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Biocide rotation and control of microbial resistance development in the food industry

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1. Preface

The project was carried out by the National Food Institute at The Technical University of Denmark (DTU FOOD) during the years 2020-2024. This report is based on publications and manuscripts written with the co-authors listed above. The three sub-projects, 1-3, are written so that they can be read independently from each other. Sub-projects 1 and 2 are published in peer-reviewed journals, whilst sub-project 3 is similarly intended for publication in a peer-reviewed journal and may therefore be found in an updated version once reviewed. The project was funded by the Danish Ministry of Environment's Research Program for Pesticides (MST no. 2019-15097). The project work was followed by a steering group led by Henrik F. Brødsgaard (Danish EPA) composed of participants in other projects and reviewed by Birte Fonnesbech Vogel and Charlotte Cleyton Jørgensen.

2. Summary

The research project “Biocide rotation and control of microbial resistance development in the food industry” had two overall specific research questions:

1. Do foodborne bacterial pathogens (e.g., *Listeria monocytogenes*) develop tolerance to biocides?
2. Can the rotation of different biocides help to prevent the development of biocide tolerance?

The two main research questions were investigated in three interconnected sub-projects.

The first sub-project aimed to evaluate the possibility of biocide tolerance development by studying the biocide sensitivity of 240 different (53 sequence types, 32 clonal complexes) isolates of *L. monocytogenes* from different sources, years and countries. The impact of residual organic matter (soils) or biofilm on biocide efficacy and the ability to adapt to biocides was also evaluated. The isolates were screened for genetic elements associated with biocide tolerance and tested for their minimum inhibitory concentration (MIC) to benzalkonium chloride (BC (Alkyl dimethyl benzyl ammonium chloride)), peracetic acid (PAA), sodium hypochlorite (SH) and ethanol (ET). Lastly, 19 representative isolates were tested for their tolerance in broth suspensions test (BST) at higher biocide concentrations.

Main findings from this work showed that:

- Among the 240 *L. monocytogenes* isolates, MIC for PAA (62 mg/L) showed no variation, while for SH (47–94 mg/L) and ET (4.7–9.4% v/v) MIC values differed twofold among isolates.
- For BC, an 8 × difference (0.3–2.5 mg/L) was observed in MIC values and 87 of 88 BC tolerant isolates (MIC of 2.5 mg/L) harboured one of the known BC tolerance genes (*qacH, bcrABC, emrC, emrE*).
- Soiled conditions mimicking surfaces with poor cleaning caused an increase in MIC-values by 8 to 33-fold for BC and 8-fold for SH but not for PAA and ET.
- Biofilm formed prior to biocide treatment meant that, compared to tests without biofilm, the minimum bactericidal concentrations increased by 4–8 × for BC but not for PAA and SH.
- Survival of isolates with BC tolerance genes was not significantly ($P > 0.05$) improved in BSTs with either PAA, SH or BC.
- Adaptation to BC (8 × MIC increase) occurred exclusively for originally sensitive isolates, while no adaptation to SH and PAA was observed.

In conclusion, *L. monocytogenes* isolates showed limited variation in sensitivity to biocides in MIC assays and BSTs, and it was clear that generally, biocide efficacy was more affected by residual organic matter and/or biofilm, demonstrating the need for proper cleaning prior to biocide use with PAA being less affected. Importantly, from an industrial and safety perspective, results revealed that *L. monocytogenes* isolates with known biocide tolerance genes *qacH, bcrABC, emrC* and *emrE* did not exhibit increased survival when the BC concentration was increased to more industrially relevant concentrations.

The second sub-project used floor drain samples from different food industries to obtain different bacterial isolates and test their biocide tolerance. Selected isolates were then used to make a realistic multispecies (31 species, 24 genera) biofilm model to study the effect of repeated biocide treatment using the approved concentrations, which academically are referred to as the manufacturer-recommended concentrations (MRC). The size and composition of the surviving biofilm and the survival of *L. monocytogenes* were also investigated. The biofilm model was exposed on days 3 and 6 to water or to BC, PAA or SH at either MRC or 2×MRC. Highlights of the results are summarized below:

- Culturomics and shotgun metagenomic analysis of 14 floor drains and 214 bacterial isolates from the three industrial food production environments revealed microbiomes showing great diversity and complexity but with the dominance of a few highly abundant taxa, including *Pseudomonas*.
- Efflux pumps conferring increased tolerance to antibiotics and QACs were frequently detected in the microbiomes of floor drains within the tested fish, shrimp and cheese production facilities.
- The representative drain biofilm model was able to represent 47-58% and 76-81% of the microbial abundance observed in the drain metagenome and viable microbiota, respectively.
- In the biofilm model, the species diversity decreased following repeated biocide treatments, but without any change in biocide tolerance as seen in log reductions (CFU/cm^2).
- The use of different biocides did, however, exert significantly different selective pressures on the microbiomes, as *Citrobacter*, *Acinetobacter*, *Aeromonas* and *Pseudomonas* dominated the biofilm after treatments with SH or PAA, while *Serratia* and *Moraxella* dominated after BC.
- The dominance of *Serratia marcescens* could be explained by the carriage of a BC efflux pump (*oqxB*) and the highest (20 mg/L BC) MIC result of the drain isolates.
- In contrast, despite carrying a BC efflux pump (*qacH*), *Listeria monocytogenes* ST121 did not show increased survival or presence in the biofilm after BC treatments as could be explained by a BC MIC of just 2.5 mg/L BC.
- Only the highest tested concentration of PAA was able to completely eradicate *L. monocytogenes* from the biofilm model, whilst BC and SH could not do so.

In conclusion, the developed representative biofilm model and the repeated biocide treatments showed how biocides affect the biofilm microbiome differently. This model and knowledge were used in the third sub-project, testing biocide rotation strategies to control biofilm regrowth and inactivation of persistent foodborne pathogens in floor drains.

The third sub-project used the biofilm model developed in the second sub-project to evaluate the effect of rotation of different biocide schemes simulating the disinfection process over a ten-day period within a food production environment. This last sub-project thus aimed to investigate the extent to which rotation between biocides improves the efficiency of biocides and if it helps to prevent biocide tolerance in biofilms, including potential horizontal gene transfer (HGT) of biocide tolerance genes. The biofilm model was treated with biocides Monday (Day 1) to Friday (Day 5) and again the following Monday (Day 8) using different schemes with rotation or no rotation of biocides. In total twelve scenarios were tested at both MRC and 2×MRC of the biocides.

- The surviving biofilm concentrations (average of 1.5 and 2.5 log CFU/stainless steel coupon (SSC) for MRC and 2×MRC) were lowest on day 5 for both tested biocide concentrations because of daily biocide treatments.
- For just one of the 24 tested biocide schemes, a significant increase in biofilm survival was seen on day 8 when the rotation of SH and PAA at 2×MRC was applied. However, the same rotation scheme did not result in any difference when applied at the MRC or when the same rotation of PAA and SH were used starting with PAA.
- No biocide tolerance build-up was seen for the remaining 23 tested biocide schemes, and in fact three biocide schemes saw a significant increase in biocide sensitivity on day 8 compared to day 1.
- The comparison of biocide treatment schemes across both applied concentrations revealed that there were no significant differences between the application of three biocides in rotation and the application of just one biocide (SH or PAA) through the entire week.
- Biocide schemes using BC more than once were the least effective, but biocide rotations schemes using BC just once a week were among the best schemes.

- The surviving biofilms were especially dominated by *Pseudomonas cremoris* making up 38% (311/819) of the surviving colonies identified by MALDI-TOF MS.
- *Stenotrophomonas rhizophila* 12.5% (102/819) and *Serratia marcescens* 12.0% (98/819) were equally dominant in the post-biocide treatment microbiomes.
- *L. monocytogenes* was only detected once in the water controls (0.4%, 1/277), while making up 2.8% (23/819) in the surviving biofilm microbiomes after biocide treatments.
- The use of biocides at either MRC or 2×MRC did not confer significant differences in the overall microbiome composition regrowing around the biofilms after treatments.
- The suspension that regrew around the surviving biofilms following biocide treatments from Monday to Friday (Day 5) was dominated by *Pseudomonas* (40-52%) and *Serratia* (17-33%) on day 8.
- Biocide rotation schemes with heavy use of BC or PAA caused *Serratia* (66%) and *Pseudomonas* (78%), respectively, to dominate. In contrast, SH-based biocide schemes had a less clear selective effect on the regrowing microbiome.
- No HGT transfer of either the efflux pumps *oqxB* or *qacH* was identified after analysis of the metagenomes from day 8.
- Without positive HGT events, we could not conclude if biocide rotation can prevent HGT of biocide tolerance genes.
- Despite carrying QAC efflux pumps, neither *L. monocytogenes*, *S. rhizophila*, *Pseudomonas anguilliseptica*, nor *Pseudomonas chengduensis* were able to dominate in BC-treated biofilms or suspensions in the way *S. marcescens* and *P. cremoris* did.

In conclusion, it was observed that rotation between two or three biocides at industrial concentrations is not necessary to prevent a build-up of increased biocide tolerance. However, rotation schemes that were based on a combination of multiple uses of either PAA and SH with BC used once a week were among the best-performing biocide schemes, but close in performance to simpler schemes using the same biocide (SH or PAA) throughout the entire week. As none of the biocide schemes resulted in detectable HGT of the efflux pumps oqxB or qacH it is not possible to conclude that rotation helps to prevent HGT or build-up of biocide tolerance. In addition, it should thus be noted that increasing biocide concentrations above the recommended and approved manufacturers' concentrations was therefore not necessary to prevent biocide tolerance. In cases with surviving biofilm, additional mechanical and chemical cleaning steps should instead be performed/repeated before application of new/increased biocide concentrations.

Throughout the entire project, in our results and in recent works published by others, it has become clear that while *L. monocytogenes* and other bacteria are able to increase their biocide tolerance to QACs, this increased tolerance only results in MICs increasing by a few mg/mL. This slight increase in biocide tolerance would have no effect on the survival when the actual high industrial concentrations (hundreds to thousands mg/ml) are applied. However, as seen in this project, different biocides affect which bacteria survive and dominate in regrowing biofilms, which can affect the biofilms that may form between production cycles. Introducing a monitoring program, especially in factories with persistent problems, can be a solution in combination with the rotation of biocides. It is, however, crucial to highlight that we saw how soiled conditions and biofilms had a greater negative impact on biocide efficacy than genetic determinants. This means that equipment and surfaces that are inadequately cleaned before biocide treatments are the biggest issues when it comes to inefficient cleaning and disinfection schemes. This means that before using resources on changing biocide rotation schemes or strategies, it is more important to properly educate staff and cleaning personnel in good hygienic practices and allocate sufficient resources for cleaning and disinfection. This includes maintaining surfaces and replacing poorly hygienically designed equipment or surfaces to minimize niches where dirt and biofilm may build up. Biocides are effective, but even the best-designed biocide schemes will be inefficient if the biocide does not reach the biofilm in the necessary concentration.

3. Introduction

3.1 Background

Background and rationale for the proposed research.

Food companies will, regardless of their size, rely on the use of biocides to reduce the microbiological contamination of the processing environment after completion of the daily production cycle. If the cleaning and disinfection (C&D) program does not sufficiently remove all microorganisms from the surfaces of production equipment, then there is a risk of contamination as bacteria and fungi may unintentionally survive and then become transferred to foods being produced in the next production cycle (Carpentier & Cerf, 2011; Yu et al., 2018). Such cross-contamination events are thought to be a major contributor to the repeated outbreaks of listeriosis (2952 confirmed cases and 335 deaths within the European Union (EU) in 2023) (ECDC EFSA, 2024). These cases can be related to consumption of ready-to-eat (RTE) foods contaminated with the foodborne pathogenic bacterium *Listeria monocytogenes* (Koutsoumanis et al., 2024). In a RTE meat outbreak (Boelaert et al., 2019) *L. monocytogenes* levels of 270,000 CFU/g were found in products leaving the plant, strongly indicating a serious problem with the C&D program. Long-term use of the same disinfectant (a.k.a., biocide) creates conditions which may promote the evolution of tolerance, as has been reported for benzalkonium chloride (BC) biocides (He et al., 2022; Kim et al., 2018). This can lead the C&D program no longer working optimally due to loss of efficacy or can lead to the application of increasing concentrations of disinfectants to regain control with food safety. From an environmental perspective, an increased use of disinfectants in the food industry will lead to increased emissions of disinfectant residues into the waste stream, wastewater treatment plants and, if not removed there, into the receiving environment (Chen et al., 2021). This may further introduce different selective pressures in these environments if residuals remain bioactive (Møretrø et al., 2017). The concern for the development of resistance or tolerance to biocides is also engrained in the Biocidal Products Regulation (EU 538/2012), which requires information on “possible occurrence of the development of resistance and appropriate management strategies” for every biocide for which approval is sought. In the food industry, different strategies have loosely been formulated to counter the build-up of resistance to biocides, including a broad and unspecific recommendation to rotate biocides (Tompkin, 1999). This recommendation has been repeated by authorities and in scientific publications but with limited efforts to test if the rotation of biocides is more efficient for preventing the development of potential biocide tolerance (FDA, 2017; He et al., 2022; Koutsoumanis et al., 2024). We, therefore, lack the scientific foundation to advise the industry on how often the biocides should be switched and whether a particular sequence is favourable to diminish resistance and tolerance development. Moreover, we lack systematic knowledge about possible tolerance development for many “broad range” type biocides as SH and PAA as well as a determination of which method to assess the formation of tolerance to biocides (Maillard, 2018).

Sanitation programs in the food industry

The purpose of a C&D program in the food industry is to create hygienic conditions that allow production of food, ingredients and additives, which are safe to consume. In the European Union food industry this is governed under the Hygiene Regulation (EC 852/2004). The types of biocidal products with approved active substances that can be safely used in an efficient C&D program without adverse effects to the environment and consumers are described in the Biocidal Products Regulation (EU 528/2012). This means that all biocidal products are evaluated for safety and efficacy before being made available in the EU. Ideally, use of chemicals should be limited to a minimum to minimize the environmental impact and the impact on human health.

Step one in a C&D program is a cleaning step to remove soils and microorganisms to the highest extent possible. The next step involves the application of a biocide whose purpose is to reset the microbiome to a hygienic acceptable level in the cleaned production environment. Ideally, the dosage of the biocide should be at the lowest possible concentration to achieve this goal. In practical terms, however, the concentration used by the industry should be the concentration that has been evaluated to be effective and approved according to Regulation (EU) No 528/2012. Different biocides target different parts of the microbial cells, including features of the cell membrane, and intracellular macromolecules such as structural proteins, DNA, enzymes, etc. (Maillard, 2018). Consequently, the microorganisms may evolve different mechanisms to evade the adverse effects of biocides, especially efflux pumps to remove biocides, but possibly also by reducing penetration through altered membrane structure and enzymatic degradation of the biocide (Alonso et al., 2022). The efficacy of biocides can further be challenged by poor removal of soils due to insufficient cleaning, and poorly designed equipment or facilities. Moreover, the biocidal effect can be challenged by the unintended presence of microbial biofilms, which are irreversibly surface-attached communities of microbes covered in a slime layer consisting of exopolymeric substances (EPS) (Flemming & Wingender, 2010; Maillard & Centeleghe, 2023).

Biofilms and tolerance to cleaning and disinfection in the food industry
Biofilms are widely found in food processing facilities including in cracks, drains, corners of equipment, etc. Inhabitants in the biofilms will mostly consist of innocent commensal microorganisms but can also include pathogenic bacteria, which is of great concern for food safety (Galié et al., 2018). Even on seemingly clean equipment surfaces, a variety of different bacteria may remain with the potential to form a biofilm (Langsrud et al., 2016; Møretrø & Langsrud, 2017). Numerous research reports have shown that bacteria lodged in biofilms become more tolerant to biocides (Carpentier & Cerf, 2011; Fagerlund, Møretrø, et al., 2017; Ortega Morente et al., 2013). Specifically, formation of biofilm has repeatedly been shown to increase tolerance to disinfectants such as sodium hypochlorite (SH) (Norwood et al., 2000), peracetic acid (PAA) (Bridier et al., 2011; Ibusquiza et al., 2011a) and BC (Fagerlund, et al., 2017; Piercey et al., 2017)

However, it is not known if the presence of biofilms exclusively increases the tolerance to disinfectants in a transient manner (i.e., the biocide tolerance of the planktonic cells would still be the same). Alternatively, the selective pressure from sub-lethal biocide concentrations may enable the bacteria to develop inherent or permanent tolerance to biocides, which could seriously impact our control options. It should be noted that poor removal of soils will serve to “dilute” the disinfectant applied, as will the EPS layer surrounding biofilm cells, meaning that a low to sub-lethal disinfectant dose will reach the target cells. This is exactly the type of scenario that may foster the “breeding” of increased tolerance to biocides. It is, therefore, one of the hypotheses in this research project that inefficient removal of soils and biofilms, together with continuous use of the same disinfectants, leads to the propagation of resistant microorganisms. It is also a hypothesis that intelligent application of different biocides in sequence can halt this development.

Development of biocide tolerance in *Listeria monocytogenes*
L. monocytogenes is a pathogenic bacterium that causes the foodborne illness listeriosis (Donnelly, 2001). The bacterium was responsible for 57% (225) of the deaths caused by foodborne and zoonotic pathogens within 18 EU countries in 2024 (ECDC EFSA, 2024). Outbreaks of listeriosis are characterized by a high case fatality (~10-20%), making the observed rise in listeriosis cases within the EU since 2008 (ECDC EFSA, 2024; EFSA ECDC, 2018) a serious concern for the health of vulnerable population groups and necessitating further research into the control of the organism. *L. monocytogenes* may enter food processing facilities via agricultural commodities (Todd & Notermans, 2011; Vivant et al., 2013) or water (Stea et al., 2015;

Vivant et al., 2013), however, contamination of foods during processing is thought to lead to most of the *L. monocytogenes* outbreaks (EFSA BIOHAZ Panel, 2018). The contamination of food contact surfaces with *L. monocytogenes* increases the risk of cross-contamination of foods with epidemiological data pointing to *L. monocytogenes* derived from the food processing environment being the main cause for contamination of RTE foods (Midelet al., 2006; Rodríguez & McLandsborough, 2007).

L. monocytogenes persistence in the food industry has been described as the relationship between introduction, ability to survive, grow, and resist cleaning and sanitation (Carpentier & Cerf, 2011; Ferreira et al., 2014). An individual isolate of *L. monocytogenes* can be considered persistent if it is isolated from the same food processing facility over a period of several months or years (Fagerlund et al., 2022; Wulff et al., 2006). It may be hypothesized that persistence is related to the isolate's ability to survive and factors such as biofilm formation, which is known to enhance the resistance of cells to biocides, or hinder physical removal (Maillard & Centeleghe, 2023). The ability of *L. monocytogenes* to become resistant to quaternary ammonium compounds (QACs) such as benzalkonium chloride is well known and thought to contribute to its persistence in the food industry (Fagerlund et al., 2022; Martínez-Suárez et al., 2016; Møretrø et al., 2017; Pasquali et al., 2018). Some of the differences in biocide tolerance among different *L. monocytogenes* strains are genetic in nature and vary greatly among the different sub-types of *L. monocytogenes*. The efflux pump *qacH*, which has many homologs in other bacterial genera, is known to confer increased tolerance to low concentrations of QACs and is located within the Tn6188 transposon (Alcock et al., 2020; Müller et al., 2013). Another example is the efflux pump *emrE* located on the Listeria Genomic Island I (LGI1) which isolates from the Canadian 2008 RTE meat outbreak were found to possess (Kovacevic et al., 2016). The carriage of *emrE* resulted in significantly ($P < 0.05$) greater tolerance to benzalkonium chloride (BC) for both planktonic and sessile biofilm cells as compared to LGI1-negative *L. monocytogenes* isolates (Piercey et al., 2017). Similarly, Muhterem-Uyar et al. (2018) observed the presence of the *bcrABC* cassette and heavy metal resistance genes conferring enhanced resistance to disinfectants for the *L. monocytogenes* ST5 isolate that persisted for several years in a cheese processing plant. In addition, Hurley et al. (2019) found that 55 out of 100 *L. monocytogenes* whole genome sequenced (WGS) isolates contained tolerance genes to BC. Interestingly, isolation of the *L. monocytogenes* isolates carrying BC tolerance genes occurred during a period where the surveyed processing plants used the biocide in their sanitation program, strongly indicating that use of the membrane acting QAC biocides selects for tolerance development. The last and fourth known BC tolerance gene known for *L. monocytogenes* is the rarer emrC, which is carried on a plasmid that could be transformed into sensitive isolates and increase their tolerance to BC (Kremer et al., 2017).

While there is thus great evidence on decreased sensitivity towards BC for *L. monocytogenes*, specific genetic tolerance markers for other biocides such as PAA, hydrogen peroxide and SH have so far not been identified. These biocides act as oxidizing agents, creating reactive oxygen species, which lead to damage of cellular components (Palma et al., 2022; Wessels & Ingmer, 2013). However, a growing body of research is pointing to the increased tolerance of *L. monocytogenes* to these biocides when present in biofilms, especially in multi-species biofilms (Ibusquiza et al., 2011b; Norwood & Gilmour, 2000; Oxaran et al., 2018; Thomassen et al., 2023; Van der Veen & Abee, 2011). In summary, it is important in the fight against *L. monocytogenes* (and other pathogenic and spoilage microorganisms) that the C&D program effectively lowers the contamination level. Incomplete sanitation can foster development of biofilms as well as disinfectant tolerant and/or resistant isolates. To prevent tolerance to biocides, the industry is recommended to pursue a scheme to change biocide chemistry on a regular basis. However, the scientific basis for this recommendation is currently lacking. The current concern of development of biocide tolerance is possibly based on the observations on development of antibiotic resistance, where the gap between minimum inhibitory concentrations (MICs) and the clinical concentrations is much smaller. This implies that even slight changes in sensitivity

due to acquisition of antibiotic resistance genes through horizontal gene transfer (HGT) could cause great clinical treatment problems (Chen et al., 2021; Jutkina et al., 2018; Skandalis et al., 2021). However, for biocides, the industrial concentrations are often 100 to 1000-fold higher than MICs for both sensitive and less sensitive biocide tolerance gene-carrying isolates (Bland et al., 2022; He et al., 2022). It should be noted that in the literature and within food authorities the terms "biocide tolerance" and "biocide resistance" are often used interchangeably. For antibiotics, resistance is defined as the concentration where the treatment has no effect at the used concentration. However, in reports on biocides, observed biocide "resistance" is invariably based on concentrations that are much lower than the actual industrial manufacturer-recommended concentrations (MRC) (Bland, Brown, et al., 2022; Willmott et al., 2024). In this report, we have therefore decided to consistently use the term "biocide tolerance" to denote increases in MIC values and hence in survival when exposed to sub-industry level concentrations.

3.2 Aim of Project

The overall objective of this research project was to close important gaps in our knowledge on biocide rotation and biocide tolerance by investigating if and how fast tolerance is developed to commonly applied biocides in the food industry and whether biocides can be rotated in a way so that their efficacy is maintained without tolerance development. The outcome of the proposed research will aid the industry and the Danish Veterinary and Food Administration (DVFA) in developing protocols for more efficient and sustainable usage of biocides in the food industry. The new scientific knowledge and method will also support DEPA's work to approve biocides and guide the industry.

The research project "Biocide rotation and control of microbial resistance development in the food industry" had two hypotheses upon which the specific aims are built upon.

Hypotheses

- Inefficient removal of soils and biofilms and/or continuous use of the same biocides lead to increased tolerance and propagation of resistant microorganisms.

- Intelligent or knowledge-based application of different biocides in sequence can halt the development of biocide tolerance and minimise biocide consumption and emission.

Project aim

The research project "Biocide rotation and control of microbial resistance development in the food industry" had two overall specific research questions:

- Do foodborne pathogens (e.g., *L. monocytogenes*) develop tolerance to biocides?

- Can the rotation of different biocides help to prevent the development of biocide tolerance?

The two main research questions were investigated in three interconnected sub-projects. The first sub-project aimed to evaluate the possibility of biocide tolerance development by studying the biocide sensitivity of 240 different isolates of *L. monocytogenes* from different sources, years and countries. The second project did the same analysis but with different bacterial isolates from floor drains in the food industry, which were subsequently used to make a realistic multispecies biofilm to study the effect of repeated biocide treatment on the composition of the surviving biofilm and the survival of *L. monocytogenes*. In addition, the samples from the food industry were metagenomically sequenced to assess the presence of known biocide and antibiotic resistance genes in the microbiome of floor drains in different food production facilities. The third sub-project used knowledge from the first sub-project and the model developed in

the second sub-project to evaluate the rotation of different biocide schemes, simulating the potential biofilm growth and disinfection process over a ten-day period within a food production environment. This last sub-project thus aimed to investigate to which extent rotation between biocides improves the efficiency of biocides and if it helps to prevent biocide tolerance in biofilms, including potential horizontal gene transfer (HGT) of biocides.

4. Sub-project 1:

Sensitivity of 240 *Listeria monocytogenes* isolates to common industrial biocides is more dependent on the presence of residual organic matter or biofilm than on genetic determinants

4.1 Introduction

L. monocytogenes is a psychrotolerant environmental bacterium and a foodborne pathogen that constitutes a challenge in the ready-to-eat (RTE) food industry due to its contamination of raw materials, processing facilities and ultimately food products. The incidence of infections with *L. monocytogenes* is low, but due to high hospitalization and fatality rates it remains a serious public health concern and a major challenge in the food sector (EFSA and ECDC, 2021). In Europe, food manufacturers are responsible for preventing food products from containing high concentrations of *L. monocytogenes* (European Commission, 2005). To secure a Listeria-free food production environment, food manufacturers rely on cleaning and disinfection procedures. However, despite these efforts genetically identical *L. monocytogenes* (i.e., ≤ 20 single nucleotide polymorphisms (SNP) differences among isolates) continue to survive and persist in RTE food processing facilities for years (Fagerlund et al., 2022; Takeuchi-Storm et al., 2023). The inability of cleaning and disinfection to eradicate *L. monocytogenes* has been suggested to imply the selection for isolates with inherent or adapted tolerance to biocides (Lundén et al., 2003; Meyer, 2006; Møretrø et al., 2017; Vidovic et al., 2022).

Biocides, which are commonly used in the food industry, include chlorine-releasing agents, hydrogen peroxide, peracetic acid, alcohols and quaternary ammonium compounds (QACs) (Bland, Brown, et al., 2022). Among these, variations in tolerance of *L. monocytogenes* to QACs, e.g., benzalkonium (BC), are known to be related to harbourage of different efflux pumps (*bcrABC*, *qacH*, *emrE*, *emrC*), which cause increased minimum inhibitory concentrations (MICs) towards BC compared to isolates without these genetic determinants (Elhanafi et al., 2010; Kovacevic, Ziegler, Walecka-Zacharska, et al., 2016; Kremer et al., 2017; Müller et al., 2013). However, there is a lack of understanding to which degree there is a variation in the sensitivity among *L. monocytogenes* isolates to other commonly used biocides such as sodium hypochlorite (SH) and peracetic acid (PAA), for which no specific genetic determinants of tolerance have been reported. For PAA and SH, some studies report limited variation among tested isolates with MICs that are well below industrially recommended concentrations (Poimenidou et al., 2016; Roedel et al., 2019). In contrast, others report minimum bactericidal concentration (MBC-) and MIC-values close to manufacturer-recommended concentrations for some but not all isolates tested (Bansal et al., 2018; Stoller et al., 2019).

