgroups, none of them was statistically significant. No significant variation

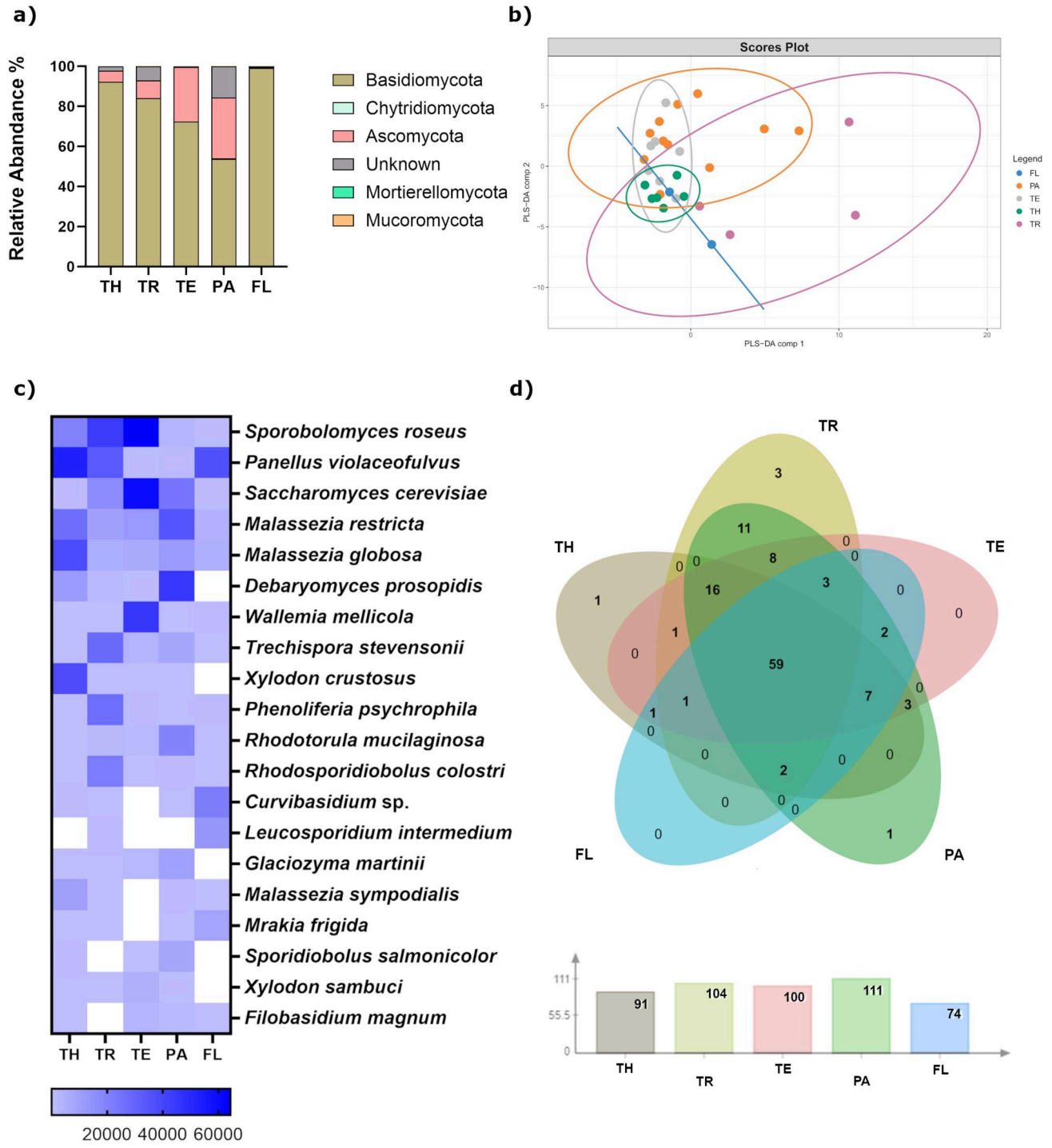

Figure 2. Fungal abundance and distribution stratified by workgroups (a) relative abundance of fungal phylum. (b) PLS-DA score plot of fungal communities showing group separation within the 95% confidence interval (circled area). Each point represents a community profile, with cluster proximity indicating compositional differentiation. (c) Heatmap showing the twenty most abundant fungal species. (d) Venn diagram showing unique and shared fungal species between workgroups. TH – thawers ($n = 6$), TR – truck drivers ($n = 4$), TE – technicians ($n = 7$), PA – packers ($n = 11$), and FL – flour production workers ($n = 2$). Full shift (8–9 hr) samples taken from the worker's breathing zone.

was shown between before and after cooking (Supplementary Figure S6). Similarly, the PLS-DA plot showed no meaningful separation, indicating strong workgroup similarity (Figure 2b).