The persistent nature of *L. monocytogenes* in food processing facilities has been proposed to be due to the adapted tolerance of *L. monocytogenes* isolates as they become exposed to sublethal concentrations of e.g., QACs in the food processing environment (López-Alonso et al., 2020; Ortiz et al., 2016; Pasquali et al., 2018). However, longitudinal studies have shown that there is low evolution of *L. monocytogenes* tolerance to BC, and PAA in the food processing environments over periods of three to four years (Møretrø et al., 2017; Stoller et al., 2019), indicating that biocide adaption of different *L. monocytogenes* isolates has no influence on persistence. The risk of such adaptation should be tested with subinhibitory biocide concentrations in environmentally relevant conditions, which could occur in niches within the food processing environment.

Other factors can negatively affect the efficacy of biocides in the food industry including the presence of biofilm, interfering organic matter and compromised integrity of surfaces (Best et al., 1990; Overney et al., 2017; Rodríguez-López & Cabo, 2017). Such factors may lead to insufficient exposure and low concentrations of biocide, which may result in survival and contribute to microbial adaptation i.e., increased tolerance to the biocide (Guérin et al., 2021; Martínez-Suárez et al., 2016; Møretrø et al., 2017).

The many different protocols, which have been applied to test the efficacy of biocides on *L. monocytogenes* make meta-studies and comparison of results reported in the literature challenging (Bland et al., 2022). Therefore, to allow for a solid comparison of variations in tolerance to biocides and ability to adapt to sublethal biocide concentrations among *L. monocytogenes* belonging to different lineages, serogroups, clonal complexes and sequencing types, it is necessary to conduct large-scale studies using the same experimental platform. Results could contribute to recommendations for the food industry and without interfering uncertainties coming from the meta-analysis of tolerance and adaptation studies carried out under different experimental conditions.

This sub-project aimed to investigate if the tolerance to four different types of biocides differs among 240 *L. monocytogenes* isolates belonging to different sequence types (STs), and whether genetic determinants, biofilm formation, organic matter and adaptation affect this tolerance. Understanding if certain STs or harbourage of genetic markers predict the biocide tolerance of *L. monocytogenes* can guide the design of improved cleaning and disinfection interventions to reduce the persistence of *L. monocytogenes* in the food processing environment. In addition, this can help to answer critical questions as to whether there is a basis for potential development of biocide tolerance in lab-scale experiments. If this is not possible, then evidence of phenotypic diversity in biocide tolerance could alternatively confirm whether *L. monocytogenes* has evolved biocide tolerance in different environments outside the laboratory.

4.2 Materials and Methods

Bacterial isolates and standardized cell suspensions

A total of 240 isolates of *L. monocytogenes* were analyzed and included the reference isolates Scott A, ATCC 19115 and EGD-e (Appendix: Supplementary Table S1). The isolates originated from seven countries and were isolated during the period of 1997 to 2019 from various foods, food processing environments, clinical cases, and freshwater environments. For long-term storage, isolates were kept in a 20% (v/v) glycerol peptone medium (TS/80, Technical Service Consultants Ltd., Heywood, UK) at −80 °C. Standardized cell suspensions were made by reviving *L. monocytogenes* from (−80 °C) on Tryptone Soy Agar (TSA, 37°C, 1 d) before sub-culturing three separate colonies in 4 ml of 10-times diluted Tryptone Soy Broth (0.1× TSB, Merck, Darmstadt, Germany) at 15 °C for 48 h to simulate the lower nutritional conditions and temperatures found in food production environments. Concentrations of cells were standardized by measuring the absorbance of 620 nm (Multiskan FC, Thermo Fisher Scientific, Roskilde, Denmark) and adjusting to an absorbance of 0.100 ± 0.01 by addition of 0.1× TSB. This corresponded to a cell concentration of approximately 10^8 CFU/ml as confirmed by spot plating (3 × 10 µl) of suitable ten-fold dilutions in peptone saline (PS, 0.85% NaCl, 0.1% Peptone) on TSA followed by enumeration after incubation for 24 h at 37°C. Depending on the experiment, standardized cell suspension was additionally diluted using 0.1× TSB. Each biological independent experiment used fresh cultures on TSA obtained from long-term stock cultures stored at -80°C.

WGS and bioinformatics

Genomic DNA from isolates (n=176), which lacked published sequencing data, were extracted from fresh overnight cultures (TSB, 37°C) using the DNeasy Blood & Tissue Kits (Qiagen, Hilden, Germany) and DNA concentrations were determined using a Qubit dsDNA BR assay kit (Invitrogen, Carlsbad, CA, US). Library preparation was conducted according to the Illumina

protocol followed by sequencing on an Illumina NextSeq platform using 150-bp paired-end reads. The raw sequencing data were subjected to quality check using FastQC v0.11.5 (Andrews, 2010), trimmed with bbtools v36.49 (Bushnell, 2023) and assembled by SPAdes v3.15.3 (Bankevich et al., 2012). Quality of assemblies were evaluated with Quast v5.0.2 (Gurevich et al., 2013). Illumina data from previously sequenced DNA (64 isolates) are available at The European Nucleotide Archive (ENA) under BioProject PRJEB38828 while sequences from the remaining 176 isolates are available under BioProject PRJEB55547. Serotypes and seven gene multilocus sequence type (MLST) profiles, lineages and core genome MLST (cgMLST) were defined using the BIGSdb-Lm database at Institute Pasteur (Moura et al., 2016). Single nucleotide polymorphism (SNP) analysis of the whole genome sequence data was performed for all 240 isolates using the CSI phylogeny (Kaas et al., 2014) with trimmed fastq as input files and *L. monocytogenes* EGDe (GenBank accession no. AL591824.1) as a reference genome. Based on preliminary SNP matrix analyses it was decided to leave out excess clones from clone clusters composed of more than three isolates with same cgMLST if these had less than 10 SNPs in difference and came from same source and year. From such clone clusters three representative isolates from each cluster were included. IQ-TREE v2.1.4-beta was used to create a Maximum Likelihood (ML) tree from the concatenated and aligned SNPs produced by CSI phylogeny under the GTR+G substitution model with ultrafast bootstrapping (--ufboot 1000) (Nguyen et al., 2015). Finally, the phylogenetic ML tree was visualized using the online webtool Microreact (Argimón et al., 2016). ABRicate v1.0.1 (Seemann, 2020) was used to screen isolates for known QACs tolerance genes with a custom blastn database consisting of *qacH* (Accession no. HG329628.1 (Müller et al., 2013), *emrC* (Accession no. MT912503.1:2384-2770 (Kremer et al., 2017)), *emrE* (Accession no. NC_013766.2:c1850670-1850347 (Kovacevic, Ziegler, Wałecka-Zacharska, et al., 2016)) and *bcrABC* (Accession no. JX023284.1 (Elhanafi et al., 2010) with minimum identification and coverage of 90% and 70%, respectively. The BIGSdb-Lm database at Institute Pasteur (Moura et al., 2016) was used to screen isolates for presence/absence of Stress Survival Island, *Listeria* Genomic Island and antibiotic resistance genes.

Determination of minimum inhibitory concentrations

All isolates of *L. monocytogenes* (n=240) were experimentally tested for their MIC to benzalkonium chloride (BC, 500 g/L, Thermo Fisher, Kandel, Germany), peracetic acid (PAA, Peroxyacetic acid, 35% w/v, Acros Organics BV, Geel, Belgium), sodium hypochlorite (SH, 10% w/v, Scharlau, Barcelona, Spain) and ethanol (ET, 99.8%, Acros Organics BV, Geel, Belgium). Sodium hypochlorite and peracetic acid were included due to their widespread use in the Danish food industry, while benzalkonium chloride is used in many food industries around the world. Ethanol is used frequently as hand and surface disinfectant in the industry (Fagerlund et al., 2020). Biocides were diluted daily in 0.1× TSB from fresh original undiluted concentrated stocks until desired working concentrations were obtained, i.e., BC (26.7 mg/L), PAA (2667 mg/L), SH (4000 mg/L), ET (99 % v/v). Biocides were further 2-fold diluted in sterile flat-bottom 96-well polystyrene microtiter plates (In Vitro, Kratbjerg, Denmark) using 0.1× TSB as dilutant leaving each well filled with 150 µl of 0.1× TSB with biocides. Two columns were left and filled with 0.1× TSB only for use as positive (inoculated without biocide) and negative controls (no bacteria, no biocide), respectively. Standardized cell suspensions of *L. monocytogenes* (see above) were diluted to 10^6 CFU/ml. Aliquots of 50 µl was used to inoculate each well, except for the negative control which received 50 µl sterile 0.1× TSB, resulting in a final well volume of 200 µl and a cell concentration of 2.5×10^5 CFU/ml and biocides in concentration ranges seen in Figure 2. Plates were sealed with sealing tape (Thermo Fisher Scientific, Rochester, NY, USA) and incubated for 72 h 15°C. Growth was measured at an absorbance of 620 nm (Multiskan FC) with a no-growth cut-off defined as absorbance readings below two times the absorbance of the negative control (0.038). The MIC was defined as the lowest biocide concentration with no growth. Isolates were picked randomly for testing with 32 isolates tested in each batch. MIC-values were tested in triplicates in two biological independent experiments

(n=6). If there was a MIC-value with discrepancy of more than 2-fold, a third or even fourth biological experiment was conducted to conclude determine the correct MIC-value (n=9).

Selection of a representative subset of isolates

Further biocide susceptibility studies including broth suspensions tests, MIC testing under soiled conditions, MBCs of biofilms and adaptation to biocides were conducted on a subset of the 240 isolates, leading to the selection of 19 representative isolates to ensure genomic and phenotypic diversity of isolates. The selection considered representation of all four major serotypes (1/2a, 1/2b, 1/2c, 4b), carriage of one of the four known BC tolerance genes (*emrE*, *emrC*, *bcrABC*, *qacH*) combined with isolates showing either low or high MICs towards BC and inclusion of as many different STs as possible.

Determination of minimal inhibitory concentrations in soiled conditions

Soiled conditions were simulated to assess how inadequate cleaning would affect the efficacy of biocides towards *L. monocytogenes*. The MIC determination protocol described above was modified to simulate soiled conditions with residual organic matter by inclusion of Bovine Serum Albumin (BSA, Merck, Darmstadt, Germany). Biocides were diluted in 0.1× TSB and 2-fold diluted in the microtiter plates using a suspension of 0.1× TSB with 4 g/L BSA, resulting in a BSA concentration of 3 g/L after inoculation with cell suspensions. Incubation and MIC determination (MIC_{soiled}) was done as described above. MIC_{soiled}-values were determined for the subset of 19 representative isolates of *L. monocytogenes* in two biological independent experiments with triplicates (n=6).

Determination of minimal bactericidal concentrations without and with biofilm

The MIC protocol was used to determine the minimal bactericidal concentration (MBC) for the subset of 19 isolates of *L. monocytogenes* in suspensions. Briefly, after assessing the microtiter plates for growth to determine the MIC-value, 3 × 10 µL from each well were spot plated on TSA (37 °C, 48 h). MBC-values were recorded as the biocide concentration where no survivors were observed after 48 h.

The MBC for *L. monocytogenes* in biofilms ($MBC_{biofilm}$) was assessed differently. *L. monocytogenes* biofilms were created by inoculating 180 µL of 0.1× TSB with 20 µL of 100× diluted standardized cell suspensions resulting in a final cell concentration of 10^4 CFU/ml. Biofilms were formed in 96-well polystyrene microtiter plates (Thermo Fischer Scientific, Roskilde, Denmark) with peg lids (Thermo Fischer Scientific) sealed with parafilm around the edges and incubated at 15 °C in a small sealed chamber with a stable relative humidity (RH) of 100%. Peg lids were moved to a new 96-well plate with fresh 0.1× TSB after three days. After three more days of incubation, the formed biofilm on the peg lids were exposed to biocides (BC, PAA, SH, ET) for 1 h. Biocides were prepared, diluted and filled into 96-well plates as described in the MIC protocol. After biocide exposure, each peg lid was washed twice in 200 µL PS by moving the peg lid into two new 96-well plates filled with PS. Finally, after washing, the peg lids were placed in a new 96-well plate with 200 µL 0.1× TSB and incubated (15 °C, 48 h). Growth was measured at an absorbance of 620 nm (Multiskan FC) with a no-growth cut off ($A620\ nm < 0.076$) defined as absorbance readings below two times the absorbance of the negative control (0.038). $MBC_{biofilm}$ was defined as the lowest concentration without growth since this would correlate to all cells of the 200 µL biofilm being inactivated after exposure to biocides.

Broth suspension test towards BC, PAA and SH

The 19 representative isolates were tested for their survival in broth suspension tests after exposure for 30 s to BC, PAA and SH. *L. monocytogenes* in 0.1× TSB were prepared as above to a cell concentration of 10^8 CFU/ml. Biocide concentrations were prepared less than one hour prior to use by dilution in peptone buffered saline (PBS, Merck Life Science, Søborg, Denmark) and pH adjusted using 6M NaOH or 6M HCl to same pH-values as were used in previous studies (Wiedmann 2020; Bolten et al., 2022). The following concentrations and pH-

values were used in the assay: BC (67 mg/L, pH 8.0), SH (267 mg/L, pH 6.0), PAA (134 mg/L, pH 5.0). When adjusting for the volume of cell suspensions this resulted in effective biocide concentrations of BC (50 mg/L), SH (200 mg/L) and PAA (100 mg/L). In brief, the broth suspension tests were done by adding 50 µL cell suspensions to the first column of 96-well microtiter plates, while 180 µL Dey-Engley neutralizing broth (D/E, Merck Life Science) were added to the second column and 180 µL PBS to columns three, four and five. Aliquots of 150 µL of biocides were added to the cell suspension in column 1 followed by exposure to the biocide for 30 s. The cell and biocide suspensions were mixed three times in the beginning and twice at the end by pipetting. After 30 s of exposure, 20 µL were transferred to wells with D/E and mixed twice before being incubated for 5 min. Finally, the neutralized suspensions were serially diluted 1:10 in PS and spot plated on TSA (24 h at 37°C). Untreated controls were tested in the same manner using PBS pH 7.2. Cell concentrations (log CFU/mL) were calculated for control and treatments and log CFU/mL reduction was estimated. Each isolate was tested in duplicate in four biologically independent experiments (n=8).

Adaptation to biocides

Fifteen of the 19 representative isolates of *L. monocytogenes* were sought adapted to BC, PAA and SH (isolates 01-7210, 06-6837, 08-5578 and LIS26 were not included). Adaptation was conducted with standardized cell suspensions of 10^5 CFU/mL at 15 °C for 48-72 h in 4 mL of diluted TSB with sub-inhibitory concentrations (SIC, half the MIC) for the biocides BC, PAA and SH. Biocides were diluted in 0.1× TSB as described above. After incubation, cell density was recorded at A620 nm followed by sub-culture of 40 µL of each *L. monocytogenes* isolate in new tubes with 4 mL of 0.1× TSB and the biocide at SIC. In total, the 15 *L. monocytogenes* isolates were sub-cultured six times over a period of 18 days in each of the three biocides (BC, PAA and SH). Controls without biocides were similarly sub-cultured. Successful or failed adaptation to biocides was assessed using the final adaptation cultures as inoculum in the MIC protocol described on page 16. The stability of any adaptation was investigated by sub-culturing adapted cells in 0.1× TSB without biocides for two days before repeating the MIC assessment.

Statistical analysis

The log CFU/mL reductions recorded in the broth suspensions test were compared using an unbalanced one-way ANOVA with the Tukey post hoc test at a significance level of $p < 0.05$ to compare the reductions across isolates in groups based on serotypes and linages. The statistical analysis was performed using GraphPad Prism (GraphPad Software) version 9.

4.3 Results

WGS in silico typing and characterization

In silico serotyping using WGS revealed that the 240 isolates were distributed among the four major serotypes of *L. monocytogenes* with 60% (n=138) from 1/2a, 23% (n=55) from 1/2b, 11% (n=32) from 4b and 6% (n=15) from 1/2c (Figure 1). Further sub-typing of all isolates revealed a diversity composed of 53 different STs clustered into 32 CCs (Appendix: Supplementary Table S1). The predominant STs (>5%) were ST121 (19.2%, n=46), ST5 (7.1%, n=17), ST6 (6.3%, n=15) and ST9 (5.4%, n=13). Higher sub-typing resolution based on the cgMLST genotyping method, divided the isolates into 137 cgMLSTs (CTs) (Appendix: Supplementary Table S1). Using the SNP analysis, three clone clusters, all within ST121, were identified in the form of 10 isolates of CT909, 7 isolates of CT9711 and 11 isolates of CT9710. For each clone cluster, three isolates were kept (n=9) for further phylogenetic visualization. The remaining isolate collection (n=221) still contains 27 isolates of ST121 in the phylogenetic tree (Figure 1).

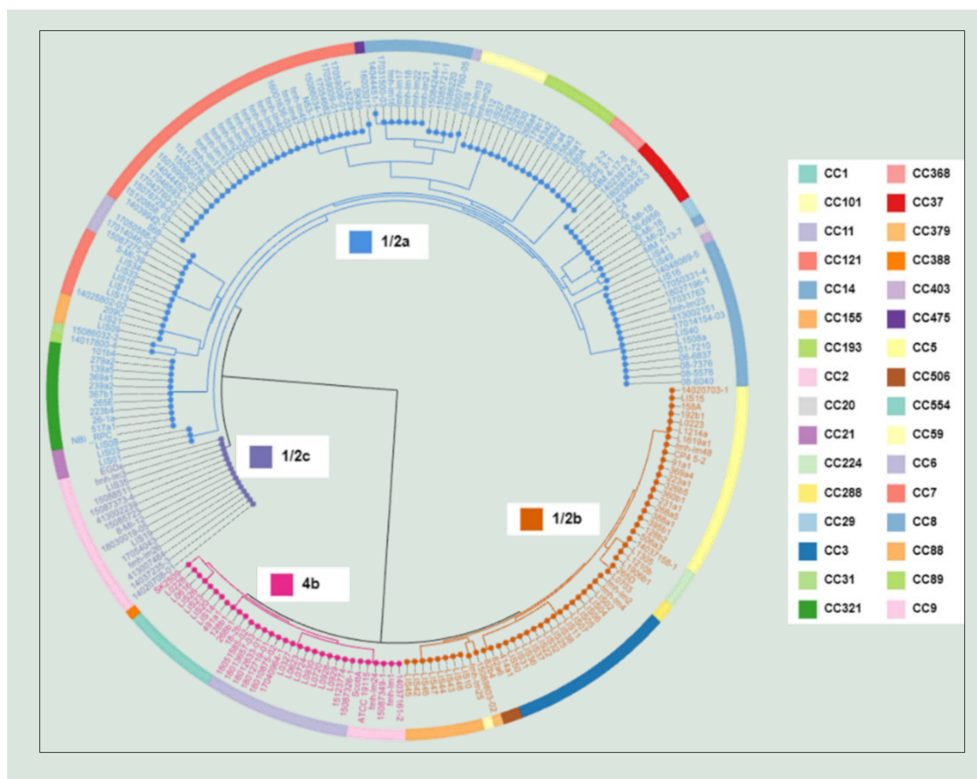


FIGURE 1. Phylogenetic visualization of 221 *L. monocytogenes* isolates. Isolate nodes are co-loured based on serotypes (1/2a, 1/2b, 1/2c or 4b) with isolate names connected to the node. The tree is surrounded by a coloured ring indicating corresponding clonal complexes (CC, n=32) for each isolate.

Screening for genetic features previously related to persistence and tolerance of *L. monocytogenes* to stress encountered in the food processing environment revealed carriage of the Stress Survival Islet I (SSI-1) in 101 isolates from serotypes 1/2a, 1/2b and 1/2c across 29 STs, while 50 isolates belonging to serotype 1/2a and four STs (ST121, ST193, ST236, ST504) carried the stress survival islet II (SSI-2, (Appendix: Supplementary Table S1). Eighty-nine isolates including all serotype 4b isolates carried neither SSI-1 nor SSI-2. None of the isolates carried both. Listeria Genomic Islands (LGI) were less prevalent among the isolates with LGI-1, found only in five serotype 1/2a CC8 isolates (ST120, ST292) from Canada. LGI-2 was found in seven isolates from 1/2a (ST14) and seven isolates from serotype 4b (ST1, ST2, ST290) derived from foods, FPE and one human clinical case. Seven serotype 1/2a isolates (ST38, ST101) from foods in Denmark and Italy possessed LGI-3. Interestingly, no isolates contained more than one LGI. However, the five CC8 isolates carrying LGI-1 also harboured SSI-1. Fmh-lm3 (ST9) was the only isolate carrying both SSI-1 and LGI-2.
Antibiotic resistance genes (*fosX*, *lmo0919*, *norB*, *lmo0441*, *sul*) conferring resistance to fosfomycins, lincosamides, cephalosporins and sulfonamides were found in all isolates, whereas *tetM* and *tetS* giving tetracycline resistance were exclusively found in one ST7 isolate (LIS13). Finally, six isolates (ST3, ST5 (1/2b) and ST31 (1/2a)) all carried the aminoglycoside resistance conferring gene *aacA4*. All 240 isolates carried three intrinsic efflux pumps (mdrL, idE, *mdtH*), whereas the QAC biocide tolerance efflux-pump encoding genes (*qacH*, *bcrABC*, *emrC* and *emrE*) were found in 87 isolates. Forty-six CC121 isolates (ST121 (n=44), ST236 (n=2)) carried *qacH*, while the three gene *bcrABC* cassette was found in 31 isolates from seven STs (ST3, ST5, ST14, ST155, ST321, ST391, ST820), representing both serotypes 1/2a and 1/2b. In contrast, *emrC* was observed in just three isolates (ST9 (1/2c), ST21 and ST691 (1/2a)), while *emrE* was only found in the five 1/2a CC8 Canadian isolates carrying LGI-1, as *emrC* is a gene within this genomic island (Kovacevic, Ziegler, Wałecka-Zacharska, et al., 2016).

MIC distributions and genotypic correlations

Sensitivity of *L. monocytogenes* to PAA, SH and ET showed a narrow distribution of MIC-values and therefore limited variation among isolates (Figures 2a, b and c). In contrast, isolates exhibited an 8× MIC difference for BC MIC-values, as the most sensitive isolates (n=94) were unable to grow at a BC concentration of 0.31 mg/L while 88 isolates exhibited MIC-values of 2.5 mg/L (Figure 2d). Based on this MIC distribution, the cut-off for BC tolerance was determined to be MIC > 1.25 mg/L. In contrast, it was not possible to define a cut-off for tolerance to PAA, SH or ET. For PAA, all isolates (n=240) shared the same MIC-value (63 mg/L). For SH (47 mg/L (n=31), 94 mg/L (n=209)) and ET (4.7% (n=46), 9.4% (n=194)) most isolates were centered on one MIC-value with 13-19% of the isolates being 2× more sensitive (Figures 2a, b and c).

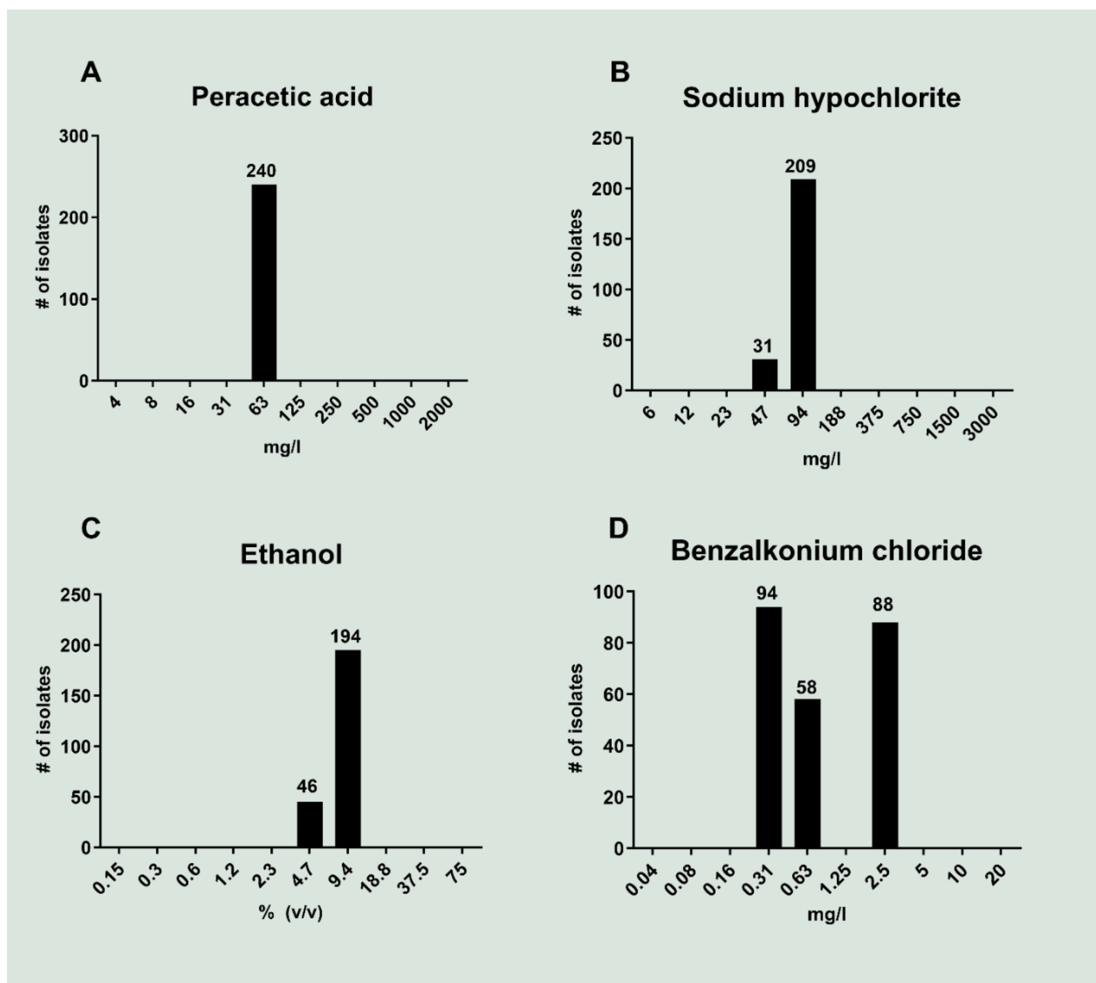


FIGURE 2. Distribution of MIC-values for 240 *L. monocytogenes* isolates. MIC-values were determined in a minimum of two independent biological experiments with triplicates for the biocides A) peracetic acid, B) sodium hypochlorite, C), ethanol and D) benzalkonium chloride.

The presence of BC tolerance genes correlated with tolerance to BC at low concentrations as 87 of 88 BC tolerant isolates (MIC of 2.5 mg/L) carried a known BC tolerance gene (*qacH*, *bcrABC*, *emrC*, or *emrE*). In contrast, none of the BC sensitive isolates (n=152) carried any of the known BC tolerance genes. Interestingly, one BC tolerant food isolate (LIS26, ST324, 4b) contained none of the known BC tolerance genes (Appendix: Supplementary Table S1). While BC sensitive and tolerant isolates were found within the same STs, the tolerant phenotype always correlated with the presence of a BC tolerance gene, i.e., *bcrABC* in ST5 (n=15) and

ST14 (n=7) or *qacH* in ST121 (n=46). Similarly, *emrC* appeared to confer BC tolerance to three of 17 isolates from three different STs (ST9 (n=13), ST21 (n=3), ST691 (n=1), which otherwise harboured BC sensitive isolates without *emrC* or any of the known QAC tolerance genes. In CC8, *emrE* resulted in BC tolerant phenotypes in isolates belonging to ST120, and ST292 (n=5). However, ST8 isolates (n=9) also from CC8 exhibited a sensitive BC phenotype and did not contain *emrE* indicating that *emrE* carriage is not dependent on clonal complex. With no known ET tolerance genes and a narrow MIC distribution (Fig. 2) it was not possible to determine a specific genetic feature related to ET sensitivity of *L. monocytogenes*. A closer look at the least prevalent serotype 1/2c (n=15) revealed that all 15 isolates from CC9 (ST9, ST35, ST580) exhibited the higher ET MIC-value of 9.7% (Appendix: Supplementary Table S1). Similar observations were made for CC37 (n=7) from 1/2a and CC88 (n=8) from 1/2b. Using the overall phylogenetic visualization (n=221) to look for evolutionary adaptational trends revealed that the ET sensitive phenotypes are distributed across the serotypes, STs and CCs (Appendix: Supplementary Figure S1). For SH, 87% of the isolates exhibited the same MIC-value, which makes any potential correlations between genetic determinants and SH sensitivity of limited value. Indeed, the phylogenetic visualization (n=221) shows how the isolates (13%) with the lower SH MIC-value were spread across the whole phylogenetic tree and unrelated to specific subtypes (Appendix: Supplementary Figure S2). As all isolates showed the same MIC-value to PAA, it was not possible to further examine genetic determinants of PAA sensitivity solely based on phenotypic MIC observations.

TABLE 1. Comparisons of minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) for commonly used biocides under different conditions (soiled and biofilm) for a representative subset of 19 *L. monocytogenes*.
[a] – BCRG is benzalkonium chloride tolerance genes.

						Peracetic acid (mg/l)				Sodium hypochlorite (mg/l)				Ethanol (%, g/100 ml)				Benzalkonium chloride (mg/l)			
ID	LINE-AGE	SERO TYPE	CC	ST	BCRG[a]	MIC	MIC soil	MBC	MBC biofilm	MIC	MIC soil	MBC	MBC biofilm	MIC	MIC soil	MBC	MBC biofilm	MIC	MIC soil	MBC	MBC bio-film
01-7210	II	1/2a	CC8	120	*emrE*	62	62	62	62	94	750	375	375	4.7	9.4	33	33	2.5	20	5	40
06-6837	II	1/2a	CC8	120	*emrE*	62	62	62	62	47	750	375	375	9.4	9.4	33	33	2.5	20	5	40
08-5578	II	1/2a	CC8	292	*emrE*	62	62	62	62	94	750	375	375	4.7	4.7	33	33	2.5	20	5	40
LIS18	II	1/2a	CC7	691	*emrC*	62	62	62	62	94	750	375	375	9.4	9.4	33	33	2.5	20	5	40
LIS29	II	1/2a	CC3	38	-	62	62	62	62	94	750	375	375	9.4	9.4	33	33	0.3	10	5	10
18027196-1	II	1/2a	CC8	8	-	62	62	62	62	94	750	375	375	9.4	9.4	33	33	0.3	10	5	10
15112778-2	II	1/2a	CC121	121	*qacH*	62	62	62	62	94	750	375	375	9.4	9.4	33	33	2.5	20	5	10
15076990-02	II	1/2a	CC121	236	*qacH*	62	62	62	62	94	750	375	375	9.4	9.4	33	33	2.5	20	5	40
14044451-1	II	1/2a	CC14	14	*bcrABC*	62	62	62	62	94	750	375	375	9.4	9.4	33	33	2.5	20	5	20
LIS19	II	1/2c	CC9	9	*emrC*	62	62	62	62	94	750	375	375	9.4	9.4	33	33	2.5	20	5	40
14020708-01	II	1/2c	CC9	580	-	62	62	62	62	94	750	375	375	9.4	9.4	33	33	0.3	10	5	10
413002239	II	1/2c	CC9	9	-	62	62	62	62	94	750	375	375	9.4	9.4	33	33	0.3	10	5	10
LIS15	I	1/2b	CC5	5	*bcrABC*	62	62	62	62	94	750	375	375	9.4	9.4	33	33	2.5	20	5	40
LIS37	I	1/2b	CC3	3	-	62	62	62	62	94	750	375	375	4.7	4.7	33	33	0.3	5	5	10
14037158-1	I	1/2b	CC224	224	-	62	62	62	62	94	750	375	375	9.4	9.4	33	33	0.3	5	5	10
LIS20	I	4b	CC1	1	-	62	62	62	62	94	750	375	375	9.4	9.4	33	33	0.3	10	5	20
18012633-01	I	4b	CC6	6	-	62	62	62	62	94	750	188	375	9.4	9.4	33	33	0.3	10	5	10
14037161-2	I	4b	CC2	2	-	62	62	62	62	94	750	375	375	9.4	9.4	33	33	0.3	5	5	10
LIS26	I	4b	CC1	328	(-)	62	62	62	62	94	750	375	375	4.7	4.7	33	33	2.5	20	5	20
Fold differences between isolates						None	None	None	None	2×	None	2×	None	2×	2×	None	None	8×	2-4×	None	2-4×
Fold differences to MIC							None	None	None		8×	2-4×	4×		None	4-8×	4-8×		8-33×	2-16×	8-64×

Selection of a subset of isolates

A selection of 19 isolates was used to represent the phenotypic and genotypic diversity seen among the 240 isolates (Table 1). All four serotypes were included with serotype 1/2a being represented by nine isolates to reflect the higher proportion of this serotype in the full set of isolates (Figure 3). For the remaining serotypes the distribution was, however, more uniform to ensure that at least three isolates were included from each serotype to avoid misinterpretation of results due to too small sample sizes. The 19 isolates represent 17 STs in 11 CCs, as to have each BC tolerance gene covered by more than one isolate it was necessary to include more than one isolate from same CC. In total, 10 isolates carrying one tolerance gene (*emrE*, *emrC*, *bcrABC*, *qacH*) were included, except for isolate LIS26 which carried no known BC tolerance gene but showed tolerance to BC in multiple repetitions of the MIC determinations (Appendix: Supplementary Table S1). Nine isolates sensitive to BC were included covering 9 different STs. As seen in the phylogenetic tree the selected isolates were generally from different clusters except for isolates from same CC as described above (Figure 3).

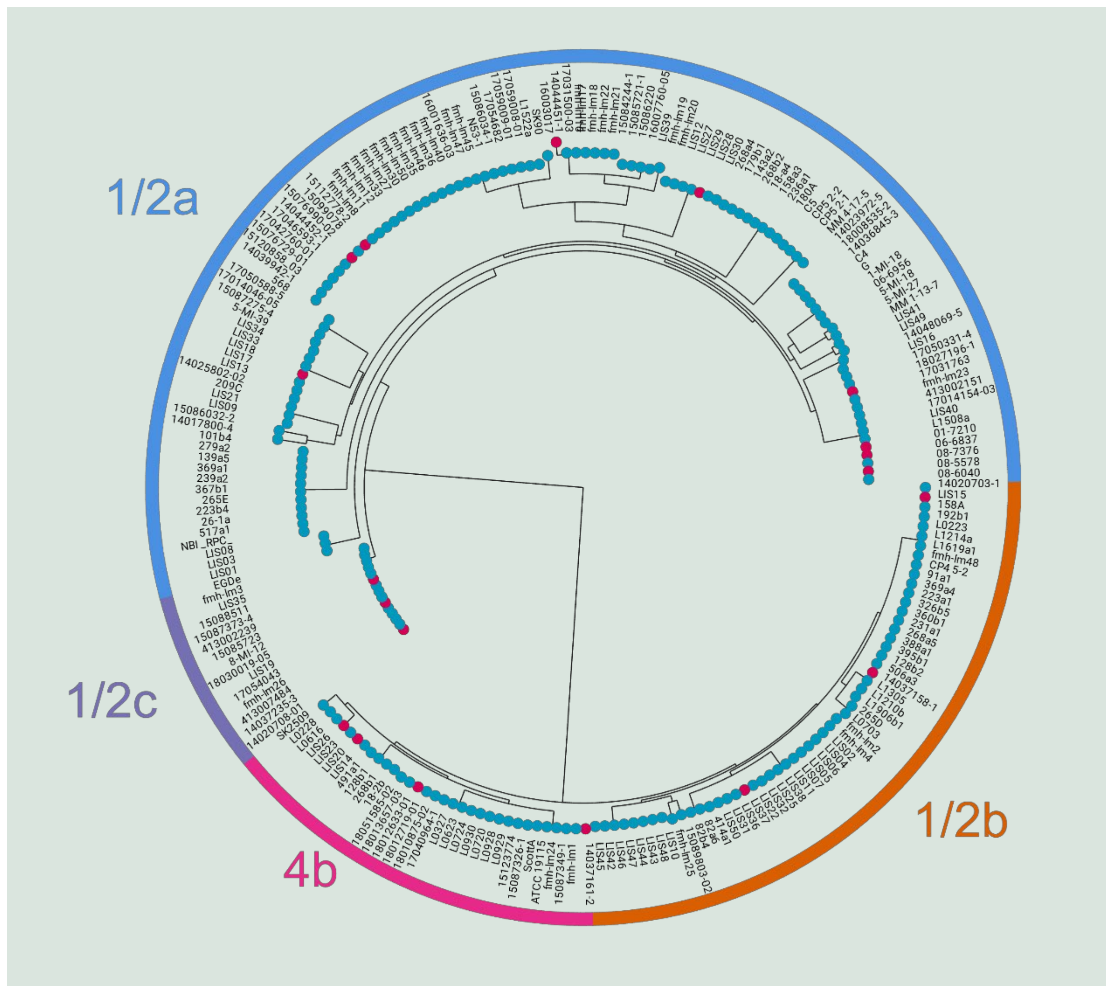


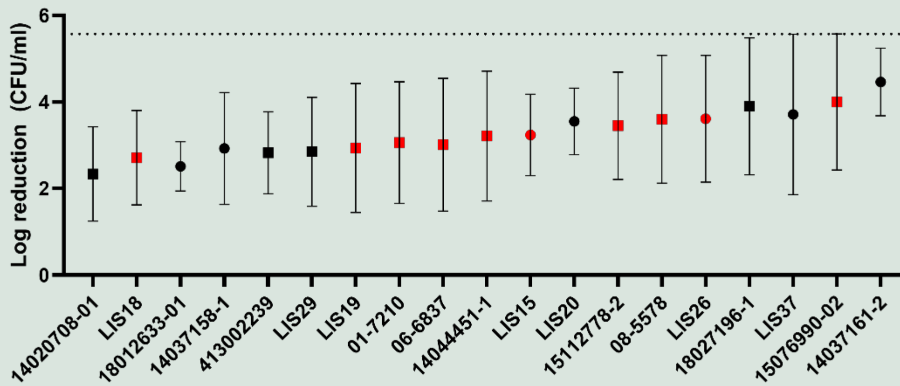
FIGURE 3. Phylogenetic visualization of 221 *L. monocytogenes* isolates. Isolate nodes in red were selected as representative isolates for further investigation in phenotypic tests. The tree is surrounded by a colored ring indicating corresponding serotype.

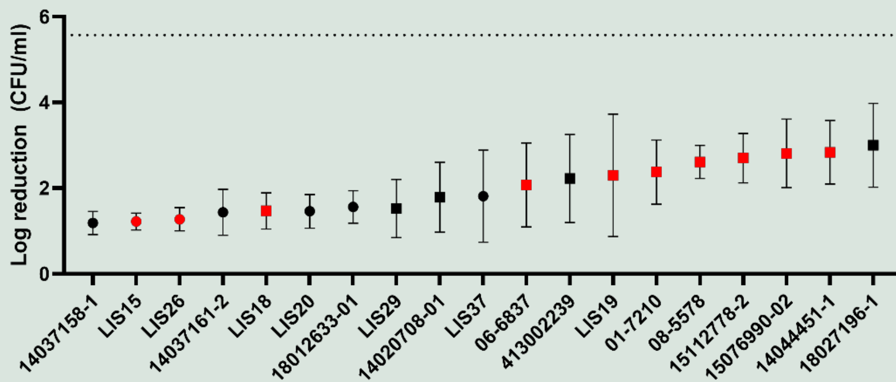
MIC under soiled conditions and MBCs without and with biofilm

The MIC under soiled conditions (MIC_{soil}) and the minimum bactericidal concentration with ($MBC_{biofilm}$) and without (MBC) biofilm were assessed for the 19 isolates of *L. monocytogenes* and compared to the initial MIC observations (Table 1). This comparison showed the MIC_{soil}, MBC and $MBC_{biofilm}$ for PAA were equal to the MIC-value of 63 mg/L for all isolates showing

that the efficacy of PAA were unaffected by organic matter or biofilm. In contrast, SH was the biocide for which efficacy was reduced the most by organic matter as evident by the 8× higher MICsoil (750 mg/L). The concentrations needed to kill all cells (MBC, $MBC_{biofilm}$) with SH or ET were 4× and 4-8× times higher, respectively, than the corresponding MIC-values (Table 1). While SH was heavily reduced in efficacy by soiled conditions, this was not the case for ET as MIC_{soil}-values were identical to the MICs. Soiled conditions, however, affected BC efficacy on *L. monocytogenes* as MIC_{soil} were 8-33× fold higher, with the most sensitive isolates showing the highest fold change resulting in limited difference among isolates. $MBC_{biofilm}$ for BC were 8-64× higher than MBC, indicating the negative impact of biofilm formation on BC efficacy. Importantly, the response of the 19 isolates to PAA, SH and ET showed limited variation with no more than 2-fold differences recorded for their MIC, MIC_{soil}, MBC, and $MBC_{biofilm}$. In contrast, BC sensitivity differed among isolates with increased values of MIC, MICsoil, and $MBC_{biofilm}$ correlating with the presence of known tolerance genes. The overall fold differences among isolates were, however, lower (2-4×) for MIC_{soil} and $MBC_{biofilm}$ compared to the differences observed in MIC (8×). Interestingly, MBC and $MBC_{biofilm}$ values were the same for all 19 isolates for all biocides excluding BC.

Broth suspension tests – survival after short time exposure to BC, PAA and SH
Initial tests showed that none of the 19 representative isolates survived exposure for 30 s in broth suspension tests (BSTs) using biocide concentrations several folds lower than recommended industrial concentrations (data not shown). The manufacturer-recommended concentrations vary among commercial industrial biocide products and studies with MRCs for BC at 200-1000 mg/L, PAA ≥ 500 mg/L and SH at 800 mg/L being reported (Cruz & Fletcher, 2012; Møretrø et al., 2017; Stoller et al., 2019). Since the purpose of the BSTs was to compare variation in survival among isolates, lower concentrations of the biocides BC, PAA and SH were applied. When exposed to 50 mg/L BC for 30 s, the 19 isolates were on average reduced by 3.3±1.3 log CFU/mL. Surprisingly, having a higher recorded BC MIC or the possession of BC tolerance genes did not confer any increase in survival as average reductions were 3.3±1.3 log CFU/mL for isolates labelled sensitive in the MIC test and 3.3±1.4 log CFU/mL for isolates labelled tolerant (Figure 4A). Similarly, there were no significant differences ($p>0.05$) in the survival of different serotypes. The three best surviving isolates (LIS18, 14020708-01 and 18012633-01) had different genotypic traits as LIS18 contains both *emrC* and SSI-1, whereas 14020708-01 contains SSI-1 without a BC tolerance gene and finally 18012633-01 has neither SSI, LGI nor a BC tolerance gene (Appendix: Supplementary Table S1). These observations were in line with MBC determinations showing the same value for all 19 isolates and indicating that actual isolate diversity for BC tolerance might be low. However, a significant difference ($p<0.05$) between the PAA induced log reductions for lineage I (1.4±0.5 log CFU/mL) and II (2.3±0.9 log CFU/mL) were seen (Figure 4B). This contrasted with the observations for MIC and MBC for PAA, where all isolates had the same values (Table 3). None of the analyzed genotypic traits appeared associated with an increased PAA tolerance, except the higher number of BC tolerance genes in lineage I isolates. Broth suspensions test with SH at a concentration of 200 mg/L showed differences in the survival of *L. monocytogenes* with an average reduction of 2.5±0.7 log CFU/mL across all 19 isolates, with the best surviving isolate LIS26 reduced by 2.0±0.7 log CFU/mL (Figure 4C).





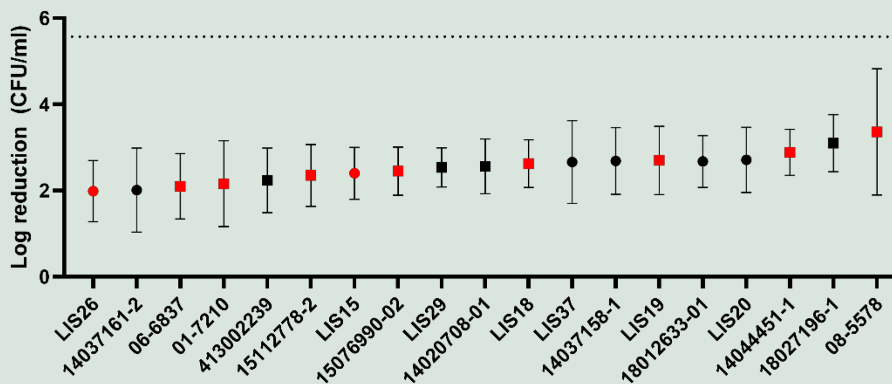


FIGURE 4. Inactivation of *L. monocytogenes* using commonly used biocides in a broth suspension test. A representative subset of 19 *L. monocytogenes* from lineage I (circle) and lineage II (square) with (●■) or without (●■) one of the known tolerance genes (*qacH*, *bcrABC*, *emrC*, *emrE*), were exposed for 30 s to biocides A) 50 mg/l benzalkonium chloride, B) 100 mg/l peracetic acid and C) 200 mg/l sodium hypochlorite. Mean log reduction (CFU/ml) is given for each isolate with standard deviations based on four independent biological experiments with duplicates. The detection limit depicted by dashed line denotes a reduction of >5.77 log CFU/ml.

Adaptation of *L. monocytogenes* to biocides
Repeated exposure to sub-inhibitory concentrations (SIC) of either PAA or SH for 18 days did not increase MIC-values of isolates (n=15). Isolates grown without biocide (controls) similarly showed unchanged MIC-values for PAA, SH and BC. For the originally BC tolerant isolates (n=6) carrying BC tolerance genes there were no increase in MIC (2.5 mg/L) to BC at the end of the adaption period. The originally BC sensitive isolates (n=9) increased their MIC-value 8× to 2.5 mg/L to achieve the same tolerance to BC as the isolates carrying BC tolerance genes. This change was, however, not permanent as after subculturing the isolates in media without BC, MIC-values dropped to 1.25 mg/L (4× their original MIC). After six additional subcultures over 12 days, this 4× increase in MIC (1.25 mg/L) was stable for three isolates (LIS20, LIS29 and 18012633-01), while the six other isolates showed inconsistent MIC-values 2-4× higher than their original MIC-value (Appendix: Supplementary Figure S3).

4.4 Discussion

The present study evaluated the sensitivity of a genetically characterized collection of 240 *L. monocytogenes* isolates to four different biocides, which are commonly used in the food industry. A major finding is that MIC-values determined at an industrially realistic incubation temperature of 15 °C showed no or limited variation for PAA, ET and SH while MIC-values for BC (a QAC) showed up to 8× differences. Below we will discuss the implications of these findings as well as the role of soils, biofilms, and adaptation on the effect of biocides.

In our study, tolerance to BC varied 4-8× among the 240 *L. monocytogenes* isolates with MIC-values ranging from 0.31 to 2.5 mg/L (Figure 1A, (Appendix: Supplementary Table S1). Previous studies similarly report finding isolates of *L. monocytogenes* showing decreased sensitivity to low concentrations of BC with tolerant isolates having 2-8× higher MIC-values (5-10 mg/L) (Luque-Sastre et al., 2018; Meier et al., 2017; Møretrø et al., 2017; Palma et al., 2022; Roedel et al., 2019). In contrast, Rodríguez-Melcón et al. (2022) observed more than 20× differences with the highest BC MIC-value at 20 mg/L, while Meier et al. (2017) observed that 6 of their 392 isolates of *L. monocytogenes* grew in the presence of 30 mg/L BC. The latter study used BC containing agar plates incubated at 37°C to test MIC-values, while the other studies use a microbroth dilution methods in full strength media and incubation at 37°C. It is possible that the lower MIC-values observed in the present study could be explained by the lower incubation temperature and use of diluted media with less organic matter to affect the inhibitory effect of BC (Table 1).

Despite MIC-values of tolerant isolates in our and other studies being much lower than manufacturer-recommended concentrations (200-1000 mg/L) for the industry, these isolates may still have a competitive growth advantage in niches with low concentrations of residual BC (Møretrø et al., 2017). This may imply that isolates carrying BC tolerance genes tend to be classified as persistent isolates in environments where QAC based disinfectants are used regularly as suggested by (Fagerlund et al., 2022; Palaiodimou et al., 2021)
Out of the 19 representative isolates, the nine, which harboured one of the specific BC tolerance genes (*qacH*, *bcrABC*, *emrC*, *emrE*), were scored as tolerant with MIC-values of 2.5 mg/L BC (Table 1). However, MBC-values and survival following a short exposure (5 min) to 50 mg/L BC were identical among the 19 representative isolates regardless of the presence/absence of known BC tolerance genes (Table 1, Figure 4). This indicates that the findings of higher tolerance at low concentration, as shown by the MIC tests in most studies, do not correlate with *L. monocytogenes* eradication through industrial sanitation as also recently discussed by Bland, Brown, et al. (2022). Moreover, Møretrø et al. (2017) noted that neither *qacH* nor *bcrABC* confer increased tolerance at higher BC concentrations. Our results show for the first time this to be the case for all four known BC tolerance genes (*qacH*, *bcrABC*, *emrC* and *emrE*) in suspension tests with 50 mg/L for 5 min (Figure 4). In contrast, the MBC

for biofilms tended to be higher for isolates with BC tolerance genes. The assay used in the present study relies on regrowth of the biofilm cells after BC exposure and despite washing, minimal BC residuals may remain in the media. Low residual concentrations of BC left after QAC disinfection in the food industry have been observed and may select for BC tolerant strains (Møretrø et al. 2017). Interestingly, there were no differences in the immediate reduction in survivors (metabolic activity) in 6-d old biofilms formed by 14 isolates with or without the *emrE* gene, following exposure to BC concentrations up to 80 mg/L (Piercey et al., 2017), highlighting the effect of BC is not completely disrupted by the tolerance genes.
Isolates with original higher tolerance to BC (MIC = 2.5 mg/L) were not able to obtain a higher MIC-value through adaptation trials (data not shown). Sensitive isolates (original MIC = 0.3 mg/L) were able to increase their MICs to 2.5 mg/L, however, this adapted MIC was not stable (Figure S3). This 4-8× increase through adaptation was also reported by Bolten et al. (2022), however, only for *bcrABC* and *qacH* carrying isolates. Moreover, these authors documented through SNP-analysis that a mutation in *fepR* leads to a permanent overexpression of an efflux pump in isolates without *bcrABC* or *qacH*.

MIC_{soil}-values in simulated dirty conditions with residual organic matter demonstrated the marked decrease in BC efficacy (8-33×, Table 1), indicating that the presence of residual organic matter impact sensitivity to BC more than harbourage of tolerance genes (Figure 4) or ability to increase MIC tolerance through adaptation trials (Appendix: Supplementary Table S1). This means that the 8× BC tolerance variation (Table 1) as seen in MIC tests may be considered a smaller challenge compared to lack of proper cleaning before sanitation. In terms of practical considerations for risk management this implies that the monitoring of isolate variation and genetic determinants for BC tolerance is less valuable compared to sanitation efforts directed towards battling biofilm build-up and securing removal of organic interfering matter before disinfection.

L. monocytogenes displays low isolate variation, adaptation and tolerance to PAA, SH and ET. In contrast to BC, limited variation in the sensitivity of 240 *L. monocytogenes* was observed to the other biocides, PAA, SH and ET (Figure 2ABC), coinciding with the lack of known genetic tolerance markers (Palma et al., 2022). Roedel et al. (2019) also observed the same PAA MIC-value for 93 German *L. monocytogenes* isolates while some variation was observed for SH. Their MIC-values were similar to several reports (Guérin et al., 2021; Poimenidou et al., 2016; Rodríguez-Melcón et al., 2022) and significantly higher than those observed in the present study. Also, one study of 197 *L. monocytogenes* from different ecological niches observed up to 4× differences in MIC for both PAA, SH and ET with *L. monocytogenes* MIC-values at 625 mg/L, 1250 mg/L and 50% w/v for PAA, SH and ET, respectively (Palma et al., 2022). As previously mentioned, direct comparisons of MIC-values across studies are difficult due to protocol variations including dilutants, media, incubation temperature and pH of biocide assays. Nevertheless, it is clear that, overall, the majority of studies report MIC-values for PAA, SH and ET below concentrations applied by industry for disinfection (Bland et al., 2022). We observed no adaptation to PAA or SH, which is consistent with the lack of differences in MIC-values (Figure 2). There are only few reported cases of adaptations of *L. monocytogenes* to PAA or SH in the literature with few isolates increasing their MIC-values 20% or less, which is well below the 8× increase for BC adaptations (Bansal et al., 2018; Rodríguez-Melcón et al., 2023). Others have, as in the present study, reported failed adaptations to PAA and SH when using 2× MIC increase as the cut off (Aarnisalo et al., 2007; Kastbjerg & Gram, 2012; Riazi & Matthews, 2011). The lack of diversity in PAA, SH and ET MIC-values for 240 isolates (Figure 2) from 53 STs and lack of differences in MBC-values (Table 1), means that either these widely used tests were not suited for differentiating *L. monocytogenes* biocide tolerance or that adaptation or biocide tolerance variation are not present. However, the broth suspension test showed improved survival of lineage I to higher concentrations of PAA at shorter exposure times (Figure 4B). This observation is supported by (Manso et al., 2020) where lineage I isolates exhibited higher oxidative stress survival than lineage II after exposure to H_2O_2. The

broth suspension test is more labor-intensive and comes with high standard deviations but gives continuous values, rather than discrete values as in the MIC tests. The broth suspension tests may offer better differentiation of isolates at higher biocides concentrations and shorter exposure times, a scenario which is more industrially relevant and a recommended focus for future adaptation studies. Recently, re-exposure experiments at higher biocide concentrations with *Listeria innocua* found either no or increased sensitivity upon re-exposure to PAA, SH or BC (Rahman et al., 2022). The lack of significant differences among isolates at higher concentrations of SH (Figure 4C) could imply that SH acts instantaneously in a multi-targeted manner that disallows development of isolate variation. This has previously been proposed as the explanation for lack of successful adaptation to SH and PAA (Kastbjerg & Gram, 2012). In this context, it is important to highlight the effect of soiled conditions on the SH efficacy, which was reduced 8× (Table 1). SH is known to be greatly affected by organic matter (Best et al., 1990). Therefore, based on the results from the present study, it appears that the biggest concern with application of the widely used SH is sufficient removal of organic matter and biofilm prior to disinfection with the biocide.

In contrast to SH, the effect of neither ET nor PAA were affected by soiled conditions (Table 1). However, a relative increase in concentration was seen in MBC for ET when biofilms were pre-formed (Table 1). In the present study, the efficacy of PAA were evenly good in clean and dirty conditions as well as when biofilm was present with no variation in sensitivity among genetically diverse isolates (Figure 2A, Table 1). PAA has previously been shown to be less affected by organic matter compared to other biocides. This could be due to its high redox potential and stronger reaction with *L. monocytogenes* and other bacteria relative to organic matter in soils (Burnett et al., 2005; Finnegan et al., 2010; Cruz & Fletcher, 2012). However, the observation of better PAA tolerance of lineage I isolates in the broth suspension assay indicates that further investigation into linage and isolate variation at higher concentrations at shorter exposure times would be of great interest. PAA is reported to trigger sublethal injuries and the viable-but-nonculturable state (Arvaniti et al., 2021; Gu et al., 2020), which could be part of the explanation for the lineage difference (Figure 4B) observed in the present study for PAA sensitivity at higher concentration. While it is possible to observe differences in isolate sensitivity to sublethal PAA concentrations, i.e., those used in the broth suspension assay, it is clear that the ability of the industry to apply markedly higher manufacturer-recommended concentrations of biocides during disinfection procedures, means that neither adaptation nor low level tolerance to biocides is as important as proper cleaning prior to disinfection. Still a higher incidence of BC genetic determinants in pervasive *L. monocytogenes* has been reported (Fagerlund et al., 2022) and thus it cannot be excluded that the adaptation to low BC concentrations (Appendix: Supplementary Figure S1) plays a role in survival within the food industry if encountering low residual concentrations of BC (Møretrø et al., 2017).