The three most abundant genera identified were *Malassezia* (17.8%), *Sporobolomyces* (13.8%), and *Panellus* (13.2%) (Supplementary Figure S7b). *Sporobolomyces roseus* (14%), *Panellus violaceofulvus*

(14%), and *Saccharomyces cerevisiae* (11%) were the three most abundant species identified. Among the technicians, *Sporobolomyces roseus* and *Saccharomyces cerevisiae* were frequently identified, whereas *Panellus violaceofulvus* was prevalent among the thawers. (Figure 2c). In total, 118 fungal species were identified, 59 shared by all the workgroups, with five unique species identified among thawers, truck drivers, and packers (Figure 2d).

Allergenic and human pathogenic species identified from NGS data

Of the 118 fungal species identified, 10 allergenic fungi were present (Table 2). These species are described as causing or contributing to allergenic sensitization or are identified as allergens on the Allergome platform. Except for *Ganoderma applanatum* and *Rhizopus microspores*, these allergenic species were found in all five workgroups, indicating a widespread presence. *Ganoderma applanatum* was detected in four workgroups, while *Rhizopus microspores* were identified in three workgroups (Table 2). Furthermore, four of these species had relatively higher percentages of the total reads: *Malassezia globose* (7.2%), *Malassezia restricta* (9.5%), *Saccharomyces cerevisiae* (10.9%), and *Sporobolomyces roseus* (14.2%) (Table 2).

According to the GESTIS and ePATHogen databases, 15 bacterial species and one fungal species were identified as risk group 2 (Table 3). Among these species, *Rhizopus microsporus* stands out as both allergenic and pathogenic, posing a potential health risk. *Roseomonas gilardii* and *Streptococcus parauberis* were found in all five workgroups, representing 7.2% and 0.5% of the total reads. *Prevotella bivia* and *Prevotella melaninogenica* were present in four out of the five workgroups. All other species were present in three or fewer workgroups (Table 3). Although *Prevotella nigrescens* was identified in only two workgroups, it had a higher percentage (17.2%) of the total reads.

Discussion

This study has thoroughly characterized microbial exposure in shrimp processing plants by endotoxin

Table 2. Fungal allergenic species identified in personal air samples.

Species	Percentage of the total reads	Number of workgroup species identified in	Workgroups	Ref.
Malassezia globose	7.2	5	TH, TR, TE, PA & FL	(Fukutomi and Taniguchi 2015)
Malassezia restricta	9.5	5	TH, TR, TE, PA & FL	(Fukutomi and Taniguchi 2015)
Malassezia sympodialis	1.3	5	TH, TR, TE, PA & FL	(Levetin et al. 2016)
Rhodotorula mucilaginosa	2.6	5	TH, TR, TE, PA & FL	(Kurup 2003; Levetin et al. 2016)
Saccharomyces cerevisiae	10.9	5	TH, TR, TE, PA & FL	(Kurup 2003; Levetin et al. 2016)
Sporobolomyces roseus	14.2	5	TH, TR, TE, PA & FL	(Allergome 2024; WHO 2022)
Tricholoma sp.	0.02	5	TH, TR, TE, PA & FL	(Allergome 2024)
Scleroderma sp.	0.01	5	TH, TR, TE, PA & FL	(Allergome 2024)
Ganoderma applanatum	0.13	4	TH, TR, TE & FL	(Allergome 2024)
Rhizopus microsporus	0.01	3	TE, PA & FL	(Levetin et al. 2016)

TH – thawers ($n = 6$), TR – truck drivers ($n = 4$), TE – technicians ($n = 7$), PA – packers ($n = 11$), and FL – flour production workers ($n = 2$).

Table 3. Risk group 2 – human pathogens identified in personal air samples.

Species	Percentage of the total reads	Number of work processes species identified in	Work processes
Bacteria			
Roseomonas gilardii	7.23	5	TH, TR, TE, PA & FL
Streptococcus parauberis	0.53	5	TH, TR, TE, PA & FL
Prevotella bivia	0.54	4	TH, TR, PA & FL
Prevotella melaninogenica	0.11	4	TR, TE, PA & FL
Empedobacter brevis	0.04	3	TR, TE & PA
Prevotella nigrescens	17.2	2	TE & PA
Prevotella corporis	0.12	2	TR & PA
Prevotella disiens	0.04	2	TR & PA
Myroides odoratus	0.21	2	TE & PA
Prevotella denticola	0.96	1	PA
Prevotella intermedia	0.23	1	TH
Prevotella nanceiensis	0.05	1	PA
Bacteroides pyogenes	0.07	1	PA
Porphyromonas gingivalis	0.03	1	TR
Prevotella pallens	0.01	1	PA
Fungi			
Rhizopus microsporus	0.01	3	TE, PA & FL

TH – thawers ($n = 6$), TR – truck drivers ($n = 4$), TE – technicians ($n = 7$), PA – packers ($n = 11$), and FL – flour production workers ($n = 2$).

level, microbial load, TLR activation, and investigation of the working environment microbiome to identify work processes more susceptible to adverse exposure.