In conclusion, this study aimed to investigate if the tolerance to commonly used biocides differ among a large collection of genetically diverse *L. monocytogenes*. The results revealed limited variation in biocide tolerance among 240 *L. monocytogenes* isolates, whilst organic matter or preformed biofilms simulating insufficient cleaning had a pronounced effect on the efficacy of BC, SH and ET. This implies that the considerable interfering effect of organic matter and preformed biofilms are a greater daily concern in food processing facilities than variation in tolerance and adaptation to biocides. However, as *L. monocytogenes* showed no ability to adapt to low concentrations of neither PAA nor SH. Importantly, as PAA was not affected by organic matter or biofilm, it demonstrates the value of this biocide to the food industry. To note, the lack of relationship between MIC-values and log-inactivation in broth suspensions test, means that results from these assays should be interpreted differently, i.e., MIC-values reflect ability to grow in low concentration of disinfectant residuals in the food processing environment while BSTs measure the effect of disinfection routines with high concentrations. This assay discrepancy was most clearly observed when isolates carrying BC tolerance genes did not show better survival at higher concentrations compared to non-carrying isolates and when isolates with

same MIC-value to PAA showed significantly different ability to survive higher concentrations of PAA in the BST.

5. Sub-project 2

Repeated biocide treatments cause changes to the microbiome of a food industry floor drain biofilm model

5.1 Introduction

Food manufacturers depend on the removal and inactivation of microbes through sanitation programs with cleaning and disinfection schemes to keep a food production environment suitable to produce safe food. Lately, there has, however, been an increased interest in the potential development of microbial tolerance or adaptation to biocides due to repeated application of the same biocide (Bland et al., 2022a; He et al., 2022; Rahman et al., 2022). Case studies with biofilms in hospital sinks and food conveyor belts have shown how difficult eradication of biofilms may be even when using multiple biocide treatments with increasing concentrations (Fagerlund et al., 2020; Ledwoch et al., 2020; Anantharajah et al., 2024). In general, drains and sinks constitute difficult-to-clean sites in both clinical settings and food production. Floor drains are continuously fed with a diverse pool of bacteria that have travelled through the food processing environment (FPE) (Dzieciol et al., 2016) after being introduced via different raw materials, ingredients, workers, equipment and vehicles depending on the quality of zoning within the food processing plant (Berrang et al., 2010; Nicaogáin and Byrne, 2016). Logically, drains also serve as the exit and collection point of cleaning and biocide residues together with dirt, residual organic matters, and non-viable and viable bacteria, some of which may adhere and form biofilms on surfaces within sinks and drains (Wagner & Stessl, 2014; Dzieciol et al., 2016). Therefore, drains may become a reservoir where pathogenic bacteria and bacteria with strong biofilm-producing capabilities may persist to create opportunities for re-introduction into the FPE through splashing caused by wrongful cleaning and trolleys (Berrang and Frank, 2012; Murugesan et al., 2015; Magdovitz et al., 2020).

The foodborne pathogen *Listeria monocytogenes* is a ubiquitous microbe that may cross-contaminate food products due to its persistence in food production environment for years (Ferreira et al., 2014; Stessl et al., 2020). Routine sampling for *Listeria* spp. has shown drains to be one of the sites with the highest probability of positive samples (Estrada et al., 2020; Magdovitz et al., 2020; Bardsley et al., 2024). In addition, depending on the microbiota of drains the positive ratio for *Listeria* spp. may increase due to cooperative and competitive interactions within biofilms (Fox et al., 2014; Møretrø and Langsrud, 2017; Fagerlund et al., 2021). In recent years, it has been shown that *L. monocytogenes* persistence may be partly aided by the possession of genes conferring increased tolerance to quaternary ammonium compound (QAC) based biocides and/or gaining tolerance through repeated exposure to low biocide concentrations (Møretrø et al., 2017; Luque-Sastre et al., 2018; Fagerlund et al., 2022; He et al., 2022). However, a search in the publicly available whole genome sequences of 37,897 *L. monocytogenes* isolates showed that less than 30% carry QAC tolerance genes, indicating that survival in food production environment is also affected by other factors (Ivanova et al., 2024). Indeed, formation of biofilm was more disruptive for the effect of benzalkonium chloride, a QAC biocide, on *L. monocytogenes* isolates than harbourage of one of the QAC tolerance genes (Kragh et al., 2024). Similar to other pathogens such as *E. coli* O157:H7 and *Salmonella enterica*, survival of *L. monocytogenes* following biocide exposure is aided when embedded in multi-species biofilms dominated by genera such as *Acinetobacter*, *Pseudomonas*, *Stenotrophomonas* (Fagerlund et al., 2017; Dass et al., 2020; Maillard and Centeleghe, 2023;

Thomassen et al., 2023a; Rolon et al., 2024). Since biofilms interfere with the efficacy of biocides, it is important to use realistic biofilm models when studying the effect of biocide treatments on microbiomes in FPEs and survival of pathogens such as *L. monocytogenes*.
The era of microbiome and metagenome techniques has enabled studies of the complexity, diversity and resistome of biofilms in food production environments and the potential spread of biocide tolerance genes and antimicrobial resistance genes (AMR) within biofilms (Alvarez-Molina et al., 2023; Flores-Vargas et al., 2023; Nimaichand et al., 2023; Xu et al., 2023). By comparing the core microbiome among different samples, it is furthermore possible to define the most abundant genera across samples, productions, or specific niches, e.g., drains in beef productions were shown to be dominated by *Pseudomonas*, *Psychrobacter* and *Acinetobacter* (Palanisamy et al., 2023). This approach opens for the development of realistic and representative biofilm models to study the effect of biocide treatments on these niches in the laboratory.

The objectives of the sub-project were to i) create a representative drain biofilm model based on culturomic and shotgun metagenomic characterization of floor drains from cheese, shrimp and fish roe food production environments, ii) use the representative drain biofilm model to test the effect of repeated biocide treatments on the composition of the surviving and regrowing microbiome, and specifically on the survival of *L. monocytogenes*, and iii) characterize the isolates of the model by biocide MIC testing and whole genome sequencing (WGS) to improve the understanding and evaluation of the observed biofilm results.

5.2 Materials and Methods

A brief overview of the methodology can be seen in Figure 5. A representative drain biofilm model was designed based on the characterization of floor drain microbiomes from three different FPEs by metagenomic sequencing and identification of representative isolates from the viable drain microbiota. This resulted in the selection of 31 whole genome sequenced isolates for the drain biofilm model (Figure 5a). For the testing of the effect of biocides, biofilms were formed (three days, 15 °C) on stainless steel coupons before treatments on day 3. Surviving biofilm bacteria were regrown (15 °C) for an additional three days before being treated on day 6 using the same biocide concentration (Figure 5b). The biofilm microbiomes of the initial biofilm (untreated, day 3) and regrown biofilms (day 6, three days after treatment with a biocide) were analyzed using 16S rRNA amplicon sequencing. Survivors were enumerated by plate counts and colonies identified using MALDI-TOF before and after the first and second biocide treatments (Figure 5).

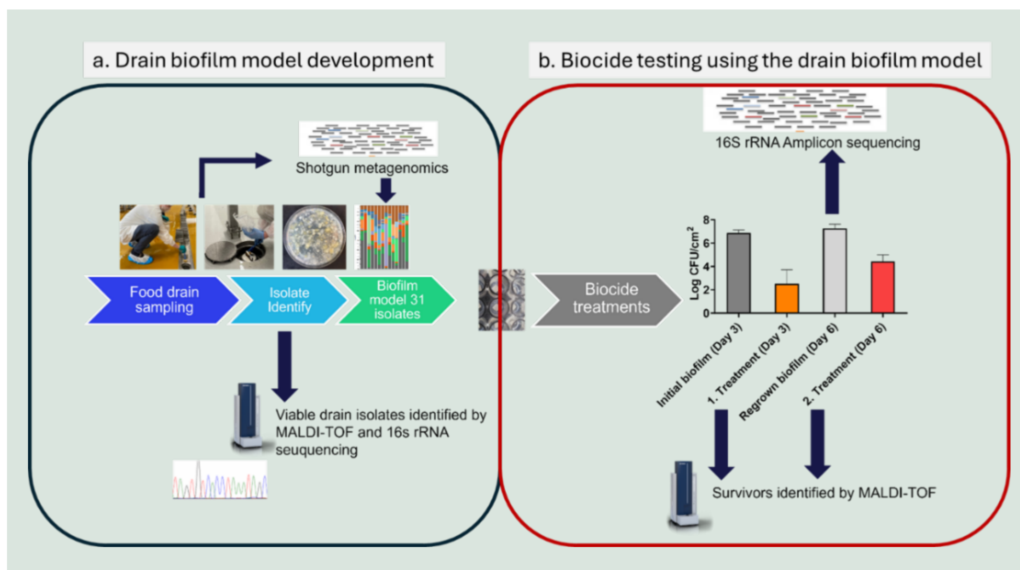


FIGURE 5. Methodology in brief, showing (a) the analysis and selection of representative isolates for the drain biofilm model and (b) the scheme for biocide testing using the drain biofilm model and ensuing analysis of the changes in the microbiome caused by the treatments. See section 5.2 for details.

Sampling of food production drains

Three food production sites were visited for sampling of drains within food production environments (Table 2). A cheese-producing company was visited two times with nine months between each visit, whilst a seafood company with separate roe and shrimp production facilities was visited once. Samples from drains (n=14) were taken using industrial surface sampling sponges pre-moistened with Dey/Engley neutralizing buffer (VWR, Søborg, Denmark). From each drain, at least triplicate samples were obtained from individual unique spots of 10 cm^2. Swaps were transported refrigerated (< 5 °C) in separate sterile plastic bags to the laboratory for analysis. Within 30 hours of sampling each sponge sample was mixed using a stomacher for 30 sec before the liquid (≈ 5-10 mL) from each sponge was squeezed into 15 mL tubes. Each sample was diluted in peptone saline (PS, 1g/L peptone, 8.5 g/L sodium chloride) and volumes of 100 µL from suitable dilutions were spread on Plate Count Agar (PCA) and incubated aerobically for six days at 15 °C for determination of the aerobic plate count (APC). The remaining sample (> 5 mL) was frozen at −80°C and used for extraction of DNA. After incubation, random colonies (n >15) were selected from PCA plates for each drain sample using a template while ensuring all different colony morphologies were included. Colonies were re-streaked on Tryptic Soy Agar (TSA, Merck, Darmstadt, Germany) plates and pure colonies were stored at −80°C in 20% (v/v) glycerol peptone medium (TS/80, Technical Service Consultants Ltd., Heywood, UK) and used for identification.

TABLE 2. Overview of drain samples from seafood and dairy production environments.

Drain ID	Food type	Year	Category	Drain placement
1	Fish roe	2022	Seafood	Raw roe mixing room
3	Fish roe	2022	Seafood	Raw roe glass filling
4	Fish roe	2022	Seafood	Ingredients mixing room
6	Fish roe	2022	Seafood	Raw fish roe separation
7	Shrimp	2022	Seafood	Shrimps on convoy belts (High-risk area)
8	Shrimp	2022	Seafood	Mixing of brine to shrimps (High risk area)
9	Shrimp	2022	Seafood	Small shrimp unpacking (Inlet to high-risk area)
10	Shrimp	2022	Seafood	Big shrimp unpacking (Inlet to high-risk area)
11	Cheese	2021	Dairy	Milk pasteurization room
12	Cheese	2021	Dairy	Cheese production area
13	Cheese	2021	Dairy	Smearing room
14	Cheese	2022	Dairy	Milk pasteurization room
15	Cheese	2022	Dairy	Cheese production area
16	Cheese	2022	Dairy	Smearing room

Culturomic characterization of the viable microbiota in floor drains

A total of 213 drain isolates were sought identified using matrix assisted laser desorption ionization – time-of-flight mass spectrometry (MALDI-TOF). Briefly for MALDI-TOF identification, protein extraction was done with fresh colonies from TSA plates (two days at 25 °C) following the ethanol/formic acid/acetonitrile protocol from Nonnemann et al. (2019) with the description by Bizzini et al. (2010). Isolates were analyzed in triplicates on a Sirius Biotyper (Bruker Daltonics, Bremen, Germany) where log scores >1.7 provided genus identification, while identification results below <1.7 were rejected. Unidentified isolates were further analyzed using Sanger sequencing of partial 16S rRNA genes. Isolate DNA was obtained using the boiling lysis method where a fresh colony from TSA plates was resuspended in 1 mL PS before centrifugation at 9.900 × g for 1 min. The supernatant was discarded and pellet resuspended in 200 µL nuclease-free water and boiled for 10 minutes before centrifugation at 9.900 × g for 5 min after which the supernatant with DNA was transferred to new tubes. Each 16S rRNA PCR reaction (50 µL) contained: 17 µL nuclease free water, 25 µL DreamTaq TM PCR Master Mix, 2 µL 10 µM forward primer (27F: AGAGTTTGATCCTGGCTCAG) 2 µL 10 µM reverse primer (1492F: GGTTACCTTGTTACGACTT) and 4 µL supernatant (DNA). The PCR was composed of an initial pre-denaturation step at 95°C for 10 min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 51°C for 60 sec and elongation at 72°C for 90 sec followed by a final elongation at 72°C for 7 min. PCR products were purified using the ExoSAP-IT™ PCR Product Cleanup Reagent (Applied Biosystems, Waltham, Massachusetts, USA) following the manufacturer's protocol. Aliquots of 5 µL purified PCR product and 5 µL primer (1492R, 5µM) were sent for Sanger sequencing at Eurofins Genomics (Konstanz, Germany). Results from the sequencing were blasted using Nucleotide BLAST at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to determine genus of each isolate. The culturomic results from identification using MALDI-TOF and 16S rRNA sequencing were combined to determine the abundance of each genus in the drains of the seafood and dairy food productions.

Determination of isolates' minimum inhibitory concentrations (MIC) towards biocides

A subset of drain isolates (n=73), representing 31 genera and the diversity of the viable drain microbiota were tested for their MIC to benzalkonium chloride (BC, 500 g/L, Thermo Fisher, Kandel, Germany), peracetic acid (PAA, Peroxyacetic acid, 35% w/v, Acros Organics BV, Geel, Belgium), sodium hypochlorite (SH, 10% w/v, Scharlau, Barcelona, Spain) and ethanol

(ET, 99.8% v/v, Acros Organics BV, Geel, Belgium). The protocol is described in detail in Kragh et al. (2024). In short, biocides were 2-fold diluted in ten times diluted (×0.1) Tryptic Soy Borth (TSB, Merck) as dilutant and inoculated using standardized cell suspension based on dilutions of cell cultures grown three days at 15°C in ×0.1 TSB. The test was conducted in a volume of 200 µL in 96-well polystyrene plates (In Vitro, Kratbjerg, Denmark) with an initial cell concentration of ≈2.5×10^5 CFU/mL and biocides in concentration ranges seen in Supplementary Figure S1. Plates were sealed and incubated for 72 h at 15 °C. Growth was measured at an absorbance of 620 nm (Multiskan FC, Thermo Fisher Scientific, Roskilde, Denmark) with a no-growth cut off defined as absorbance readings below two times the absorbance of the negative control (0.038). The MIC was defined as the lowest biocide concentration with no growth. MIC-values were tested in triplicates in two biologically independent experiments (n=6).

Metagenomic sequencing and analysis of drain swaps

Forty-two drain samples were thawed with triplicate samples from each drain being pooled (n=14) for DNA extraction. Briefly, each pooled drain sample of ≈15 mL was centrifuged at 7.400 × g to obtain a pellet which was resuspended in 200 µL of the remaining liquid in the tube and transferred to a DNeasy PowerBead tube from the Qiagen DNeasy PowerSoil Kit (Qiagen, Hilden, Germany). DNA extraction was carried out following manufacturer's protocol. Extracted DNA was quantified using Qubit 3.0 (Invitrogen, Carlsbad, CA, United States) with the Qubit DNA HS Assay Kit (Invitrogen). Due to low DNA concentrations (median = 0.6 ng/µl), DNA samples were whole genome amplified (WGA) using the REPLI-g UltraFast Mini Kit (Qiagen) following the manufacturer's protocol which relies on the Phi29 polymerase to obtain enough DNA to allow shotgun metagenomic sequencing. DNA purity was assessed using NP80 NanoPhotometer (Implen, Westlake Village, CA, USA). Amplified DNA samples of >200 ng with concentrations of >2 ng/µL were sent on ice to Eurofins Genomics for Illumina 150 bp paired-end sequencing. The raw metagenomics data was submitted to the NCBI Sequence Read Archive under BioProject: PRJNA1194757 (Supplementary Table S11). The raw sequencing data of the 14 drain samples were initially processed with CLC Genomics Workbench 23.0.4 (Qiagen, Aarhus, Denmark) to trim away adapters and low-quality reads with quality scores below 0.05. After trimming, 62.6-136.2 million reads remained for each sample with an average of 101.2 million reads. Taxonomic profiling of the filtered reads was performed with the CLC Microbial Genomics Module 23.0.2 (Qiagen) using the Qiagen Microbial Insights - Prokaryotic Taxonomy Genus Database (QMI-PTDB) as reference. This database is curated and based on genome sequences and annotations from the NCBI Reference Sequence Database (NCBI RefSeq) with annotations from the Genome Taxonomy Database (GTDB). Taxonomic classification results were filtered to remove non-complete taxonomic classifications and additional sequencing and WGA noise were removed by removing low abundance species classifications with less than 0.1% abundance combined across all 14 drain samples. The filtered taxonomic abundance tables were merged and used as input for calculations of chao1 bias corrected alpha diversity and beta-diversity using Bray-Curtis distance matrices in CLC Microbial Genomics Module. Beta-diversity PCoA plots from CLC were enhanced visually to improve dot, text and label size with Inkscape (inkscape.org). Permutational multivariate analysis of variance (PERMANOVA, 9999 permutations) using abundance tables was applied to check for significant differences in the beta-diversity between the drains from the different processing environments (dairy, shrimp and fish roe). Antibiotic and biocide resistance genes in the drains were identified using ShortBRED (Kaminski et al., 2015), which quantifies the abundance of resistance genes in the metagenomes by identifying unique peptide marker sequences in resistance genes and mapping reads to only those markers. The Qiagen Microbial Insight - Antimicrobial Resistance (QMI-AR) Peptide Marker Database was used for ShortBRED blast, identification and quantification as this is curated with peptide markers from all four major databases (CARD (Alcock et al., 2020), ARG-ANNOT (Gupta et al., 2014), NCBI Bacterial Antimicrobial Resistance Reference Gene Database (Feldgarden et al., 2021) and ResFinder (Bortolaia et al., 2020)). Resistance genes were included in results if identity was >95%, alignment length >95%, and read length ≥ 90 bp.

Whole genome sequencing and analysis of drain isolates

Thirty-one isolates (Table 3) were selected for whole genome sequencing (WGS) based on: 1) high abundance in the metagenomic drain data, 2) abundance in isolation in the viable microbiota of the drains, and 3) relevance in floor drains based on existing reviews of food processing microbiota. Isolates were grown two days in 4 ml TSB at room temperature (~20 °C) before centrifugation at 7.400 × g to obtain a pellet, which was resuspended in 200 µL of the remaining liquid in the tube and subsequently used for DNA extraction using the Qiagen DNeasy PowerSoil Kit (Qiagen) following the manufacturer's protocol. DNA from the 31 drain isolates were subjected to WGS by Illumina 150 bp paired-end sequencing (Eurofins Genomics). The raw sequencing data was processed with CLC Genomics Workbench 23.0.4 (Qiagen) including quality control and trimming of the sequencing reads before de novo assembly using default parameters. Assemblies were used for in silico species identification using KmerFinder v2.0 (Hasman et al., 2014; Larsen et al., 20214; Clausen et al., 2018) and The Microbial Genomes Atlas (MiGA) (Rodriguez-R, 2018). The *L. monocytogenes* isolate was in silico multilocus sequence typed (MLST) using BIGSdb-Lm (Moura et al., 2016). Assemblies of all drain isolates were analyzed using ResFinder 4.1 (Bortolaia et al., 2020) to identify resistance genes and for the presence of virulence and stress genes using VFanalyzer with the reference database for virulence factors of pathogenic bacteria (VFDB, Liu et al., 2019) using default parameters and a cut-off threshold of 90% identity and coverage. Assemblies were automatically annotated using Bakta Web (Schwengers et al., 2021) with the bacterial translation table and manually explored for genetic determinants implicated in biocide tolerance. Finally, the PLSDB web-tool (Galata et al., 2019; Schmartz et al., 2022), which is a comprehensive database including 34,513 (October 2023) bacterial plasmids, was used for a large-scale comparative analysis to detect presence of plasmids in the drain isolates. The PLSDB database was used with the Mash search setting (Ondov et al., 2016) with a default maximal p-value of 0.1 and minimum identity of 0.99. Any plasmid identification and additional identification of mobile elements were validated with results from MobileElementFinder (Johansson et al., 2021). The raw WGS data of the drain isolates were submitted to the NCBI Sequence Read Archive under BioProject: PRJNA1194757 (Supplementary Table S11).

Creation and repeated biocide treatment of a drain biofilm model

The 31 sequenced isolates were selected for the drain biofilm model based on the metagenomic drain abundance levels, the frequency in the viable drain microbiota and their relevance to mimic the drain microbiota. The drain biofilm model was created on food-grade stainless steel coupons (SS coupons, 0.5×0.5 cm, thickness of 1 mm, AISI 316, type 4 finish). The SS coupons were washed and prepared as previously described (Kragh et al., 2020). The isolates were cultured on TSA plates for one to three days (~20 °C) from stocks stored in 20% (v/v) glycerol peptone medium (TS/80, Technical Service Consultants Ltd Heywood, UK) at -80 °C. Three fresh colonies were used to inoculate each isolate individually in 4 mL TSB with incubation for two days at 15°C. Equal volumes (100 µL) of each isolate were mixed and diluted 3000× with TSB resulting in a final concentration of 10^6 CFU/mL. Aliquots of 500 µL of this inoculation mix was used to cover SS coupons placed in wells of a 48-well plate (BioLite 48 Well Multidish F-well, Thermo Fisher Scientific, Roskilde, Denmark). Biofilms were allowed to form during incubation for three days at 15°C with lids on before biocide treatments commenced. Working concentrations of biocides, i.e., BC (250, 1000, 2000 mg/L), SH (1000, 6000, 12000 mg/L), and PAA (500, 2500, 5000 mg/L), were made by dilution of freshly prepared stock solutions using sterile water. Each biocide was used to disinfect two SS coupons (n=2) with one of the three concentrations indicated above. In addition, two SS coupons (n=2) were treated with sterile water as control. Before application of biocide or sterile water, planktonic cells in suspension (~500 µL) surrounding the SS coupon were carefully removed by slow pipetting. Six hundred µL of biocide or sterile water were added to the SS coupons with an exposure time of 5 min following removal and 3× washing of both treated and non-treated (controls) SS coupons with 700 µL of PS. Following washing, each SS coupon was transferred

carefully to microcentrifuge tubes with 1000 µL PS. Sessile biofilm cells were released using sonication with 50/60 kHz in a 1000 W sonication bath (Elmasonic S 120, Thermo Fisher Scientific) for 5 min to allow for enumeration of surviving biofilm cell concentrations and detect survival of *L. monocytogenes*. Enumeration of cell concentrations were assessed by 10-fold dilutions of samples in PS with spread plating (100 µL) on TSA, counting after four days incubation at 15 °C and expressing as log CFU/cm^2 for biofilm samples. Survival of *L. monocytogenes* in the biofilm was assessed by inoculation of 10-fold diluted sample volumes of 100 µL in Half-Frazer broth (48 h, 37°C, Oxoid, ThermoFisher) before plating on PALCAM agar (48 h, 37°C, Oxoid, ThermoFisher). The limit of detection for survival of *L. monocytogenes* was 10 CFU/SS coupon. To allow repeated biocide treatments to occur on day six, all biocide treatments on day three were done on a total of six SS coupons of which two were sacrificed for day three analyses. The four remaining SS coupons were allowed to regrow and form new biofilms by adding 500 µL of fresh TSB to each well after the last washing step followed by incubation for three days at 15°C. For each of the regrown biofilms, two SS coupons were treated with water to assess the regrown biofilm cell concentration, whereas the two other SS coupons were treated with the same biocide concentration as in the day three treatment.
All biocide treatments and controls were done in duplicates with the entire experiment repeated twice. To determine the efficacy of applying the same biocide concentration on initial and regrown biofilms, the sessile biofilm cell concentrations were used to calculate the log reductions (log CFU/cm^2) after the first (day 3) and second (day 6) treatments.

MALDI-TOF identification of the surviving drain microbiota
To determine which bacteria survived the biocide treatments, MALDI-TOF was used for a growth-dependent identification of surviving bacteria as described above. Each of the drain biofilm model isolates (Table 3) was manually added to a local MALDI-TOF database for improved genus and species identification based on three independent spectra. For assessment of the identity of the most abundant genera of surviving biofilm cells following biocide treatments, five isolates were picked randomly using a template on top of the TSA plates where the most diluted biofilm samples had been spread following treatment with the highest biocide concentration (BC = 2.000 mg/L, SH = 12.000 mg/L, PAA =5000 mg/L). As each treatment was repeated four times (duplicates in two independent trials) on each sampling day, this resulted in the isolation of twenty colonies from each treatment (Control (sterile water), PAA, BC, SH) on days three and six, respectively, totalling 160 colonies for MALDI-TOF identification using the local custom database and MALDI-TOF scores as described above for species or genus identification.

Culture-independent analysis of the regrown biofilm
The remaining liquid (800 µL) from the sonicated samples (se above) containing the sessile biofilm cells was used as input for 16S rRNA amplicon sequencing to determine composition of the microbiota of the regrown biofilms after the biocide treatments (BC, PAA, SH at three concentrations each) and controls (sterile water after three and six days for the initial and regrown control biofilms, respectively). DNA was harvested by centrifuging the 800 µL (9.900 × g) and resuspending the pellet in 200 µL of the remaining liquid in the tube for DNA extraction (Qiagen DNeasy PowerSoil Kit). All biological duplicates from both biological independent experiments (n=4) were used for individual DNA extractions. The V3-V4 16S rRNA region of the sample DNA was amplified and sequenced using Illumina MiSeq 300 bp paired-end sequencing (Eurofins Genomics). The quality control and trimming of the raw sequencing data of the biofilm samples were processed with CLC Genomics Workbench 23.0.4 (Qiagen) as described above. After trimming 106.2-204.1 thousand reads remained for each sample with an average of 105.4 thousand reads. Taxonomic profiling of the filtered reads was performed with the CLC Microbial Genomics Module 23.0.2 (Qiagen) using The SILVA ribosomal RNA database (99%) reference database (Glöckner 2017) for operational taxonomic unit (OTU) clustering. Taxonomic classification results were filtered to remove non-complete taxonomic classifications and

sequencing noise by removal of OTUs with less than 1000 reads across all samples. Taxonomic classification was further manually curated after filtration to merge identical taxonomic groups. This was done *for Stenotrophomonas, Yersinia, Pseudomonas,* and *Aeromonas* (Supplementary Table S8). In addition, sequences from non-complete taxonomic classifications were blasted using blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) before merging if more than one group of bacteria belonged to same taxa. This was done for an incomplete classification of Enterobacteriaceae and Micrococcaecea, whose sequences were identified as the genera of *Yersinia* and *Rothia*, respectively. The final relative abundances were visualized as a heatmap using GraphPad Prism 10 (GraphPad Software, Boston, MA, USA). Principal component analysis was performed in GraphPad Prism 10 (GraphPad Software) to identify the effect of biocides on the microbiome composition using the relative abundance levels of each genus in samples. PERMANOVA analyses were conducted in CLC Microbial Genomics Module 23.0.2 (Qiagen) using the relative abundance tables to check for significant differences in the beta-diversity between the different treatments of the biofilm. Differential abundance analysis was also conducted using the sterile water treatment (day 6) as control to compare the fold changes caused to the abundance of each genus of isolates by the different biocide treatments. The fold changes were expressed as the mean of the $\log_2$ fold change ($\log_2$ FC) for each biocide.

Statistical analysis and data visualisation
The Student's T-test was done using Microsoft Excel (Version 2307) at a significance level of $p < 0.05$ to compare log-transformed microbial counts (log CFU/cm^2). This was done for the cell concentrations of the drain swaps to test for differences between food production types. In addition, T-tests were done for samples of the biofilm model to compare the cell concentrations and log-reductions obtained after the first and second biocide treatments.
Metagenomic and 16S rRNA amplicon sequencing data analysis and statistical comparisons were done in CLC Genomics using the Microbial Genomics Module 23.0.2 (Qiagen) with significance evaluated using Bonferroni corrected P-values ($P_{corrected}$) to correct for multiple-comparison and visualized in GraphPad Prism 10.

5.3 Results

The viable microbiota of floor drains
Samples from fourteen floor drains from seafood (fish roe and shrimp) and dairy (cheese) processing facilities were used for a culturomic characterization of the microbiota in floor drains. Forty different genera were identified from the drains based on genus identification of 213 bacterial isolates with 22 genera having an isolation frequency > 2% (Figure 6A, Supplementary Table S1). A comparison of the microbiota of seafood drains and dairy drains found 14 genera to be present in both environments (Figure 6A). The viable microbiota of seafood drains was dominated by Pseudomonas (16%) followed by *Flavobacterium* (9%), *Microbacterium* (9%), *Brevundimonas* (8%) and *Rhodococcus* (8%). For dairy drains, *Serratia* (15%) dominated the drains, together with *Chryseobacterium* (14%) and *Pseudomonas* (8%). Cumulative frequencies across the FPEs showed *Pseudomonas* (24%), *Chryseobacterium* (20%), *Serratia* (15%), *Microbacterium* (15%), *Acinetobacter* (12%), *Rhodococcus* (12%) and *Brevundimonas* (12%) to be highly present, however, with *Serratia* solely isolated from dairy drains (Figure 6A). Two isolates of *Listeria* were isolated from drains in the seafood processing environment producing pasteurized fish roe. The mean aerobic plate count of the seafood drains was 3.9 ± 1.4 log CFU/cm^2 with no significant ($P > 0.05$) difference to dairy drains concentrations of 4.7 ± 0.8 log CFU/cm^2. The APC ranged among the fourteen drains from 2 – 6 log CFU/cm^2 (Figure 6B).

Testing of the general biocide tolerance level in the viable drain microbiome as represented by 87 isolates belonging to 31 genera showed variability in tolerance towards commonly used biocides. This was especially clear for BC where a >30-fold diversity in MIC was seen with 18 isolates with MICs above 5 mg/L of which seven isolates recorded MIC values ≥ 20 mg/L (Supplementary Figure S1, Supplementary Table S2). For SH, PAA and ET, more than half of

the isolates had identical MIC values with ≤8-fold differences in tolerance between the least and most sensitive.

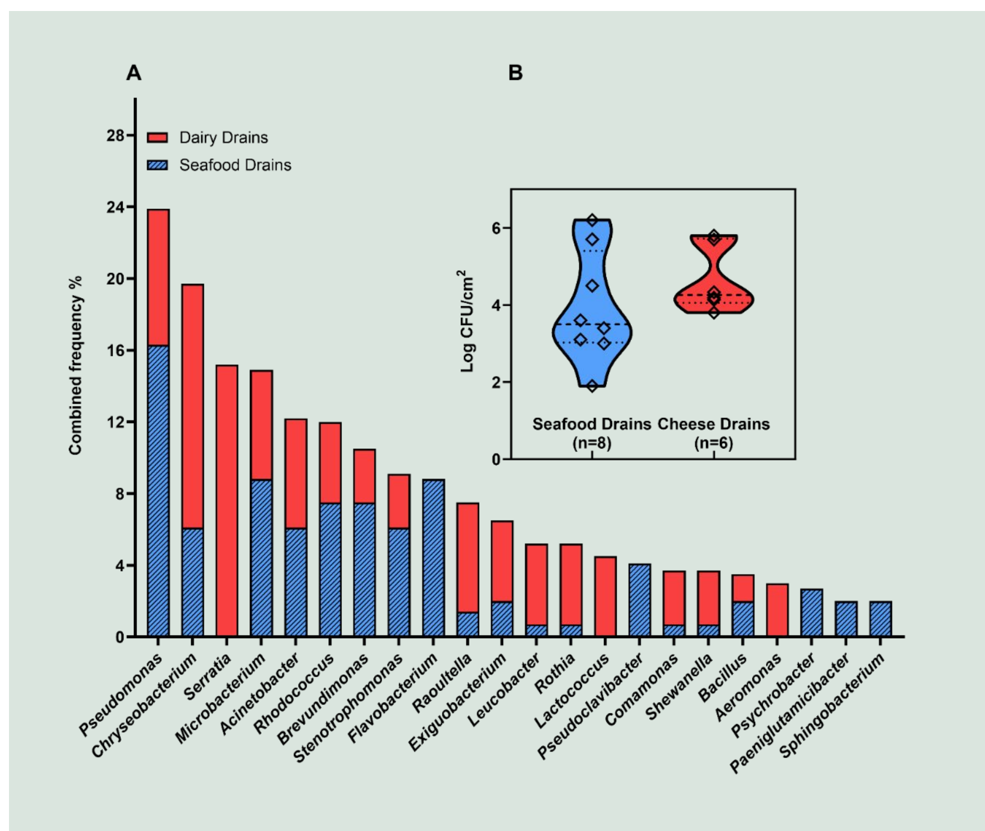


FIGURE 6. Diversity of the viable microbiota of floor drains: A) Frequency of isolation of specific genera as determined by identification by MALDI-TOF and 16S rRNA Sanger sequencing; B) the aerobic plate counts in floor drain samples. Drain isolates and drain cell concentrations were obtained by plating samples on TSA plates (6 d at 15°C).

Taxonomic profiling of the core metagenome of drains across food production types
Shotgun metagenomic sequencing of the 14 drains produced 62.6-136.2 million paired-end high-quality reads per sample with an average of 101.2 million reads. A high proportion of reads could not be classified to any taxa, while an average of 24.2 million paired-end reads were assigned to the domain of Bacteria. After filtering and removal of extremely low abundant species, a total of 665 species, 274 genera and 89 families were identified across the drains. The differences in alpha diversity based on chao1 bias-corrected richness between the food production environments were not significant (Kruskal-Wallis $P > 0.05$) at neither species, genus nor family levels (Figure 7A, B and C). The beta-diversity based on Bray-Curtis dissimilarity did not clearly separate the drain metagenomes at family or genus level (Figure 7D and E). At genus level, the taxonomical abundances caused significant differences in the metagenome between shrimp and cheese drains ($P_{corrected} < 0.05$) (Figure 7D, supplementary Table S4). The beta-diversity at the family level was in contrast not significantly different among drains from the three production types ($P_{corrected} > 0.05$) (Figure 7E, Supplementary Table S3). When grouped as either dairy or seafood production drains the difference in the metagenomes was significant ($P_{corrected} < 0.05$) at genus level. At both genus and family levels, the variation and complexity of the drain metagenomes were, however, apparent from the low PCoAs (Figure 7DE). This was additionally seen when the most abundant families from each drain were evaluated based on relative abundance (Figure 7F, supplementary Table S3), as differences

among some drains were obvious with e.g., drain 4 and drain 10 dominated by Selenomonadaceae and Caulobacteraceae, respectively, which were not dominating taxa elsewhere. Common for most drains was the high abundance of Pseudomonadaceae with an average of 17% ± 17% across all drains resulting in this family being one of the three dominating families in 8 of 14 drains along with Enterobacteriaceae (9% ± 9%) and Moraxellaceae (9% ± 11%) (Supplementary Table S3). Common for all drains was that despite the high diversity and complexity more than half of the total drain metagenome across all three processing environments could be summarized from the abundance of the six most abundant families which contain Gram-negative species (Pseudomonadaceae. Enterobacteriaceae, Moraxellaceae, Caulobacteraceae, Weeksellaceae and Burkholderiaceae), similarly the 20 most abundant families (Figure 7F) accounted for 84% of the total drain metagenome. The dominance of Gram-negative bacteria was also evident at phylum level with Pseudomonadota (former Proteobacteria) accounting for 80, 68, and 70% of the drain metagenomes in fish roe, shrimp and cheese drains, respectively (Supplementary Figure S3). Together with Pseudomonadota, Bacteroidota, Actinomycetota (formerly known as Actinobacteria), Bacillota (formerly known as Firmicutes) and Deinococcota made up > 95% of the drain bacterial metagenomes.

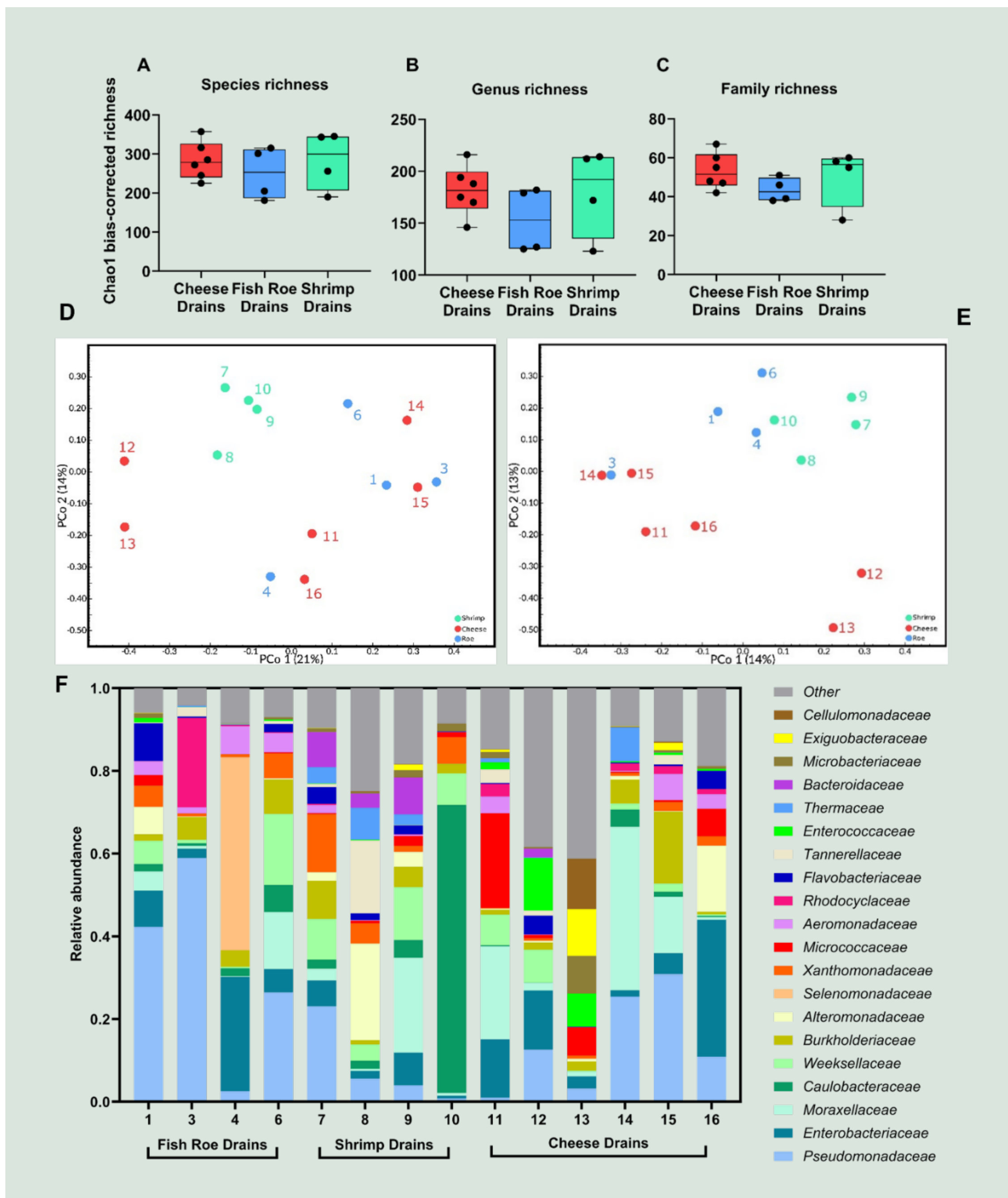


FIGURE 7. Microbial diversity and relative abundance of drain metagenomes in 14 drains from fish roe (n=4), shrimp (n=4) and cheese (n=6) processing environments. Comparison of the alpha-diversity based on chao1 bias corrected richness at A) species, B) genus and C) family taxonomic level. Beta-diversity based on Bray-Curtis dissimilarity on D) genus- and E) family-based taxonomy. F) Relative abundance of families in the 14 drains, with the three most abundant families included from each drain, resulting in 20 families included.

A total of 274 genera were present at a combined relative abundance greater than 0.1% across all drain samples after merging of identical genera and removal of incomplete taxonomic designations. Of these, a total of 131 genera were present at a cumulated relative abundance greater than 1% and 30 genera at a cumulated relative abundance greater than 10% across all drain samples (Supplementary Table S4). Of the dominating genera, 36 were present at least once at relative abundances greater than 5% and of these 22 genera were on average present at a relative abundance greater than 1% (Figure 8). Interestingly only four of these dominating genera (*Pseudomonas* (18.0%), *Brevundimonas* (6.7%), *Acinetobacter*

(4.2%) and *Stenotrophomonas* (1.8%) were present in all 14 drains with an average relative abundance as reported, while *Moraxella* (2.5%), *Aeromonas* (2.4%), *Psychrobacter* (2.5%), *Enterococcus* (1.6%) and *Escherichia* (1.4%) were present in 13 drains. *Propionispira* were found in the fish roe drain (Drain 4) in the room for mixing of raw ingredients done separately from raw fish roe processing and although abundant in that sample it was not reflected in the overall drain metagenomes. Similarly, the dominance of Caulobacteraceae in shrimp drain 10 was reflected in the high abundance of *Brevundimonas*, which were, however, widely present in other drains across all three FPEs (Figure 8).

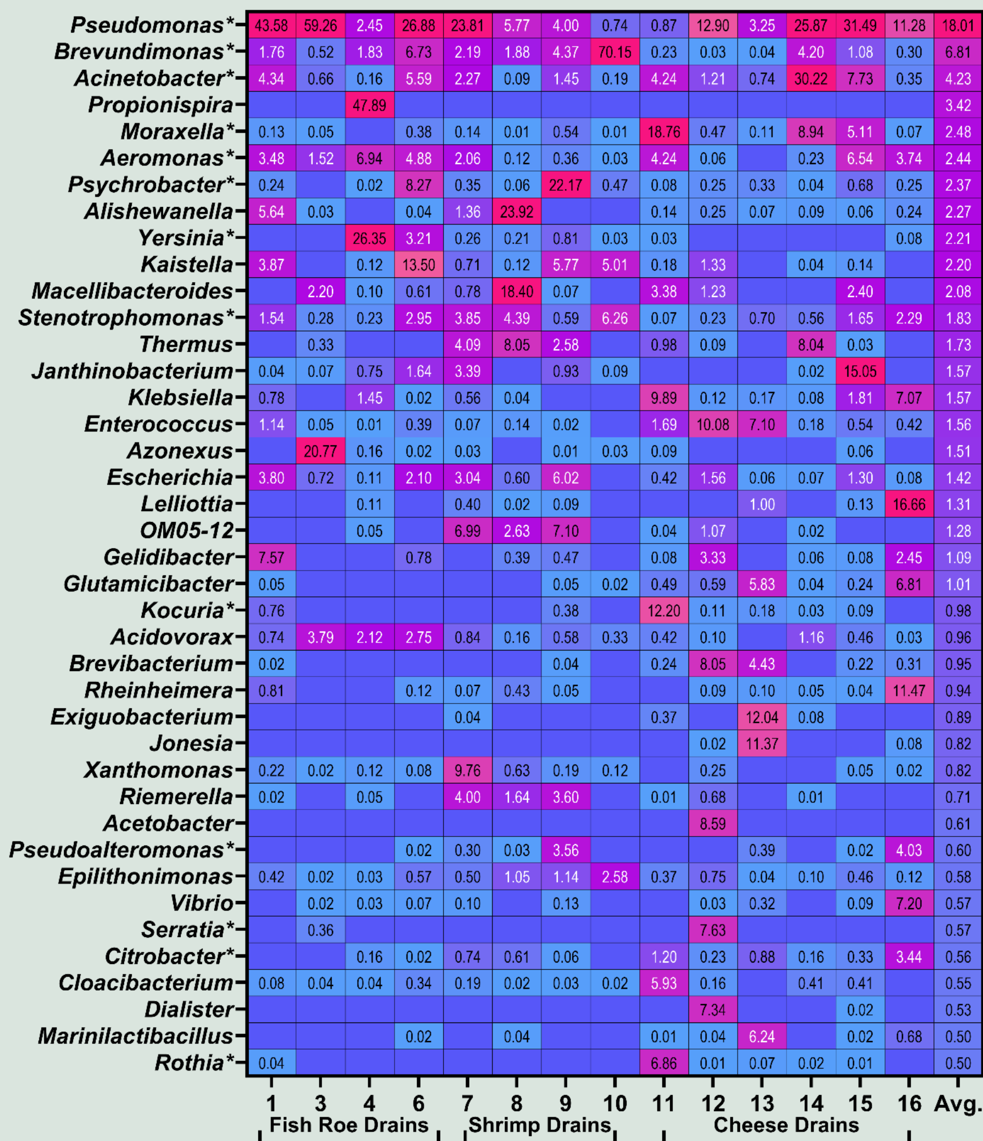


FIGURE 8. Heatmap visualizing 40 most abundant genera by highest average (Avg.) relative abundance (%) identified in the metagenomes of floor drains. Boxes without values are the result of the genera not being identified in the specific drain. Genera marked by * (n=13) are included in the biofilm drain model. Relative abundances for all identified 274 genera across all drains can be seen in Supplementary Table S4.

Prevalence of antibiotic resistance gene markers in the resistome of floor drains

A total of 699 unique antibiotic resistance gene (ARG) markers were identified using ShortBRED in the metagenome of floor drains (Supplementary Table S5). The identified genes belonged to 18 different antibiotic classes (CARD Ontology) with 259 genes linked to beta-lactam resistances as the most frequently observed ARG resistance markers (Figure 9A, Supplementary Table S5). However, the most abundant group based on normalization using Reads Per Kilobase per Million mapped reads (RPKM) were 119 markers belonging to the multi-resistance and undefined group of 'antibiotic molecules' (Figure 9A). Of these 119 markers, the majority (102) and those with highest RPKM were efflux pump complexes such as *mdtO*, *smeE* and *mexB*. Other highly abundant efflux pumps included *qacG*, *qacJ* and *qacL*, which are all related to quaternary ammonium compound (QAC) resistance and belong to the ARG class of "disinfecting agents and antiseptics" (Figure 9A). QAC resistance genes were identified in drains from all three FPEs but were most abundant in cheese drains (Figure 9B). Other QAC resistance genes identified included *mdfA*, *qacE*, *qacH* and *sdrM*. In general, the efflux pump-mediated resistance mechanism dominated along with resistance markers related to antibiotic enzyme inactivation which was driven by a high abundance of genes conferring resistance towards aminoglycosides *(ant(3"), aph(6)-Id, aph(3")-Ib)* and beta-lactams (bla_{TEM}, bla_{OXA}, bla_{MOX}). (Figure 9, Supplementary Tables S5, S6 and S7). For the overall resistome, there were no significant differences among shrimp, fish roe and cheese drains ($P_{corrected}$ = 0.085). Similarly, the difference between the resistome of shrimp and fish roe drains was not significant ($P_{corrected}$ = 0.77). However, a much lower overall ARG marker abundance was seen in shrimp drains.

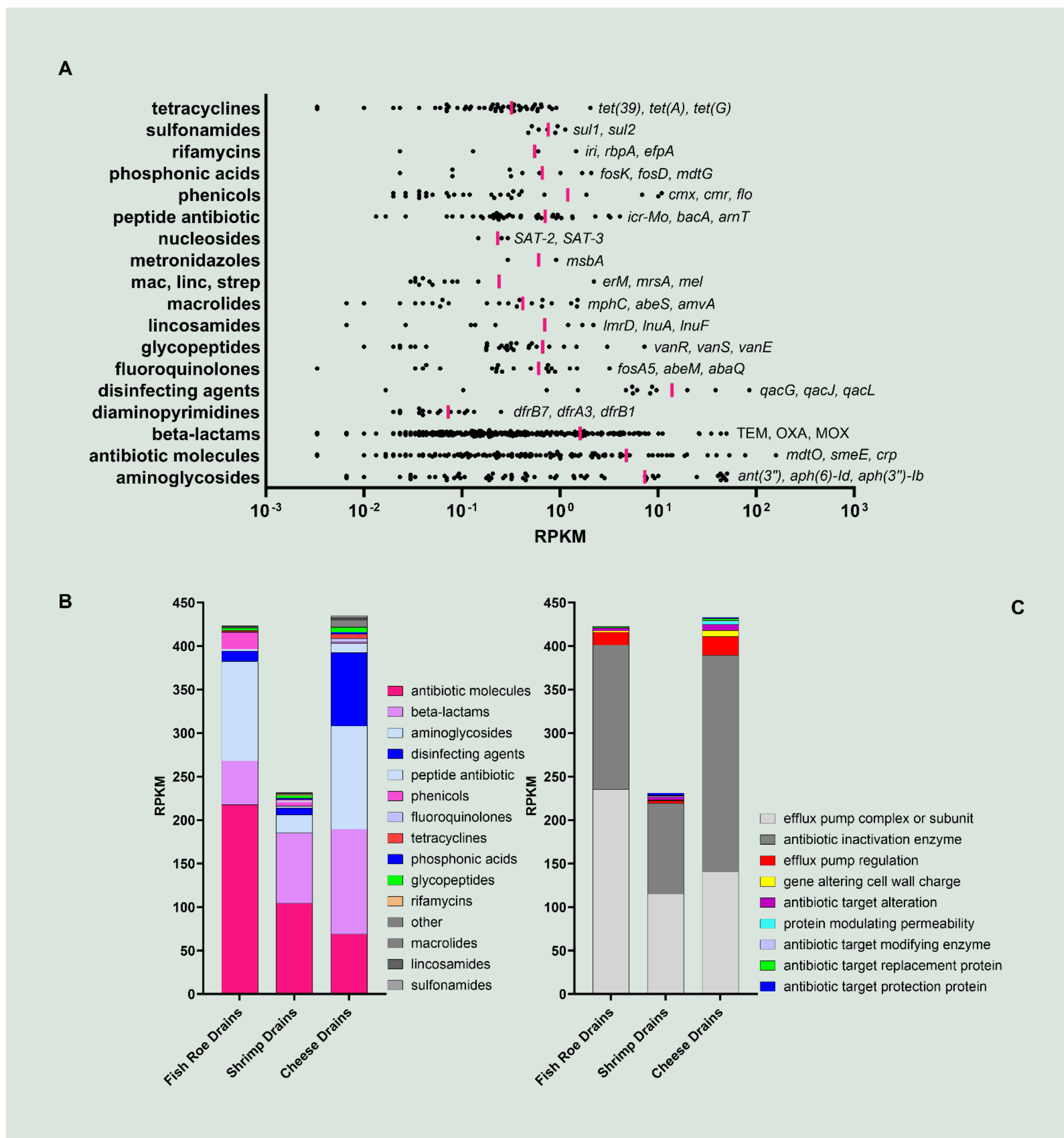


FIGURE 9. Resistome identification and normalized abundance (Reads Per Kilobase per Million mapped reads (RPKM)) of ARG markers in floor drains using ShortBred. A) The abundance of ARGs (•) to specific antibiotic drug classes with vertical pink lines marking mean RPKM for all markers in each drug class. The gene names for the three most abundant ARGs are listed for each drug class. Bar graphs showing the B) normalized abundance is summarized by drug class and (C) resistance mechanism for each drain.

Selection and analysis of representative isolates for the creation of a drain biofilm model

To study how the drain microbiota is affected by repeated biocide treatments, a multi-genera biofilm drain model was created to reflect the observed microbiome diversity of the studied food industries. Therefore, based on the metagenomic abundance and isolation frequency across the food facilities five isolates of *Pseudomonas* (*P. cremoris, P. migulae, P. anguilliseptica, P. fluorescens* and *P. chengduensis*) were included along with *Acinetobacter* (*A. guillouiae* and *A. johnsonii*) and two isolates of *Brevundimonas* (*B. vesicularis*). In addition, two isolates of *Chryseobacterium* (*C. scophthalmu*m and *C. haifense*) were included as second most abundant viable genera, while ranked as the 49th most abundant genera in the metagenomic data (Figure 6, Supplementary Table S4). Similarly, *Microbacterium* (*M. liquefaciens*)

was included due to its high frequency in the viable microbiota, while ranked 58th of the metagenomically most abundant genera. Eleven more isolates (*Moraxella osloensis*, *Aeromonas media*, *Psychrobacter alimentarius*, *Yersinia aldovae*, *Stenotrophomonas rhizophila*, *Kocuria rhizophila*, *Pseudoalteromonas translucida*, *Serratia marcescens*, *Citrobacter portucalensis*, *Rothia amarae* and *Pseudoclavibacter helvolus*) were included based on presence in the viable drain microbiota and a place among the fifty most abundant genera in the metagenome. *Flavobacterium* (*F. frigoritolerans*) were included as the second most viable abundant genera in seafood drains and identified in four of six metagenomes of cheese drains. Four isolates were included based on frequent drain isolation in other studies (Fox et al., 2014; Rodriguez-Lopez et al., 2019; 2020) combined with either co-isolation from dairy and seafood drains in present study (*Leucobacter luti*, *Rhodococcus qingshengii*, and *Shewanella oncorhynchi*) or high abundance in shrimps (*Carnobacterium*, Unpublished). In contrast, *Myroides*, and *Listeria* isolated from drains in the present study were included as model organisms based on their ability to be either multidrug-resistant (*Myroides* spp., Licker et al., 2017; Lorenzin et al., 2018) or persistent in food production environments (*L. monocytogenes*, Belias et al., 2022; EFSA, 2024). The included *L. monocytogenes* was typed as ST121. Lastly, an isolate of *Sphingobacterium* (*S. faecium*) was included due to its frequency in seafood drains and based on reports of some isolates' ability to degrade pollutants such as chlorinated pesticides and hence of interest in the context of repeated biocide treatments of a multi-species biofilm model (Abraham et al., 2013; Satti et al., 2021).