The endotoxin levels measured were generally low, with detectable maximum levels only among thawers (49 EU/m^3), truck drivers (98 EU/m^3), and technicians (28 EU/m^3). These levels are comparable to historically reported levels in the shrimp processing industry (11–100 EU/m^3) (Bang et al. 2005). The present study and the measurements reported by Bang and colleagues demonstrate that the observed levels slightly exceed the recommended occupational exposure limits (90 EU/m^3) (Torén 2011) and may be a health concern for these workgroups. Thawers and truck drivers handle raw shrimp while the technicians, packers, and flour production workers handle cooked shrimp. The endotoxin level significantly declines after cooking and becomes undetectable in packing and flour production. A similar trend was observed in a previous study that compared endotoxin exposure levels in workers handling raw and cooked crabs (Thomassen et al. 2016), illustrating higher endotoxin levels in processes handling raw material. Other seafood processing industries such as salmon and crab processing have reported up to 29 EU/m^3 and 24,000 EU/m^3, respectively (Dahlman-Höglund et al. 2012; Shiryaeva et al. 2014; Thomassen et al. 2016).

The variations in endotoxin levels observed in different studies can be attributed to factors such as variations in work processes, marine species, and the specific measurement methods employed (often using LAL). This study used the rFC assay, an animal-free and potentially superior method to LAL (Piehler et al. 2020). However, due to potential protein interference, two samples had to be re-analyzed using LAL. While both methods have equivalent sensitivity, some argue that the LAL method may produce slightly higher endotoxin values as it utilizes both factor C enzyme and factor B to determine endotoxin levels (Piehler et al. 2020).

This study is the first to investigate circulating microbial ligands in air samples from such workplaces using a HEK reporter cell assay. The results showed significant activation of the TLR2 NF-κB signaling pathway in samples from thawers, indicating the presence of microbial ligands capable of triggering an immune response. Despite detecting endotoxin among thawers, truck drivers, and technicians through rFC/LAL measurements, no significant TLR4 activation was observed among these workgroups. Several factors may contribute to the lack of TLR4 activation. It is important to note that these assays are not directly comparable, as the TLR4 assay only detects the biologically active endotoxin, whereas the rFC/LAL assay measures the total endotoxin levels. Moreover, activation of TLR4 may trigger alternative signaling pathways that are not detected by the reporter cell line used in this study.

Further, the bacterial and fungal DNA copies in the personal air samples were analyzed to assess microbial load. The measurement of bacterial DNA copies weakly correlated with endotoxin levels. The bacterial load was significantly higher before cooking than after cooking, illustrating that thawers and truck drivers are particularly susceptible to bacterial exposure. Bang et al. (2005) demonstrated that the technicians had the highest microbial load, followed by the thawers. Their findings were based on microscopic analysis and were not directly comparable to data generated in this study. Regarding fungal exposures, the highest fungal load among workers in flour production. The shrimp shell is transported in a closed pipe then dried and ground in flour production. The higher fungal exposure load in this working group can be attributed to the water-dependent processes such as thawing, cooking, and water transport that may facilitate fungal growth in the transport pipe. However, it is essential to note that these exposure levels may appear generally low compared to other work environments, mainly due to the thorough cleaning of the processing plants for product safety (Gilbert and Duchaine 2009).

The microbiome investigation in shrimp processing plants revealed that microbial species' community structures and patterns varied among workgroups. To the best of the authors' knowledge, this study is the first to employ NGS to evaluate the microbiome in this work environment. While alpha diversity did not show a significant difference for both fungi and bacteria, there was a significant difference in bacterial species richness between the workgroups. This could indicate that work processes before the cooking (thawing and truck driving) may be associated with higher variability of microbial exposure. Furthermore, the presence of specific allergenic and pathogenic species in the various work processes raises health concerns for workers.

In total, 238 bacterial genera were identified. *Staphylococcus*, *Prevotella*, *Streptococcus*, and *Roseomonas* were among the top twenty most abundant genera identified in this study. Some of the species in these genera are associated with lung inflammation and toxin production through TLR2 activations, potentially inducing respiratory problems

and other health effects (Larsen 2017; Lopes et al. 2020). In addition, species that belong to these genera play a significant role in triggering and exacerbating allergy, rhinitis, and asthma, which may contribute to chronic respiratory health problems among workers (Abdurrahman et al. 2020; Kitur et al. 2015; Miao et al. 2023; Srifuengfung et al. 2007; Xu et al. 2008).