The final 31 isolates from 24 genera collectively represented 58%, 53% and 47% of the microbial diversity seen in the metagenome of fish roe, shrimp and cheese drains, respectively (Supplementary Table S8). Similarly, 76% and 81% of the viable dairy and seafood drain microbiota are reflected by the included isolates in this model (Supplementary Table S1).
Of the 31 isolates, nine possessed ARGs mainly towards beta-lactams with *C. portucalensis*, *Se. marcescens*, *A. guillotine* and *Sh. oncorhynchi* harbouring ARGs to more than one drug class (Table 3). QAC resistance efflux pumps *sugE*, *oqxB* and *qacH* were seen in *C. portucalensis, Se. marcescens* and *L. monocytogenes*, respectively. The *qacH* found on the *L. monocytogenes* Tn6188 transposon was, together with the heat resistance gene *clpL* on plasmid *L. monocytogenes* plm5578, the only resistance genes identified on mobile genetic elements among the genomes of the 31 isolates. In addition, *P. alimentarius, Se. marcescens, R. qingshengii, C. portucalensis*, and *A. media* carried plasmids, but none contained ARGs.

TABLE 3. List of drain biofilm model isolates selected to represent the core viable microbiota and metagenome of floor drains in seafood and dairy processing environments. GMR: Genus metagenomic ranking based on their relative abundance among the 274 genera identified in the drains (Appendix: Supplementary Table S4). (-) Genera detected but below filtering limit. A full list of identified mobile genetic elements, including insertions sequences (IS), can be seen in Supplementary Table S9).

	GMR	Source	Mobile elements	Resistance genes
Pseudomonas anguilliseptica C97	1	Seafood	Tn5501	
Pseudomonas chengduensis T33	1	Dairy		
Pseudomonas cremoris C118	1	Seafood	Tn5501 + Multiple IS	
Pseudomonas fluorescens T31	1	Dairy	ISPpu14	
Pseudomonas migulae C88	1	Seafood	Tn5501 + Multiple IS	*aph(3'')-Ib*
Brevundimonas vesicularis T3	2	Dairy		
Brevundimonas vesicularis C164	2	Seafood		
Acinetobacter guillouiae C42	3	Seafood		*aph(3')-VI, bla*$_{OXA-274}$

Acinetobacter johnsonii T53	3	Dairy		$bla_{OXA-334}$
Moraxella osloensis T34	5	Dairy		
Aeromonas media T12	6	Dairy	pfekpn2511-4	$bla_{OXA-427}$ & bla_{CMY-8b}
Psychrobacter alimentarius C147	7	Seafood	pVB11737_6	
Yersinia aldovae C62	9	Seafood	MITEYpe1, IS5075	
Stenotrophomonas rhizophila C2	12	Seafood		
Kocuria rhizophila C38	23	Seafood		
Pseudoalteromonas translucida LU10	32	Shrimp		
Serratia marcescens T40	35	Dairy	pSM22	*aac(6')-Ic blaSRT-2, OqxB, tet(41)*
Citrobacter portucalensis T5	36	Dairy	pKPC-CAV1321	*qnrB9*, bla_{CMY-34}, *sugE*
Rothia amarae T2	40	Dairy	Multiple IS	
Pseudoclavibacter helvolus C31	48	Seafood		
Chryseobacterium haifense T23	49	Dairy		
Chryseobacterium scophthalmum C1	49	Seafood		
Flavobacterium frigoritolerans C105	52	Seafood		
Microbacterium liquefaciens C25	58	Seafood		
Leucobacter luti T63	67	Dairy	Multiple IS	
Carnobacterium iners MU23	94	Shrimp		
Sphingobacterium faecium C58	112	Seafood		
Myroides odoratus T56B	220	Dairy		*blaTUS-1*
Rhodococcus qingshengii C133	225	Seafood	pR85A	
Listeria monocytogenes C16	(-)	Seafood	plm5578, Tn6188	*fosX qacH*
Shewanella oncorhynchi C102	(-)	Seafood	Multiple IS	*mcr-4.3*, $bla_{OXA-549}$

Repeated biocide treatments of the drain biofilm model

The 31 isolates were mixed to a starting concentration of 10^6 CFU/mL, which resulted in an initial biofilm on the SS coupon of 6.9 ± 0.3 log CFU/cm^2 after three days at 15 °C (Figure 10A). The suspension of planktonic cells around the SS coupons with the initial biofilm had a concentration of 9.2 ± 0.2 log CFU/mL. BC treatments at low, high or double industrial concentrations (250, 1000, 2000 mg/L) were unable to reduce the biofilm by more than 2 log CFU/cm^2. Correspondingly low reductions (< 2 log CFU/cm^2) of the biofilm were also the result of treatments with low industrial concentrations of SH (1000 mg/L) and PAA (500 mg/L). Conversely, higher concentrations of both SH (6000-12000 mg/L) and PAA (2500-5000 mg/L) reduced the biofilm by 3.4-4.5 log CFU/cm^2 with the greatest effect achieved at the highest concentrations for both biocides (Figure 10A). After each biocide treatment, surviving biofilms regrew to cell concentrations of 7.3± 0.6 log CFU/cm^2 after three days at 15 °C, which were in all but one case higher than in the initial biofilm. For controls treated with water, the regrown biofilms reached a concentration of 7.0 ± 0.6 log CFU/cm^2 similar to the initially formed biofilm. The cell concentrations in regrown biofilms tended to be highest for those treated with the lowest biocide concentrations, as seen in a 0.7 log CFU/cm^2 difference between lowest and highest concentrations of BC and PAA. No significant (P >0.05) changes in log-reductions between first and second biocide treatment were seen despite three of nine regrown biofilms showing increase tolerance to the repeated biocide treatment (6000-12000 mg/L SH and 2500 mg/L PAA), while one regrown biofilm exhibited increased sensitivity to the 1000 mg/L SH treatment (Figure 10A).

The biocide treatments only inactivated *L. monocytogenes* below the limit of detection (10 CFU/SS coupons) at the highest concentration of PAA and only after the second treatment

(Figure 10B). In total 52 of 80 treated biofilms samples (65%) were positive for *L. monocytogenes*. There was, however, no increase in the number of positive samples between the first and second treatments, as 72.5% of the samples were positive after treatment on day three, while 57.5% were positive after treatment on day six. In addition, increases in biocide concentrations always resulted in fewer *L. monocytogenes* positive samples (Figure 10B).

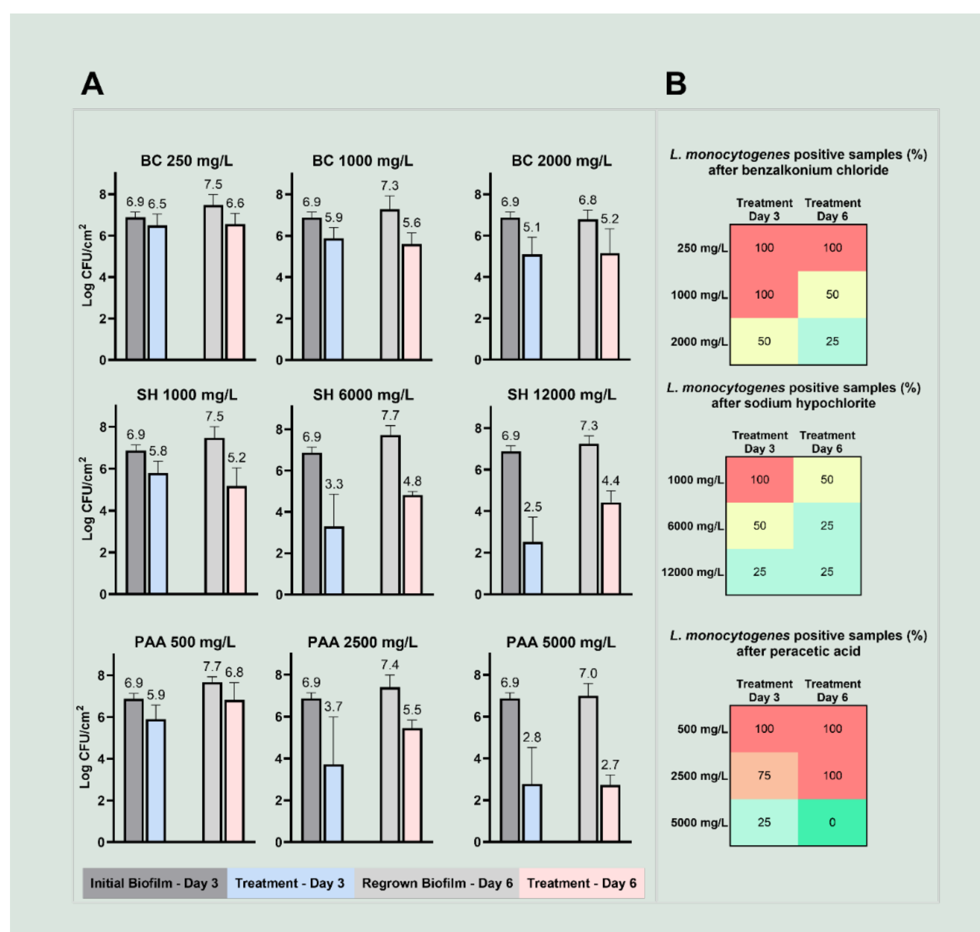


FIGURE 10. Biocide treatment of drain biofilms formed on SS coupons A) The mean cell concentrations in the biofilm (n=4) before and after biocide treatment with different concentrations of biocides (BC, SH and PAA) on days three and six with a biofilm regrowing phase between. B) The percentage of *L. monocytogenes* positive drain biofilms after treatment with biocides and selective enrichment for *L. monocytogenes* after each treatment (n=4).

Changes in the drain biofilm microbiome in regrown biofilms following biocide treatment

Culture-independent 16s rRNA amplicon sequencing identified reads mapping to 23 of the 24 genera, which were in the inoculum suspensions used for biofilm startup. Reads mapping to *Microbacterium liquefaciens* were not identified. Similarly, *Pseudoalteromonas* and *Rhodococcus* were not found in the initial biofilm (day 3) formed on the SS coupons, leaving the biofilm consisting of 28 species belonging to 21 genera with low relative abundance (>2%) of 11 genera (Figure 11A). This initial biofilm was dominated by Citrobacter (18.9%) and *Aeromonas* (18.8%), followed by *Serratia* (10.8%), *Shewanella* (7.9%) and *Pseudomonas* (7.6%) (Figure 11A, Supplementary Table S10). Following treatment with water, the diversity of the regrown biofilms (day 6) did not decrease as 12 genera had >2% in relative abundances, however, the

abundances shifted to higher content of *Myroides* (18.1%) and *Acinetobacter* (16.4%) accompanied by *Citrobacter* (14.4%) and *Aeromonas* (11.6%). The impact of the three biocides on genera diversity was biggest for BC, where increasing BC concentrations reduced the number of genera with relative abundances exceeding 2% from 12 (H_2O control) to 9, 7, and 4 for 250, 1000 and 2500 mg/L BC treatments, respectively. For increasing concentrations of SH and PAA, the selective pressure on genera was less pronounced as the number of genera >2% in relative abundance was reduced from 12 in the control to 10, 8, and 8 genera and 11, 11, and 8 genera for various concentrations of SH and PAA, respectively (Figure 11A). Specifically, the treatment with BC led to significant ($P < 0.05$) changes within the overall drain biofilm microbiome as *Serratia*, *Moraxella* and *Citrobacter* made up 57% of the regrown biofilm even after low treatments with 250 mg/L BC, while *Serratia* alone constituted 55-63% of the regrown drain biofilm microbiome after treatments with higher industrial concentrations (1000-2000 mg/L) of BC. This change was obvious in beta-diversity analysis, where regrown biofilms treated with the highest concentrations of BC clustered far apart from regrown biofilms after sterile water, PAA and SH treatments (Figure 11B). In the beta-diversity analysis, the initial biofilm clustered separately (Figure 11B), which could be explained by several genera, e.g., *Brevundimonas*, *Chryseobacterium*, *Pseudoclavibacter*, *Psychrobacter* and *Sphingobacterium*, being detected in low abundances (≤ 0.1%), but not in the water treated control biofilm from day 6. These biofilms treated with water clustered near biofilms regrown after PAA and SH treatments (Figure 11B), which mostly contained *Citrobacter*, *Acinetobacter*, and *Aeromonas*, but also, to a higher degree, *Pseudomonas* (Figure 11AB). These four genera also largely explain the difference in biofilms after BC treatment with PC loadings directly opposite to BC biofilms (Figure 11B).

Interestingly, *Pseudomonas* spp., which were represented by five different species in the inoculum (14.8% relative abundance), were not seen with high abundance in either the initial biofilm (7.6%), water control (3.1%) or after BC treatments (2.9-4.8%). The overall changes in the regrown biofilm microbiome after SH or PAA treatments were not significantly different ($P_{corrected} > 0.05$) compared to when treated with water. In addition, regrown PAA and SH treated biofilms clustered together in the PCA (Figure 11B) irrespectively of biocide concentrations, which also reflected the relative abundance increases and decreases of several genera (e.g., *Acinetobacter* and *Aeromonas* for both PAA and SH) not following the changes in biocide concentrations (Figure 11A). For *Citrobacter* and *Pseudomonas*, there was a slight decrease in their dominance with increased concentrations of SH and PAA, respectively. The relative increase of *Pseudomonas* after PAA and SH treatments and the increase of *Serratia* after BC represented the most significant ($P_{corrected} < 0.05$) changes in relative abundances when all concentrations of the same biocide (n=12) were compared against the water control (n=4) as seen in Supplementary Figure S2. Interestingly, *L. monocytogenes* recorded its highest relative abundance in the inoculum (4.7%) and in the initial biofilm (0.7%) and were never above these percentages in any of the regrown biofilms, but detectable in all biofilms (Figure 11A).

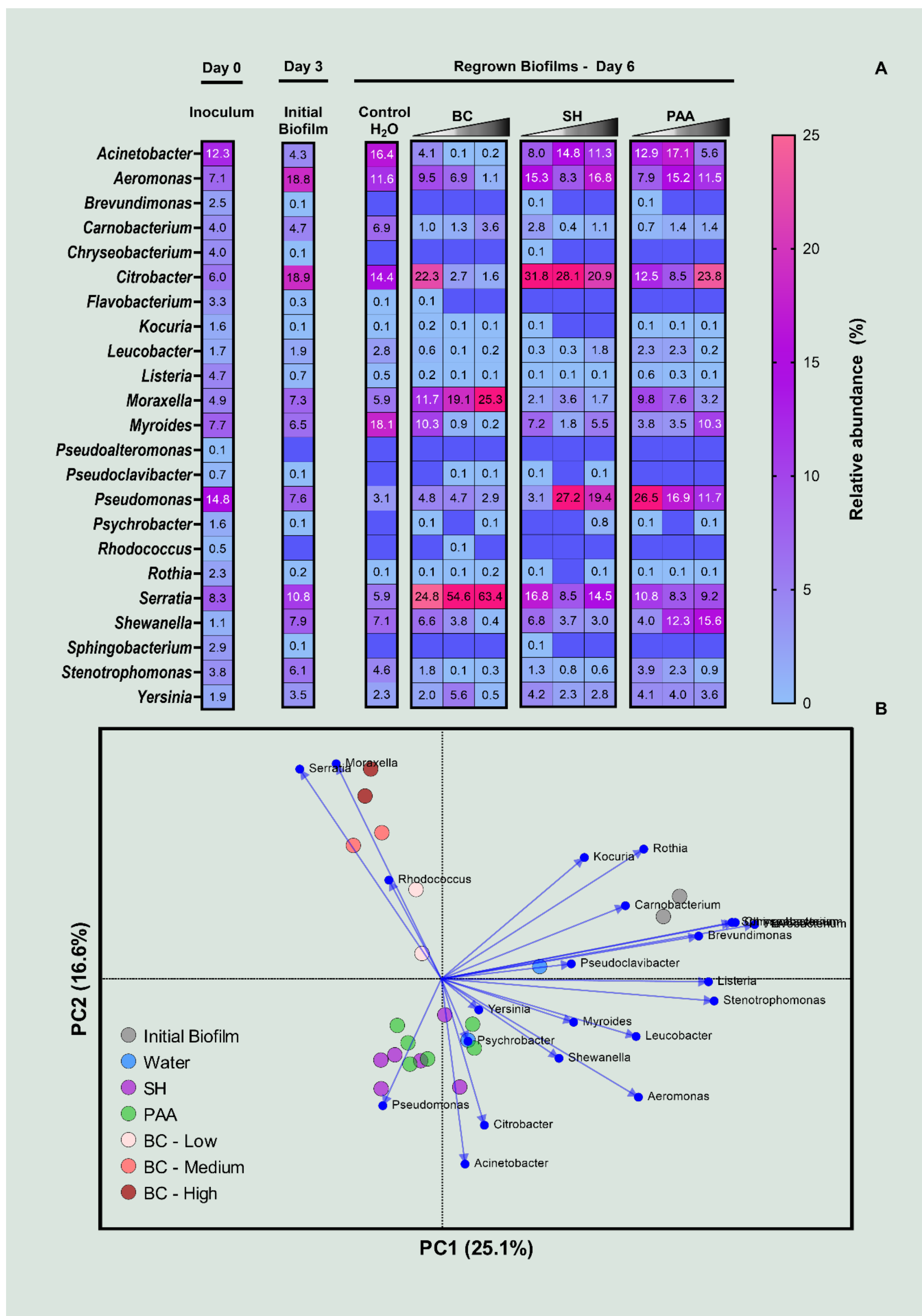


FIGURE 11. Culture-independent analysis of the drain biofilm microbiome changes after biocide treatments with each average based on 16S rRNA amplicon sequencing of biological independent replicates (n=4). A) Relative abundances of the microbiome in the inoculum, initial biofilm and regrown biofilms on day six after treatments with either sterile water, BC, SH or PAA on day three. B) PCA of microbiomes shown in the heatmap above and the differences in each genus caused by biocide treatments as shown by PC scores and PC loadings.

Changes in the surviving viable biofilm microbiota after biocide treatments

Multiple genera (>8) were observed among the colonies reflecting the dominating culturable and viable bacteria in the initial biofilms. *Pseudomonas* spp. accounted for 40% (n=8/20) of the initial control biofilm (H_2O, day 3) isolates identified (Figure 12). Similarly, more than eight genera were observed in the control biofilm on day six; however, no genera dominated as *Stenotrophomonas* with 20% (n=4) was the most frequently detected genera. The number of genera among the surviving bacteria decreased after biocide treatments (BC = 2.000 mg/L, SH = 12.000 mg/L, PAA =5000 mg/L,), e.g., BC treatment on day three led to *Pseudomonas* (*Pseudomonas migulae*) accounting for 70% (n=14) of the viable biofilm isolates. *Pseudomonas* were overall the most frequently identified viable survivors after biocide treatments and present in all surviving biofilms. Treatments on day three with SH left several surviving genera including *Shewanella* (25%, n=5), while PAA treatments on day three resulted in *Shewanella* constituting 75% (n=15) of the survivors (Figure 12). *Shewanella* was, however, not observed among the dominating survivors after treatments with BC, SH or PAA on day six and similarly occurred in low abundance of 5-10% in the initial- and control biofilms. Interestingly, *Serratia*, which harbours the QAC efflux *oqxB* gene, went from being present (5%, n=1) in the initial biofilm and not observed among the dominant survivors after first BC treatment on day three to being the dominant isolate after BC treatment on day six (40%). Similarly, strikingly were the 35% (n=7) of *Psychrobacter* after treatment with PAA on day six. For *Acinetobacter*, an increase was seen from 10% (n=2) after the first treatment with SH on day three to an abundance of 45% (n=9) after SH treatment on day six. *Listeria* were in contrast only among the dominating viable colonies after SH treatment on day six, thus reflecting it mainly being present in lower abundances of less than 5% in biofilms.

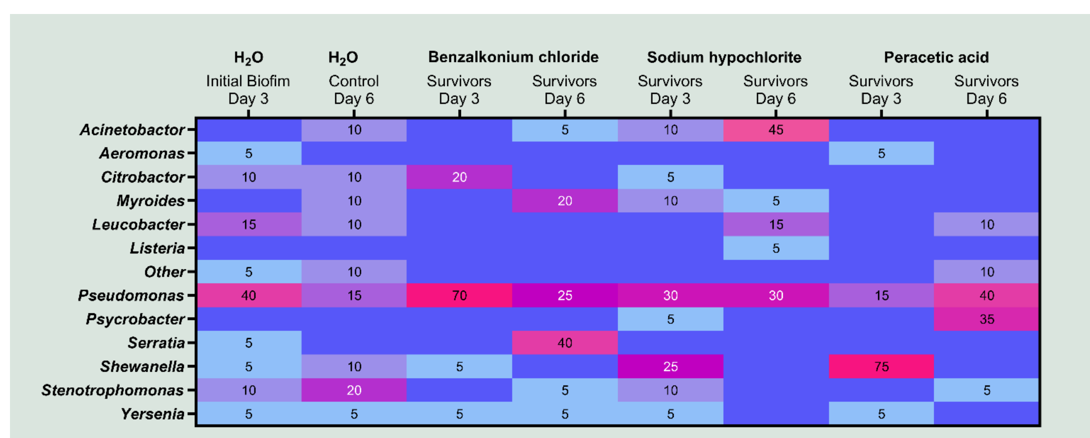


FIGURE 12. Heatmap of culture-dependent analysis using MALDI-TOF genus identification of the viable bacteria dominating in the drain biofilms after biocide treatments on days three and six. Presence is given in percentage (%) and based on a random selection of colonies (n=20) from each treatment (n_{total}=220) with colonies originating from the most diluted APC and thus reflecting the most abundant genera (≥ 5%) in the surviving biofilms.

5.4 Discussion

This sub-project aimed to define a core microbiome in drains within FPEs to create a representative biofilm model for biocide testing. Using this multispecies drain biofilm model composed of 31 bacterial isolates representing 24 genera, it was demonstrated that commonly used biocides selected for survival and regrowth of different genera after repeated exposure and that in the instance of repeated use of BC, the dominance of *Serratia*, could be explained by the carriage of a QAC efflux pump of *S. marcescens*. Below, we will discuss the characterization of the metagenome and resistome of drains and how this was used in the design of a

representative floor drain biofilm model. Finally, we will discuss results from the repeated biocide treatments of drain biofilms, and perspectives for the design of biocide rotation schemes to prevent the development of biocide-tolerant biofilms in the industry.

Floor drain microbiomes are diverse and complex but dominated by a few highly abundant taxa

Floor drains are reported to be the site with highest microbial load and the biggest contributor to microbial diversity compared to other surfaces in the FPE (Cobo-Díaz et al., 2021; Xu et al., 2023). This was reflected in diversity among the 14 floor drains of this study (Figure 7) where 36 genera were present at least once at relative abundance >5% in individual drains, while 22 of those had mean abundance levels >1% across all floor drains (Figure 8). This shared core microbiome underlines the presence of a core microbiome in different, yet microbiologically similar, wet food processing productions facilities.

Results showed Gram-negative bacteria, as represented by the phylum Pseudomonadota dominated in both cheese, shrimp and fish roe drains at levels of 68-80% despite the difference in food production type (Supplementary Figure S3). Similar 70-98% dominance of Pseudomonadota was seen in drains of three different beef-processing plants (Palansiamy et al., 2023), while 34-52% were found in metagenomes of Canadian food production facilities (Caraballo Guzmán et al., 2020). Pseudomonadaceae dominated at 17% in the present study and 22-26% in Palansiamy et al. (2023), with Moraxellaceae (>9%), being the second most abundant family in both studies together with Enterobacteriaceae (>9%) in present study (Figure 7, and 4). *Pseudomonas spp*., which is a genus consisting of species known to form biofilms and spoil food products, especially dominates wet FPEs as shown in a meta-study of microbiomes from surfaces in 39 different facilities (Xu et al., 2023). *Pseudomonas* spp. have been shown to rapidly populate new processing facilities for pork or salmon products, leading to >50% in the sequenced microbiome, (Cobo-Díaz et al., 2021; Bjørge Thomassen et al., 2023b). Interestingly, *Pseudomonas* remained the most dominant genera on cleaned and disinfected surfaces in both a salmon facility and beef industry (Bjørge Thomassen et al., 2023b; Palanisamy et al., 2023), which points to insufficient removal of this genus.

An important limitation of metagenomic analysis is the sequencing of DNA from both viable, non-culturable and dead bacteria. Therefore, the metagenomic characterization of floor drain microbiomes was supplemented by culturomic analysis, which revealed a diverse viable microbiome leading to isolation of forty different genera that largely concurred with the genera dominating in metagenomic sequencing results, including high numbers of *Pseudomonas*, *Acinetobacter*, *Brevundimonas* and *Stenotrophomonas* (Figure 6). Genera common in the viable microbiome but less abundant in the metagenome included *Chryseobacterium*, *Serratia* and *Microbacterium*. *Serratia* spp., which belong to Enterobacteriaceae, were widely present in the drains (Figure 7A). *Serratia* spp. are frequently isolated in different types of food processing facilities (Møretrø and Langsrud, 2017) and are known to be among the more tolerant bacteria to cleaners and disinfectants (Langsrud et al., 2003; Boyce, 2023). *Microbacterium* spp. have been found with abundance ≥7% in metagenomes of several drains in a beef processing facility (Palanisamy et al., 2023), while *Chryseobacterium* spp. have, in contrast, rarely been classified as part of the resident microbiome of FPEs despite being isolated from a wide range of environmental sources (Møretrø and Langsrud, 2017; Mwanza et al., 2022). Reasons for this discrepancy are not known. Interestingly both *Microbacterium* and *Chryseobacterium* failed to establish themselves in the drain biofilm model (Figure 7A).

Design of a representative floor drain microbiome for testing biocide tolerance development

Thirty-one isolates were deemed sufficient to capture the core microbiota and diversity of floor drains across FPEs, as most of the dominating genera were found in all three production environments (Figures 6 and 8). This broadens the applicability and results of the model to the whole food industry. As discussed above, *Pseudomonas* must be included in the model to reflect the drain microbiome (Møretrø and Langsrud, 2017), and lead to accurate assessment of

biocide efficacies on industry-like biofilms (Fagerlund et al., 2017; Charron et al., 2023; Rolon et al., 2024). With 22 genera metagenomically present at abundances of >1% in the FPEs, we deemed it necessary to include enough isolates to capture at least half of metagenomic abundance and >75% of the viable drain microbiome (Supplementary Table S1 andS8). To compensate for the diversity and high abundance of some genera it is reasonable to include two or more isolates from same genera (Lake et al., 2024), and this led us to include five *Pseudomonas* isolates (Table 3). Previous biofilm experiments have shown that biocide survival is, however, not necessarily linked to the complexity of the biofilm as *L. monocytogenes* performed better in mono-species biofilm on conveyor belt coupons of polyvinyl chloride (PVC), while a dual-species biofilm with *Pseudomonas spp.* on stainless steel yielded better survival than the mono-species biofilm (Fagerlund et al., 2017; Dass et al., 2020; Thomassen et al., 2023a). The composition of the complex biofilm model will evidently lead to cooperative but also competitive interactions as some genera outgrow others. The biofilm models should not be designed to limit these, as the results of interactions can vary depending on the stress conditions applied to the model (Li et al., 2021). Specifically, Lake et al. (2024) observed how *L. monocytogenes* had initial higher survival in mono-species biofilms, but when cycles of follow-up cleaning and disinfection procedures were applied again and again, they observed better survival of *L. monocytogenes* in the multi-species biofilm which might shelter the pathogen in the long term despite competitive interactions. Importantly, our biofilm model composition led to consistent levels of *Pseudomonas* survival across the different controls and biocide treatments (Figure 12), as would be expected based on its high abundance in this (Figure 8) and other studies.

The inclusion of a pathogen of interest, e.g., *L. monocytogenes*, is based on the relevance for food safety, as they are rarely found in abundances justifying their inclusion. Similarly, the initial concentrations of isolates could be discussed as their abundance in the drains of FPEs clearly were not even (Figure 8). We chose to go with a standard preparation protocol for all isolates and mixing of even volumes, which led to some isolates being present in slightly higher concentrations compared to others in the biofilm inoculum (Figure 11A). To further simulate the true biofilm conditions and abundances, microbial samples and nutrients taken directly from the environment can be used as inoculum, which, however, limits reproducibility for other researchers (Fernández-Gómez et al., 2022; Flores-Vargas et al., 2023). We chose to conduct the biofilm assays in undiluted laboratory media and use a temperature of 15 °C to represent a worst-case soiled scenario and realistic FPE temperatures, respectively. This approach gave high and stable biofilm concentrations ≈ 7.0 log CFU/cm^2 on the stainless steel (Figure 10A). It is common practice to apply full-strength media in biocide efficacy studies to investigate when, where and why cleaning and disinfection programs might fail as this often occurs in hard-to-reach niches, where nutrients and dirt build up (Fagerlund et al., 2021; Rahman et al., 2022; Thomassen et al., 2023a). These niches are typically those, where mechanical brushing and cleaning fail to reach. We chose to leave out mechanical cleaning, but it should be noted that biocide manufacturers do recommend mechanical scrubbing as part of the cleaning and disinfection and, in addition, also recommend increasing biocide concentration when faced with persistent problems (Fagerlund et al., 2020).

Biocides alters the microbiome biofilm composition but not the biocide tolerance

In our study, the simulation of a worst-case "weekend" scenario with three days allowed for undisturbed biofilm formation on stainless steel, which meant that even at the highest recommended industrial concentrations or more of PAA and SH, there was a surviving biofilm microbiome of ~3-4 log CFU/cm^2 (Figure 10A and 12). Following biocide treatments, it was observed that surviving microbiomes were altered in a manner that depended on the applied biocide, thus indicating a selective effect caused by biocides (Figure 11). Moreover, increasing biocide concentrations decreased the alpha diversity in the biofilm that regrew after the first treatment. Reducing the diversity might not be advantageous as observations from microbiome samples from drains in factories after intense and long sanitation procedures showed that

the surviving regrowing microbiomes produced a bigger biofilm mass making them more difficult to eradicate (Wang et al., 2024). We observed similarly that following PAA and SH treatments, there was an increase in overall biofilm concentration (Figure 10A) and a significant increase in *Pseudomonas* abundance (Supplementary Figure S2), whereas BC treatments caused *Serratia* and *Moraxella* to increase (Figure 11). The culture-dependent analysis of biocide-treated biofilms confirmed that *Pseudomonas* was a frequent survivor after all treatments (Figure 12), as also seen in other biofilm studies with mixes of environmental isolates from the food industry (Fagerlund et al., 2017; Lake et al., 2024). It is well known that *Pseudomonas* biofilms would potentially shelter foodborne pathogens such as *L. monocytogenes* (Thomassen et al. 2023b).
While the biocide altered the composition of the regrowing biofilm, these biofilms were not more tolerant to applications of the same biocide three days later (Figure 10A). However, *L. monocytogenes* prevalence decreased after the second treatment (Figure 10B), which means that at higher "industry" biocide concentrations, there is no adaptation of *L. monocytogenes* towards PAA and SH. This aligns with what has previously been shown for these biocides at lower biocide concentrations (Aarnisalo et al., 2007; Riazi and Matthews, 2011; Kastbjerg and Gram, 2012; Kragh et al., 2024). For intermediary biocide concentrations of 512 mg/L, Rahman et al. (2022) showed how repeated exposure of suspensions of *Listeria innocua* to PAA and SH led to no significant changes in log reductions, while repeated treatment with BC became less effective. The material to which the biofilms are attached also plays a role in development of tolerance. Lake et al. (2024) reported that repeated disinfection of multi-species biofilm on PVC caused decreased reductions, an observation not found for the same biofilms on stainless steel. For multi-species and mono-species biofilms of *L. monocytogenes* formed on PVC, Fagerlund et al. (2017 & 2020) similarly showed that repeated biocide treatments were less effective on day 7 compared to day 4. Together, this indicates that changes in biocide tolerance likely relate to the age of biofilm, biocide concentration and the material, where more porous materials such as PVC may offer better sheltering and formation options compared to stainless steel.

Regarding removal and inactivation of biofilms, PAA has previously been shown to be better at biofilm eradication than SH and BC (Cruz and Fletcher, 2012). This was also the case in the present study both quantitatively for total biofilm concentration and for *L. monocytogenes* prevalence (Figure 10). We have, in addition, previously shown how PAA was less affected by organic matter and biofilm (Kragh et al., 2024). In that regard, it is important not to only adjust the concentration of the applied biocide as we saw a very limited effect of doubling the concentration of BC and SH. These findings are in line with Barroso et al. (2019), who observed that after obtaining a 4-log reduction, a plateau was reached where further increasing biocide concentrations did not increase the inactivation. This implies that simply increasing biocide concentrations when faced with persistence problems is an inadequate solution, which, in addition, results in increased biocide expenses, equipment tear and hazard exposure for cleaning personnel.

Benzalkonium chloride residues can be detected on surfaces after disinfection and continue to exert a selective pressure (Møretrø et al., 2017). We observed that *S. marcescens* was among the most BC tolerant isolates with MIC ≥ 20 mg/L (Supplementary Table S2) and that this high MIC value could be explained by its carriage of a QAC efflux pump *OqxB* (Table 3). Interestingly, the included ST121 *L. monocytogenes* isolate carrying the *qacH* efflux pump gene had a markedly lower MIC value (2.5 mg/L) and low abundance in regrown biofilm after BC treatments (Figure 11). We and others have previously shown how the carriage of the major BC tolerance genes (*qacH*, *bcrABC*, *emrC* or *emrE*) in *L. monocytogenes* does not contribute to increased tolerance at industrially relevant biocide concentrations (Møretrø et al., 2017; Barroso et al., 2019; Kragh et al., 2024). The metagenomic assessment of drains highlighted that QAC tolerance genes are widely abundant in the FPEs with high numbers of genes associated with tolerance to QACs (*qacG*, *qacJ*, *qacL*, Figure 9B) We therefore propose that BC residuals

left in the FPE may create niches, where increased tolerance to low concentrations might induce an important selective pressure, leading to better survival in subsequent biocide treatments. Such a scenario seemed to occur in the present study where the proportion of *Serratia* spp. increased in regrown biofilms and were among the dominating survivors identified after the second BC treatment in comparison to after the first treatment (Figure 11 and Figure 12). Such a clear example of selective pressure and improved survival due to previously applied biocide begs the question of whether rotation of biocides could prevent such one-sided development of the drain biofilm. In contrast, the usage of biocides such as SH and PAA with a broad mode of action left a more diverse biofilm with multiple genera surviving and regrowing. To the best of our knowledge none of these biocides have been associated with specific biocide tolerance genes.

In conclusion, a biofilm drain model composed of 29 bacterial isolates was created based on culture-dependent and independent analysis of floor drains in different food processing environments. The biofilm model reflected >50% of the metagenomes of floor drains and >75% of the viable floor drain microbiome. While 274 different genera were identified, it was revealed that the drains were often dominated by few genera as only 22 genera were present at least once at relative abundances >5%. *Pseudomonas* were the most frequently dominating genus with an average relative abundance of 18% and the most abundant genus in the viable microbiome. Biocide treatments of the biofilm model formed on SS coupons showed how PAA and SH were more effective in reducing the biofilm compared to BC. Repeated treatment with the same biocide did not lead to increased tolerance, however, biocides selected for different bacterial genera in the surviving biofilm. This was most strikingly seen for the *S. marcescens* isolate, carrying a BC efflux pump (*oqxB*), as it became the most abundant bacteria in biofilms regrown following BC treatments. This showed how tolerance to low levels of residual biocides may provide a competitive advantage potentially leading to the domination of a niche within an FPE. Harbourage of an efflux pump (*qacH*) in *L. monocytogenes* ST121 did not in contrast lead to an increase in abundance after BC treatments, however, this isolate survived in biofilms following all biocides treatments except the highest tested concentration of PAA, confirming the protective role biofilms have on the persistence of this pathogen in the FPE. Future studies should investigate if rotational use of biocides can improve cleaning and disinfection regimes in the food industry as sequences of different biocides could be hypothesized to be able to control the biofilm better due to their different modes of action.

6. Sub-project 3

Evaluation of biocide rotation schemes to improve inactivation of microbial biofilms

6.1 Introduction

The foodborne pathogens *Listeria monocytogenes* and *Salmonella enterica* are regarded as the most relevant microbiological food safety hazards with respect to public health and persistence in food processing environments (FPEs), with many outbreaks, especially for *L. monocytogenes* linked directly to the long-term survival of the pathogen in niches or harbourage sites in the food production (Koutsoumanis et al., 2024). There is still ongoing debate and conflicting studies as to whether persistence of *L. monocytogenes* can be linked to specific genetic parameters, but the carriage of quaternary ammonium (QAC) efflux pumps has in many studies been found to be more frequent in persistent isolates (Fagerlund et al., 2022; Fox et al., 2011; Martínez-Suárez et al., 2016; Ortiz et al., 2016). In addition, increased biofilm formation as well as protection by the FPE biofilm microbiome can add to persistence (Fagerlund et al., 2021; Rolon et al., 2024). The FPE microbiome partially composed of persistent bacteria and biofilms have also been proposed to contribute to the transfer of antibiotic resistance genes to foodborne pathogens (Choy et al., 2024; Skandalis et al., 2021). Food manufacturers rely on the inactivation of microbes using biocide schemes to keep an FPE in a hygienic condition that permits production of safe foods. The numbers of outbreaks with *L. monocytogenes* linked to persistence and harbourage of QAC efflux pumps and the concerns of a post-antibiotic era have created a concern within food microbiology that biocide tolerance or resistance could evolve similarly to antimicrobial resistance (AMR) due to repeated application of the same biocide (Bland et al., 2022; Willmott et al., 2024). The current understanding seems to stem from the observations on AMR, where the gap between minimum inhibitory concentrations (MICs) and clinical concentrations is much smaller which implies that horizontal gene transfer (HGT) of antibiotic resistance genes providing small increases in resistance could cause great clinical treatment problems (Jutkina et al., 2018). For biocides, the industrial concentrations are often 100 to 1000-fold higher than recorded MICs for both sensitive and less sensitive isolates (Bland et al., 2022; He et al., 2022). Still, the concern has focused on adaptive trends around slightly increased MICs and the development and HGT of biocide tolerance genes responsible for these elevated MICs.

Concerns of foods becoming cross-contaminated with *L. monocytogenes* persisting in the FPE have existed for decades, and among many mitigative practical guidelines, it has been suggested that “rotating between different types of biocides may provide greater effectiveness against *L. monocytogenes*” (Tompkin et al., 1999). The rotation concept has also been discussed in clinical settings due to concerns of biocide resistant spores (Murtough et al., 2002). The rotation advice has since been passed on by national authorities, e.g., in the USDA Food Safety and Inspection Service guidelines for controlling *L. monocytogenes*, where it states that “rotating sanitizers will help prevent the development of microorganisms resistant to a particular sanitizer” (FSIS, 2014). The recommendation was repeated in the 2017 FDA guidance to the industry on how to control *L. monocytogenes* in ready-to-eat (RTE) foods, with references to the former guidelines from Tompkin et al. (1999) and FSIS (2014) guidelines (FDA, 2017). Recently, however, the recommendation has been removed in the newest guideline from the USDA Food Safety and Inspection Service on controlling *L. monocytogenes* in retail delicatessens (FSIS, 2023). In contrast, the latest scientific opinion on the persistence of microbiological hazards in food from the EFSA BIOHAZ panel states that “rotation of disinfectants can be

considered to avoid adaptation and development of tolerance or resistance by surviving bacteria" (Koutsoumanis et al., 2024). However, this statement is not backed by further recommendations, guidelines or references as to whether this rotation should involve multiple biocides and the frequency of rotation. Closing this clear knowledge gap will help to improve decision-making and allow better guidance for both industry and food authorities. This sub-project, therefore, aimed to investigate if there is a scientifically valid reason for the use of biocide rotation schemes to prevent the development of biocide tolerance in biofilms. Further, the sub-project aimed to assess if the rotation of biocides results in lower biofilm concentrations over time. Moreover, this sub-project aimed, using shotgun metagenomics, to investigate whether HGT of biocide genes were more likely to occur if biocides were not rotated.

6.2 Materials and Methods

Bacterial isolates and growth conditions

A total of 31 isolates covering 24 genera of bacteria found in food processing environments were included to create a representative floor drain biofilm model (Table 2). The isolates originate from various floor drains in shrimp, fish roe and dairy processing environments isolated between 2021 and 2022 (Kragh et al. 2025). For long-term storage, isolates were kept in a 20% (v/v) glycerol peptone medium (TS/80, Technical Service Consultants Ltd., Heywood, UK) at −80 °C.

TABLE 4. Isolates used for the creation of multi-species biofilm. Isolates from floor drains were isolated, characterized and described by Kragh et al. (2025) in terms of their contents of mobile genetic elements, including transposons, plasmid, insertion sequences (IS) and antimicrobial resistance genes.

Biofilm drain model isolates	Source	Mobile elements	Resistance genes
Pseudomonas anguilliseptica	Seafood	Tn5501	*oqxB homolog*
Pseudomonas chengduensis	Dairy		*oqxB homolog*
Pseudomonas cremoris	Seafood	Tn5501 + Multiple IS	
Pseudomonas fluorescens	Dairy	ISPpu14	
Pseudomonas migulae	Seafood	Tn5501 + Multiple IS	*aph(3'')-Ib*
Brevundimonas vesicularis	Dairy		
Brevundimonas vesicularis	Seafood		
Acinetobacter guillouiae	Seafood		*aph(3')-VI*, $bla_{OXA-274}$
Acinetobacter johnsonii	Dairy		$bla_{OXA-334}$
Moraxella osloensis	Dairy		
Aeromonas media	Dairy	pfekpn2511-4	$bla_{OXA-427}$ & bla_{CMY-8b}
Psychrobacter alimentarius	Seafood	pVB11737_6	
Yersinia aldovae	Seafood	MITEYpe1, IS5075	
Stenotrophomonas rhizophila	Seafood		*oqxB*
Kocuria rhizophila	Seafood		
Pseudoalteromonas translucida	Shrimp		
Serratia marcescens	Dairy	pSM22	*aac(6')-Ic* bla_{SRT-2}, *oqxB*, *tet(41)*
Citrobacter portucalensis	Dairy	pKPC-CAV1321	*qnrB9*, bla_{CMY-34}, *sugE*
Rothia amarae	Dairy	Multiple IS	
Pseudoclavibacter helvolus	Seafood		
Chryseobacterium haifense	Dairy		

Chryseobacterium scophthalmum	Seafood		
Flavobacterium frigoritolerans	Seafood		
Microbacterium liquefaciens	Seafood		
Leucobacter luti	Dairy	Multiple IS	
Carnobacterium iners	Shrimp		
Sphingobacterium faecium	Seafood		
Myroides odoratus	Dairy		bla_{TUS-1}
Rhodococcus qingshengii	Seafood	pR85A	
Listeria monocytogenes ST121	Seafood	plm5578, Tn6188	*fosX qacH*
Shewanella oncorhynchi	Seafood	Multiple IS	*mcr-4.3*, $bla_{OXA-549}$

Drain biofilm model formation
Biofilms were grown on food-grade stainless steel coupons (SSC) that were washed and cleaned before biofilm formation as previously described (Kragh et al., 2025). In short, cell suspensions for biofilm formation were made by reviving the isolates from colonies on Tryptone Soy Agar (TSA, 25°C, 3 days) before sub-culturing each isolate individually in 4 ml of Tryptone Soy Broth (TSB, Merck, Darmstadt, Germany) at 15 °C for 72 h. Biofilms were formed by mixing equal volumes (100 µL) of each isolate before 3000× dilution with TSB, resulting in a final concentration of 10^6 CFU/mL. This inoculation mix was used to cover each SSC with 500 µL of cell culture before placing each SS coupon in separate wells of 48-well plates (BioLite 48 Well Multidish F-well, Thermo Fisher Scientific, Roskilde, Denmark). Biofilm was allowed to form for three days (Friday to Monday) at 15°C to simulate the lower temperatures found in most food production environments.

Treatment of biofilm with biocides
After three days of forming an initial biofilm, different biocide schemes, including biocide rotations, were initiated with one daily treatment from Day 1 “Monday” to Day 5 “Friday”. Between each treatment, there was a regrowth phase with freshly added TSB until the following day to simulate the regrowth of a surviving biofilm in food production from day to day. After five days of daily biocide treatment, the biofilm was left to regrow with fresh TSB from Friday (Day 5) to Monday (Day 8) to simulate a weekend closure. Finally, a last biocide treatment (Day 8) was conducted to represent the new production week. Twelve different biocide schemes (Table 2) consisting of rotation among three biocides, two biocides or one biocide (no rotation from day to day). Each biocide was applied at the same concentration throughout a biocide scheme, with each scheme tested twice in different biological experiments at either manufacturer-recommended concentration (MRC) or 2×MRC, resulting in two different biological experiments each with four technical replicates. For each daily biocide treatment, fresh dilutions of the biocides benzalkonium chloride (BC, 500 g/L, Thermo Fisher, Kandel, Germany), peracetic acid (PAA, Peroxyacetic acid, 35% w/v, Acros Organics BV, Geel, Belgium) or sodium hypochlorite (SH, 10% w/v, Scharlau, Barcelona, Spain) were prepared from original undiluted concentrated stocks using sterile water until manufacturer-recommended industrial concentration (MRC) or 2×MRC were obtained, i.e., BC (1000 or 2000 mg/L), SH (6000 or 12000 mg/L) and PAA (2500 and 5000 mg/L). Each of the twelve biocide schemes was used to disinfect four SS coupons (n=4) with either of the two concentrations depending on the experiment. Before application of biocide or sterile water, the initial cell culture (500 µL) surrounding the SS coupon was removed and 800 µL of biocide or sterile water was added to wells with the SS coupons with an exposure time of 5 min following by removal of the treatment solution and two times of washing of SS coupons with 900 µL of peptone saline (PS, 1 g/L peptone, 8.5 g/L sodium chloride).

Following washing, samples were either removed for enumeration of surviving biofilm cell concentration or covered with fresh 500 µL TSB until the next biocide treatment. For enumeration

of survivors, the SS coupons were transferred carefully to microcentrifuge tubes with 1000 µL PS, and biofilms loosened from the coupons with 50/60 kHz for 5 min in a 1000 W sonication bath (Elmasonic S 120, Thermo Fisher Scientific). The sonicated samples were then used to determine the biofilm cell concentrations by dilution in PS and spread plating on TSA incubated for three days at 20°C. Cell concentrations were log-transformed and expressed as Log CFU/SSC. The limit of detection was 0.7 Log CFU/SS coupon. To allow analysis of the effect of repeated biocide treatments on survivors in biofilms to occur with destructive samplings on days 1, 5 and 8, each biocide scheme was applied to multiple additional SS coupons (n=112) for one biological experiment at one concentration with destructive sampling of 16, 48 and 48 SS coupons on days 1, 5 and 8, respectively. All biocide treatments and controls were thus done in quadruplicates with the experiment repeated twice for each biocide concentration.

TABLE 5. Overview of biocide treatment schemes applied to a drain biofilm model daily with either no rotation of biocides or rotation with two or three biocides. Biocide treatments were not applied during the weekend between Friday and Monday.

Scheme code	Monday	Tuesday	Wednesday	Thursday	Friday	Monday	# of biocides
P-P-P-P-P---P	PAA	PAA	PAA	PAA	PAA	PAA	1
S-S-S-S-S---S	SH	SH	SH	SH	SH	SH	1
B-B-B-B-B---B	BC	BC	BC	BC	BC	BC	1
P-S-P-S-P---P	PAA	SH	PAA	SH	PAA	PAA	2
S-P-S-P-S---S	SH	PAA	SH	PAA	SH	SH	2
P-B-P-S-P---P	PAA	BC	PAA	SH	PAA	PAA	3
P-S-P-B-P---P	PAA	SH	PAA	BC	PAA	PAA	3
S-B-S-P-S---S	SH	BC	SH	PAA	SH	SH	3
S-P-S-B-S---S	SH	PAA	SH	BC	SH	SH	3
B-P-B-S-B---B	BC	PAA	BC	SH	BC	BC	3
B-S-B-P-B---B	BC	SH	BC	PAA	BC	BC	3
Water	Water	Water	Water	Water	Water	Water	0

Ranking of biocide treatments based on surviving biofilm concentrations

The deviation from best biocide treatment (DFB) was calculated for each biocide scheme on days 5 and 8 for both biocide concentrations to allow comparison of the effects of biocide treatments across days and applied biocide concentration. The treatments using the same concentration (MRC or 2×MRC) resulting in the lowest surviving biofilm concentration on days 5 and 8, respectively, were used as a benchmark. DFB were thus calculated as shown below resulting in four DFBs from each biocide scheme.

DFB = Surviving biofilm concentration on a specific day – Lowest biofilm concentration on same day

To rank the 12 biocide schemes, the DFBs from each scheme were summarized (DFB_{Total}) as well as averaged ($DFB_{Avg.}$).

Culture-dependent analysis of surviving biofilm microbiome

A random selection of isolates was selected by placing a 2x2 cm square template centrally on the TSA enumeration plates, aiming to identify the most abundant species in the surviving biofilm microbiome. Using the template all isolates within the square were selected for identification. Colonies were picked from the most diluted biofilm samples, with the second most being used if less than 20 isolates were obtained from the first analysed plate. This selection was done for five biocide schemes (marked with * in Table 5) to analyse if biocide schemes using

two or three biocides had different selective impacts on the surviving biofilm microbiome after days 5 and 8. From each analysed biocide scheme, an average of 42 ± 20 colonies were re-streaked on TSA blood agar (TSBA, SSI Diagnostica, Hillerød, Denmark) and incubated for 2-4 days at 20°C before being identified using MALDI-TOF MS (MALDI Biotyper, Bruker Daltonics, Bremen, Germany) as previously described (Kragh et al. 2025). To improve the identification to species level, a custom database with the 31 biofilm isolates was used to supplement the MBT Compass reference library. For each isolate of the biofilm model, three spectra originating from TSBA were used to create new identification profiles in the MBT Compass Explorer Module. The final analysis resulted in the identification of 573 isolates from MRC treatments and 523 isolates from 2×MRC treatments to species level. Results from the identification were summarized for each treatment day and biocide concentration and expressed as relative abundances (%).

Culture-independent analysis of regrowing planktonic microbiome
Cell suspensions surrounding the SS coupons were on day 8 removed for metagenomic sequencing. From three of the four experiments (n=24 from 2×MRC and n=12 from MRC, n_{total}=36), four identical samples were pooled and centrifuged at 7.400 × g to obtain a pellet, which was frozen at -20 °C. The resuspended pellet was thawed and transferred to a DNeasy PowerBead Pro tube from the Qiagen DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany). DNA extraction was carried out following manufacturer's protocol using a FastPrep-24 (MP Biomedicals, Eschwege, Germany). Extracted DNA was quantified using Qubit 3.0 (Invitrogen, Carlsbad, CA, United States) with the Qubit DNA BR Assay Kit (Invitrogen). DNA samples of > 300 ng with >10 g/µL were sent on ice to Eurofins Genomics (Konstanz, Germany) for Illumina 150 bp paired-end sequencing. The raw metagenomics data were submitted to the European Nucleotide Archive (ENA) under project number: PRJNA1196425. The raw sequencing data of the samples were processed with CLC Genomics Workbench 24.0 and the Microbial Genomics Module 24.0 (Qiagen, Aarhus, Denmark) to trim away adapters and low-quality reads with quality scores below 0.05. After trimming, 20.5 – 39.8 million reads remained for each sample, with an average of 28.2 million reads. Taxonomic profiling of the filtered reads was performed in CLC using the Qiagen Microbial Insights - Prokaryotic Taxonomy Genus Database (QMI-PTDB) as reference. Taxonomic classification results were filtered to remove non-complete taxonomic classifications and sequencing noise by removing low abundant genus classifications that were not present with at least 1% abundance in one sample. The filtered taxonomic abundance tables were merged and exported for visualizations based on their biocide treatment scheme, biocide concentration, number of biocides and the primary biocide used.

Investigation of possible horizontal gene transfer during biocide treatment
The metagenomes of the regrowing planktonic microbiomes from day 8 were assessed using MetaCherchant (Olekhnovich et al., 2018) to extract the genomic regions around the efflux pump *oqxB* carried by *Serratia marcescens*, which is known to confer increased tolerance to BC and to be widespread among Enterobacteriaceae on both chromosomes and plasmids (Alcock et al., 2020). The contigs outputted from MetaCherchant were subsequently taxonomically classified using Kraken at a strict confidence score of 0.1, as the default would increase the risk of false hits. Due to the high similarity between *oqxB* (3144 bp) and efflux pumps in *Pseudomonas anguilliseptica* (98.0%, 3080/3144 bp), *Pseudomonas chengduensis* (98.0%, 3081/3144 bp) and *Stenotrophomonas rhizophila* (98.7%, 3104/3144 bp) the 60 bp outputs from MetaCherchant was deemed not suitable for trustworthy determination of horizontal gene transfer (HGT) events in the current biocide biofilm model. Instead, CLC Genomics Workbench was used to de novo assemble contigs from each metagenome. The resulting contigs were used for screening (blastn) for the presence of *oqxB*. Any resulting hit with a minimum identity and coverage of 95% and 80% (2515 bp) was then used to analyse for HGT by blasting the surrounding genomic region of the specific contig against a custom blastn database consisting of the isolates in the biofilm model (Table 1). The other remaining isolates (n=26) in

the biofilm model had no more than 50.5% coverage of the *oqxB* genes of *S. marcescens* prior to the experiments. The same procedure as described above for potential HGT was investigated for the known efflux pump *qacH* (372 bp) carried by *L. monocytogenes* on the Tn6188 transposon.

Statistical analysis and visualization

Four experiments with quadruplicate samples were conducted for each biocide rotation scheme of which two biological independent experiments were done using the MRC of the biocides and two other biological independent using 2×MRC. The Student's T-test was done using Microsoft Excel (Version 2307) at a significance level of $P < 0.05$ to compare log-transformed plate counts (Log CFU/SSC of the surviving biofilm after the initial biocide treatments (Day 1) and the last treatment (Day 8). This was done to determine if certain biocide schemes altered the tolerance of biofilms. Similarly, the Student's T-test on the calculated DFB values was used to test if any biocide schemes resulted in significantly lower biofilm concentrations. One-way ANOVA with the Tukey post hoc test at a significance level of $P < 0.05$ was additionally used to compare means of the surviving biofilm concentrations for schemes with rotation of one, two or three biocides after five or eight days. Principal component analysis (PCA) was performed to investigate the effect of biocide schemes and biocide concentrations on the culture-dependent surviving microbiome composition on day 5 and day 8 using GraphPad Prism 10 (GraphPad Software, Boston, MA, United States) using the relative abundance levels in the surviving biofilms. Similarly, Pearson correlations were calculated based on the relative abundance levels of species in the surviving biofilms and the concentration (Log CFU/SSC) in the surviving biofilms. Lastly, Permutational multivariate analysis of variance (PERMANOVA) using abundance tables of the regrown suspension around the biofilm on day 8 was used to check if the microbiome composition differed significantly (Bonferroni corrected P-values ($P_{corrected}$) to correct for multiple-comparison) depending on the used biocide concentration (MRC or 2×MRC) or the primary biocide (PAA, SH or BC) in the schemes.