Among the bacterial species identified, fifteen were classified as human pathogens (risk group 2). *Prevotella intermedia* was one of the species identified and is not only associated with severe asthma but is recognized as a human pathogen (Lopes et al. 2020). Based on the commonly known habitat, the various *Prevotella* species and *Porphyromonas gingivalis* identified are part of the human microbiota (workers themselves) (Alauzet et al. 2010; How et al. 2016). *Empedobacter brevis* and *Streptococcus parauberis* are from raw shrimp (Li et al. 2019; Woo et al. 2021), while *Roseomonas gilardii*, *Bacteroides pyogenes*, and *Myroides odoratus* are probably from water or soil during fishing. Although all of them are categorized as risk group 2, their pathogenicity varies. For example, many *Prevotella* species, *Porphyromonas gingivalis*, and *Bacteroides pyogenes* can cause serious illnesses such as periodontal diseases, abscess formation, and respiratory tract infections. Additionally, they have the potential to cause systemic infections such as endocarditis and bacteremia (Bertelsen et al. 2020; How et al. 2016; Jia et al. 2019; Larsen 2017; Majewska et al. 2021). Others are opportunistic pathogens, such as *Roseomonas gilardii* which can cause severe disease in immune-compromised individuals (Srifuengfung et al. 2007). Despite the presence of several Gram-negative bacterial species in the samples, the endotoxin levels measured were low. This may be due to the higher sensitivity of NGS compared to rFC/LAL assay. Additionally, the overall bacterial count might be below the threshold necessary to produce detectable endotoxin levels, or the identified bacteria may not be viable to release measurable endotoxin.

In total, 87 genera and 118 fungal species were identified. Among the twenty most abundant genera, there were *Malassezia*, *Sporobolomyces*, *Saccharomyces*, *Wallemia*, and *Rhodotorula*. *Saccharomyces* species and *Wallemia* species are capable of initiation and exacerbation of IgE-mediated allergy, while others are known for inducing harmful respiratory conditions in immune-compromised individuals (Chang et al. 2002; Cockcroft et al. 1983; Fukutomi and Taniguchi 2015; Kurup 2003). Previously *Penicillium* species and *Stachybotrys* species were reported as the dominating genera in the same work environment by culturing in V8 medium (Bang et al. 2005). However, culturing has limitations, including inaccuracies in identification and the inability to culture every species, in contrast to NGS. In the present study, ten allergenic fungal species were identified, including *Sporobolomyces roseus*, *Saccharomyces cerevisiae*, *Malassezia restricta*, and *Malassezia globose*, and these were also among the top five most abundant fungal species detected. These species originate from various environmental sources such as soil, air, and water. However, they each have the potential to trigger respiratory symptoms, allergies, and asthma, especially in individuals with underlying health conditions (Cockcroft et al. 1983; Crameri et al. 2014; Fukutomi and Taniguchi 2015). Other allergenic species identified include *Rhizopus microspores*, *Rhodotorula mucilaginosa*, and *Malassezia sympodialis*, which can trigger preexisting respiratory conditions through exposure to their spores (Chang et al. 2002; Crameri et al. 2014; Eduard et al. 1992). Furthermore, *Scleroderma* sp. and *Granoderma applanatum* were identified, both of which are described as allergens through inhalation. Most of these fungal allergenic species are identified in all five work processes, which may indicate the need for exposure prevention.

Limitations

The small sample size and the uneven distribution of samples across workgroups may limit the generalization of the study findings. While NGS offers an invaluable and comprehensive overview of species composition within the work environment, it does not provide information on the biological state of these species, such as their viability and potential infectivity. Despite this limitation, the detection of pathogenic and/or allergenic species in the work environment indicates a potential health risk among workers. Nonetheless, this study can be used as the groundwork for future research into the accurate sources of these species and their biological state and infectivity, which may be essential in the evaluation of necessary preventive measures.

Conclusion

This study has provided an in-depth insight into microbial exposure in a shrimp processing plant. Although the exposure levels were generally low, workers in the shrimp processing plant were exposed to several bacterial and fungal species, including those

that are allergenic and pathogenic. The exposure varied between the different work processes, and the cooking processes significantly reduced exposure to endotoxin and bacteria, while at the same time reducing bacterial species richness. Moreover, these findings show that thawers, truck drivers, and flour production workers were particularly susceptible to higher microbial exposure. Specifically, thawers and truck drivers are more prone to bacterial exposure, while fungal exposure is more prevalent among flour production workers. These findings can have a positive impact in facilitating improved risk assessment and the implementation of more targeted preventive measures such as improved ventilation systems and the utilization of protective equipment.

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Data availability statement

The data produced or analyzed in the present study can be obtained from the corresponding author upon reasonable request. Metagenomic data files for this study can be found at BioProject ID: PRJNA1126435 (https://submit.ncbi.nlm.nih.gov/subs/sra/SUB14537666).

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