6.3 Results

Biofilm cell concentrations after application of biocide rotation schemes

The surviving biofilm concentrations were on all enumeration days 1, 5 and 8 significantly ($P < 0.05$) lower after application of the highest (2×MRC) biocide concentration compared to MRC concentrations (Table 6). The mean surviving biofilm concentrations (1.5 and 2.5 Log CFU/SSC for MRC and 2×MRC, respectively) were lowest on day 5 for both biocide concentrations as a result of 5 rounds of daily biocide treatments. Naturally, there were higher surviving biofilm concentrations after biocide treatments on days 1 and 8 where the biofilm had been left untreated during the weekends. However, there was no observable tolerance build-up as the mean surviving biofilm concentrations were not significantly ($P > 0.05$) different on day 8 compared to day 1 for 11 of the 12 biocide schemes at 2×MRC. Significantly ($P < 0.05$) lower biofilm concentrations were seen on day 8 for 3 of 12 biocide schemes when using MRC (Table 6), while there were no significant ($P > 0.05$) differences between days 1 and 8 for the remaining schemes at MRC. Among all the 24 tested biocide schemes, only one case of a significant increase ($P < 0.05$) in biofilm survival on day 8 was seen when the rotation of SH and PAA at 2×MRC were applied. However, the same rotation did not result in any difference when applied at the MRC of the biocides or when the same rotation of PAA and SH were used starting with PAA.

TABLE 6. Surviving biofilm concentrations and comparison of biocide rotation schemes by the Deviation From Best criteria (DFB). Biofilm concentrations (Log CFU/SSC) at days 1, 5 and 8 after daily biocide treatments Monday to Friday and again Monday with either benzalkonium chloride (B), Sodium hypochlorite (S) or Peracetic acid (P) (n=8). Biocide cell concentrations on Mondays (days 1 and 8) in the same row with the same biocide concentration having different letters are significantly different, indicating increased sensitivity or tolerance towards the same biocide and concentration. Based on the daily treatment resulting in the lowest surviving biofilm concentration, the deviation from the best biocide treatment (DFB) is calculated for days 5 and 8. Numbers in the column DFB Average (Avg.) sharing a letter are not significantly different from each other. LOD = 0.7 Log CFU/SSC. The surviving biofilms from schemes marked with * were further analysed with MALDI-TOF. Each Log CFU/SSC determination is based on two biologically independent experiments with quadruplicates (n=8).

		Log CFU/SSC											
		Biocide conc.: MRC			Biocide conc.: 2x MRC			Biocide conc.: MRC		Biocide conc.: 2x MRC			
Scheme	Biocides in rotation	Day 1	Day 5	Day 8	Day 1	Day 5	Day 8	DFB Day 5	DFB Day 8	DFB Day 5	DFB Day 8	DFB Total	DFB Avg.
S-P-S-B-S---S*	3	4.9 ± 1.2	2.6 ± 1.4	3.5 ± 0.3	2.0 ± 1.2	0.8 ± 0.3	1.1 ± 1.0	1.3	0.0	0.1	0.0	1.5	0.4±0.6ab
P-S-P-B-P---P*	3	6.1 ± 0.5x	1.4 ± 0.7	4.0 ± 0.9y	2.9 ± 1.4	0.9 ± 0.5	2.5 ± 1.5	0.1	0.5	0.2	1.4	2.3	0.6±0.5a
S-S-S-S-S---S	1	4.9 ± 1.2	2.9 ± 0.5	4.5 ± 0.9	2.0 ± 1.2	0.7 ± 0.0	1.6 ± 1.7	1.6	1.0	0.0	0.5	3.1	0.8±0.6abc
P-B-P-S-P---P	3	6.1 ± 0.5x	2.0 ± 1.7	5.0 ± 0.5y	2.9 ± 1.4	2.1 ± 1.9	2.3 ± 1.6	0.7	1.5	1.4	1.2	4.9	1.2±0.3abc
P-P-P-P-P---P*	1	6.1 ± 0.5x	2.6 ± 1.4	5.1 ± 0.5y	2.9 ± 1.4	2.2 ± 1.1	1.7 ± 0.9	1.3	1.6	1.5	0.6	5.0	1.3±0.4abc
S-B-S-P-S---S	3	4.9 ± 1.2	2.3 ± 1.4	4.9 ± 0.7	2.0 ± 1.2	1.3 ± 0.9	3.2 ± 1.7	1.0	1.4	0.6	2.1	5.1	1.3±0.6bc
P-S-P-S-P---P*	2	6.1 ± 0.5	3.1 ± 1.1	5.7 ± 0.5	2.9 ± 1.4	1.5 ± 0.8	3.1 ± 1.4	1.8	2.2	0.8	2.0	6.7	1.7±0.5c
S-P-S-P-S---S*	2	4.9 ± 1.2	3.0 ± 2.0	4.6 ± 0.6	2.0 ± 1.2x	2.7 ± 1.5	4.8 ± 0.9y	1.7	1.1	2.0	3.7	8.6	2.1±1.0bc
B-B-B-B-B---B	1	5.8 ± 0.3	2.4 ± 1.4	4.5 ± 1.1	5.7 ± 1.1	3.0 ± 1.2	5.0 ± 1.2	1.1	1.0	2.3	3.9	8.3	2.1±1.2abc
B-P-B-S-B---B	3	5.8 ± 0.3	1.3 ± 0.7	5.5 ± 0.3	5.7 ± 1.1	2.8 ± 1.3	5.5 ± 1.0	0.0	2.0	2.1	4.4	8.5	2.1±1.6abc
B-S-B-P-B---B	3	5.8 ± 0.3	3.6 ± 1.7	4.9 ± 0.3	5.7 ± 1.1	1.9 ± 1.1	4.9 ± 2.0	2.3	1.4	1.2	3.8	8.8	2.2±1.0bc
Mean	-	5.3 ± 0.6	2.5 ± 0.8	4.8 ± 0.6	3.3 ± 1.5	1.5 ± 0.8	3.2 ± 1.7	-	-	-	-	-	-
Water*	0	7.3 ± 0.6	8.0 ± 0.1	7.8 ± 0.1	6.8 ± 0.7x	7.9 ± 0.5	7.8 ± 0.3y	6.7	4.3	7.2	6.7	25.0	6.2±1.1d

Ranking and comparison of biocide schemes with and without rotation

The comparison of biocide schemes based on average DFBs across both applied concentrations revealed that there were no significant ($P < 0.05$) differences between the application of three biocides in rotation and the application of just one biocide (Table 6). Specifically, these schemes, except for BC only, resulted in average DFBs ranging from 0.4 to 1.3 log CFU/SSC, with total DFBs ranging from 1.5 to 5.1 log CFU/SSC. In contrast, the switch between the two biocides SH and PAA was less efficient with significantly ($P < 0.05$) higher average DFBs of 1.7-2.1 and total DFBs of 6.7 and 8.6 log CFU/SSC. Similarly, biocide schemes with BC were less efficient when the scheme was based solely on BC or in rotation with PAA and SH with BC as the dominant biocide (Table 5). These schemes had one log CFU/SSC higher DFBs on average with total DFBs of 8.3-8.8. This overall lower efficiency of BC-based schemes was especially caused by the relatively lower efficiency of 2×MRC compared to the other biocide treatments, which was most pronounced for the 2×MRC treatments on day 8 (Figure 13).

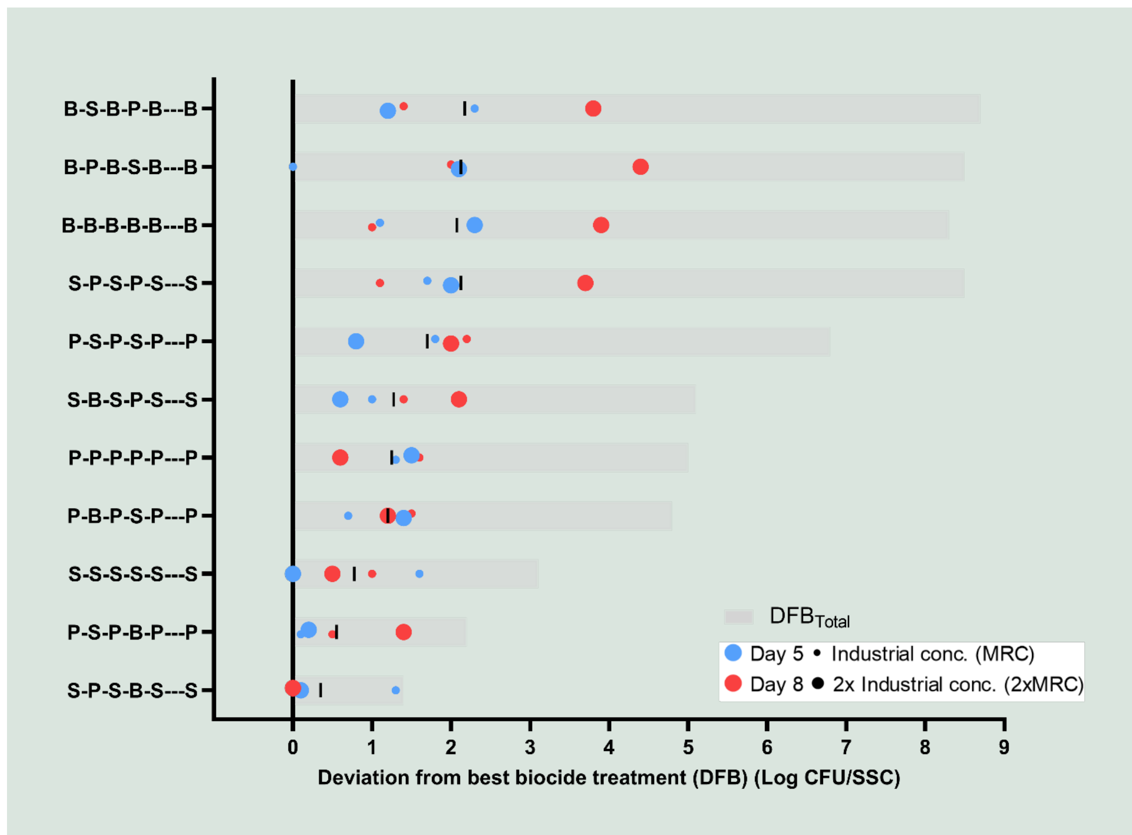


FIGURE 13. Ranking of best-performing biocide schemes using the Deviation From Best (DFP) criteria. The ranking is based on the enumeration of biofilm concentrations (Log CFU/SSC) surviving biocide treatments on days 5 and 8. For each biocide scheme the deviation (Log CFU/SSC) from the best biocide treatment is calculated and mean for each scheme presented (I) and used to rank the treatments. The summarized (DFB_{Total}) represents the total deviation of a given treatment compared to the best treatment on each given day. Each Log CFU/SSC determination is based on two biologically independent experiments with quadruplicates (n=8).

Changes in biofilm microbiome after daily biocide treatments

The surviving biofilms from assessed selected biocides schemes were especially dominated by *Pseudomonas cremoris* making up 38% (311/819) of the surviving colonies (Appendix: Supplementary Table S1). The strong survival of *P. cremoris* was seen at both MRC and 2×MRC, in contrast to the low abundance of 3.6% (10/227) of this species in the water controls (Figure 14AB). *Acinetobacter guillouiae* 9.6% (79/819), *Stenotrophomonas rhizophila* 12.5% (102/819) and S. *marcescens* 12.0% (98/819) were frequent among the survivors. In water controls, *Acinetobacter guillouiae* exhibited a high frequency of 22.7% (63/277), which was in contrast to a relative abundance of 4.3% (12/277) for both *Stenotrophomonas* rhizophila and *S. marcescens*. *L. monocytogenes* was only detected once in the water controls (0.4%, 1/277), while making up 2.8% (23/819) in the surviving biofilm microbiome after biocide treatments. The latter correlated to *L. monocytogenes* being 8-fold more present in biocide-treated biofilms, apparently due to the selection pressure, whilst *P. cremoris*, on average, increased 11-fold (Appendix: Supplementary Table S1). *Stenotrophomonas rhizophila* and *S. marcescens* were 3-fold more prevalent in biofilms treated with biocides, whereas all other species were less prevalent compared to their abundance in water controls. This was especially clear for *Citrobacter portucalensis* and *Myroides odoratus* whose prevalences were 2-6 fold lower in biocide treated biofilms compared to water control and in addition had significant ($P < 0.05$) positive correlations ($r = 0.7441$ and $r = 0.4326$, respectively) with the biofilm concentration (log CFU/SSC) (Appendix: Supplementary Table S2). On the other hand, *P. cremoris* was the only species showing a significant ($P < 0.05$) negative correlation ($r = - 0.523$) with biofilm concentration as the dominant species after biocide treatments. The dominance of *P. cremoris* was apparent at both biocide concentrations at both day 5 and 8 (Figure 14AB) and therefore had a strong clustering effect as seen in the PCA analysis of the surviving microbiomes, where most biocide schemes clustered due to high numbers of *P. cremoris* (Figure 15AB). Microbiome samples outside these clusters were outliers due to being either water controls or biocide-treated biofilm microbiomes with higher abundances of *Acinetobacter spp*., *L. monocytogenes* or *S. marcescens* (Figure 15AB).

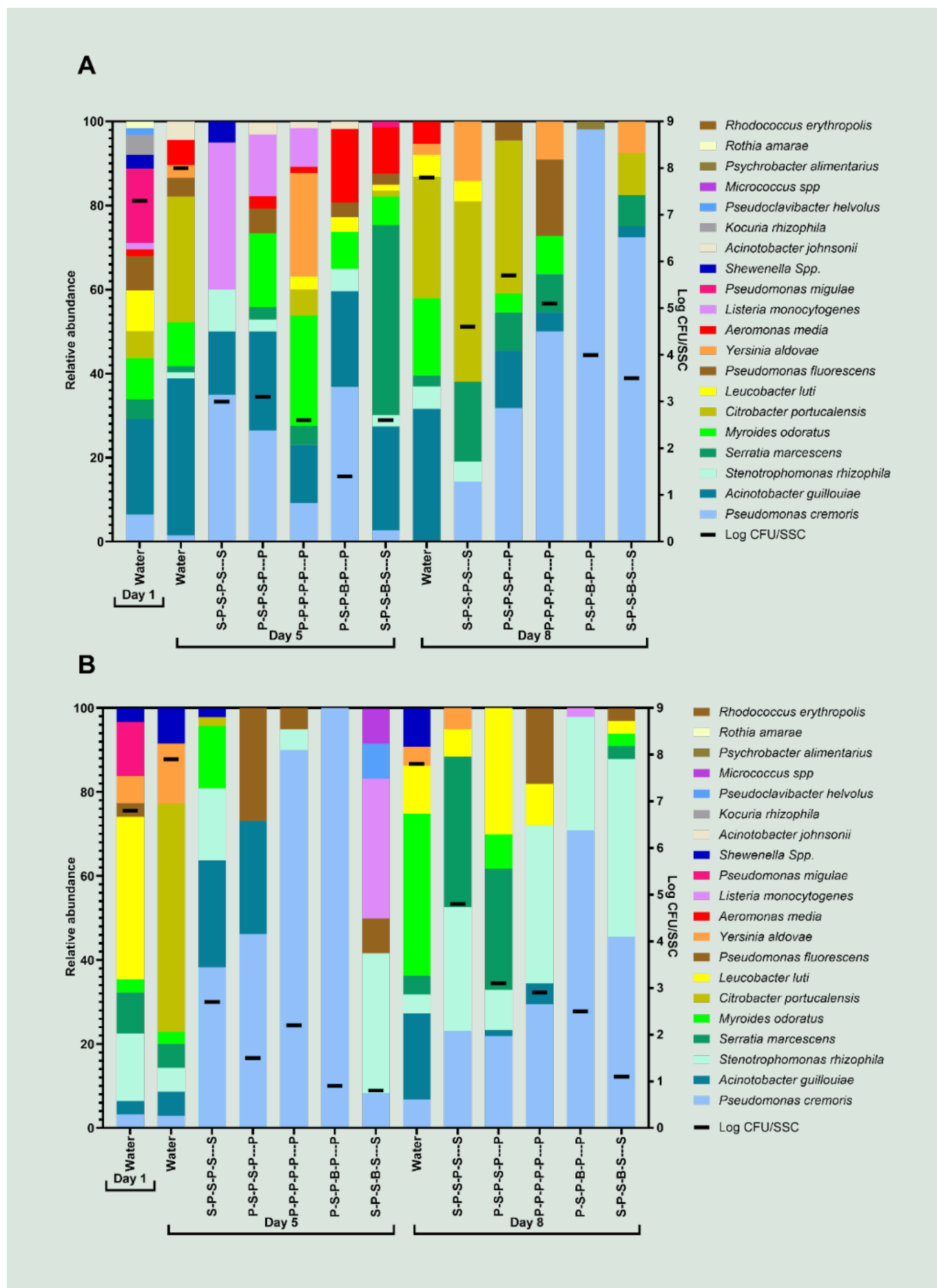


FIGURE 14. Culture-dependent analysis using MALDI-TOF MS species identification of the viable bacteria dominating after control (water) or biocide treatments on days one, five and eight after using either A) MRC of the biocides or B) 2×MRC. Species abundances are given in percentage (%) and based on a random selection of colonies (n = 42 ± 20) from each treatment (n_{total}=1096) with colonies originating from the most diluted APC and thus reflecting the most abundant species in the surviving biofilms. The surviving biofilm concentrations (Log CFU/SSC) are based on aerobic plate counts (3 days, 15 °C).

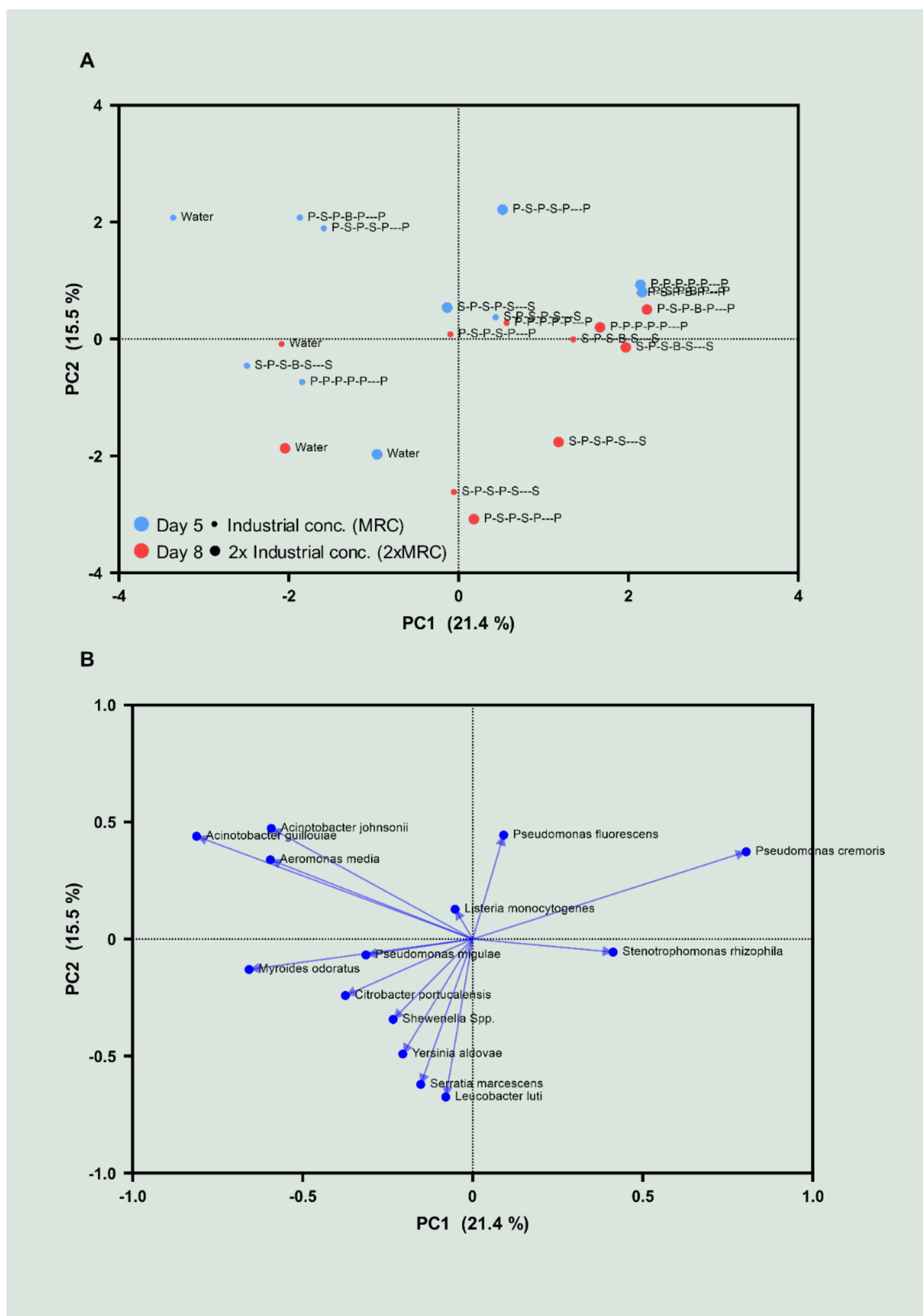


FIGURE 15. PCA of the culture-dependent microbiomes surviving biocide treatments and the differences among the microbiomes of the different biocide schemes as reflected by changes in species, as shown by A) PC scores and B) PC loadings. Microbiome composition is based on an analysis of 1096 colonies, with 42 ± 20 isolates from the selected biocide schemes.

Metagenomic assessment of regrowing planktonic microbiome

The microbiome in the suspension that regrew around the biofilms that survived the biocide treatments from Monday to Friday (Day 5) was generally dominated by *Pseudomonas* (40-52%) and *Serratia* (17-33%) regardless of the biocide concentration (MRC or 2×MRC) with no significant ($P_{corrected}$ > 0.05) difference in the overall microbiome composition (Appendix: Sup-

plementary Table S3, Figure 16A). However, the different selective pressures from the biocides could be seen when the microbiomes were grouped based on the primary biocide in the treatment (Table 5, Figure 16B). Biocide schemes dominated by BC or PAA caused *Serratia* (66%) and *Pseudomonas* (78%), respectively, to dominate (Appendix: Supplementary Table S4). In contrast, SH-based biocide schemes had a less clear selective effect on the regrowing microbiome with the biocide microbiome composition being the least different from the water controls. When microbiomes were averaged and grouped based on the number of biocides used in the scheme (Figure 16C), the dissimilarities based on the primary biocides were diminished except for a higher proportion of *Yersinia* when two biocides were used in rotation (Appendix: Supplementary Table S5). The selective nature of the biocides was, therefore, strongest seen for each individual biocide scheme, with the extremes of PAA (P-P-P-P-P) and BC (B-B-B-B-B) used without rotation for a week causing nearly sole dominance of *Pseudomonas* and *Serratia*, respectively (Figure 16D). Only the water controls and the biocide scheme (P-B-P-S-P) had *Listeria* abundances ≥ 1%, however, with the similar biocide scheme (P-S-P-B-P) with no *Listeria* (Appendix: Supplementary Table S6).

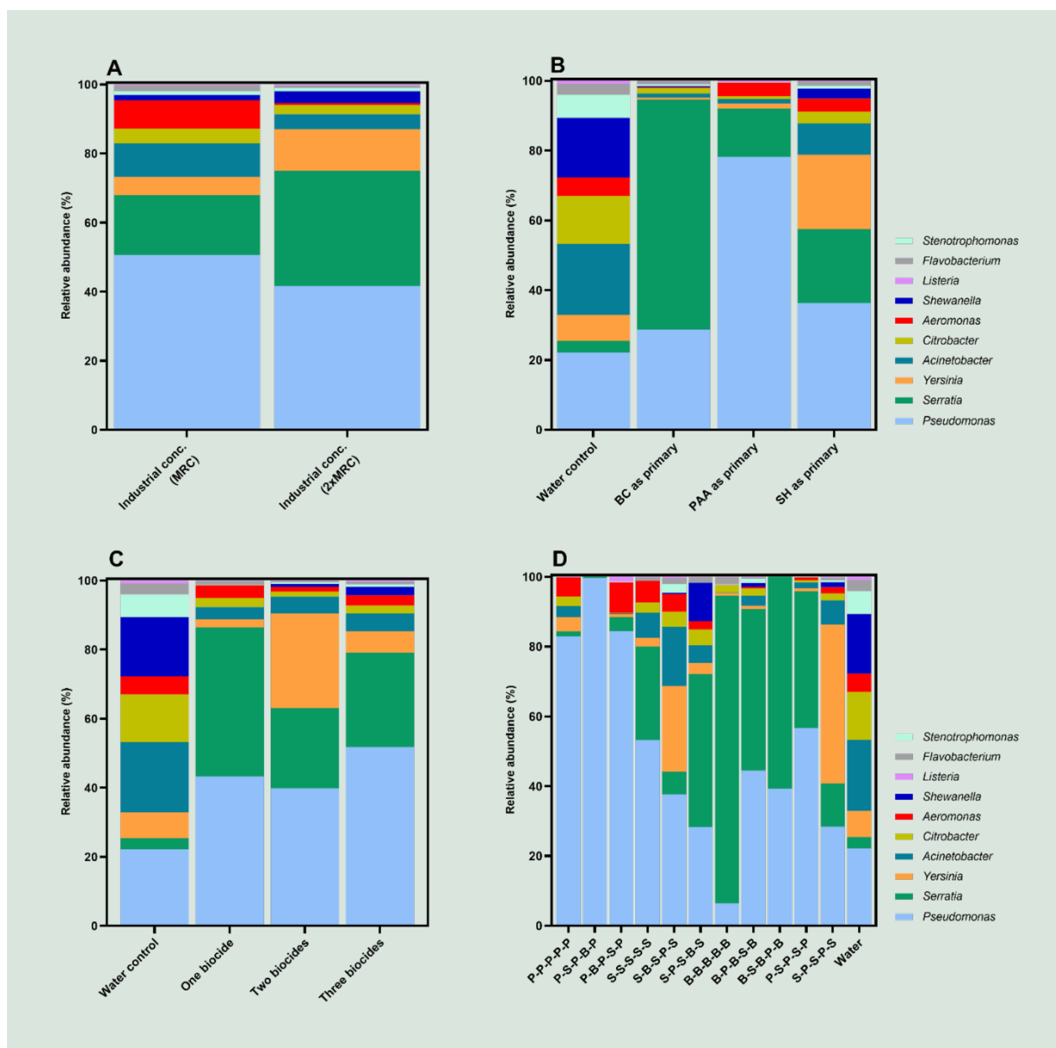


FIGURE 16. Culture-independent analysis of the microbiome regrowing in the fresh suspensions surrounding the biofilms reflecting cells detaching from the biofilms and growing between days 5 and 8. Genus abundances are based on shotgun metagenomic sequencing of biological independent replicates from each biocide scheme where four samples from the same scheme were pooled before sequencing (n=24 from 2×MRC and 12 from MRC, n_{total}=36). Metagenomes were analysed, and abundance was grouped and visualized based on A) Biocide concentration, B) Biocide treatment, C) Number of biocides in rotation and D) Biocide scheme.

Metagenomic assessment of potential resistance gene transfer in biofilms

The analysis of assembled contigs from the 36 metagenomes of the suspensions regrowing around the biofilms on day 8 revealed that none of the efflux pumps *oqxB* or *qacH* were found in contigs other than contigs which could be identified as *S. marcescens* and *L. monocytogenes*, respectively (Appendix: Supplementary Table S7 and S8). The analysis did, however, show that even before the start of the biocide rotation experiments, *Pseudomonas anguilliseptica*, *Pseudomonas chengduensis* and *Stenotrophomonas rhizophila* all carried an efflux pump with >98% similarity to the *oqxB* gene in *S. marcescens*.

6.4 Discussion

This sub-project showed that rotation between two or three biocides at industrial concentrations is not necessary or better to prevent a build-up of increased biocide tolerance, as just one of 24 biocide schemes using 2×MRC resulted in increased tolerance, and with very similar biocide schemes showing similar or decreased tolerance after ten days it is questionable if a

specific tolerance was developed (Table 6). Indeed, it has previously been shown in suspension tests using *Listeria innocua* that preexposure to SH, BC and PAA did not confer neither increased tolerance to the same nor to other biocides (Rahman et al., 2022). In contrast, (Fagerlund et al., 2020) saw increases in tolerance to biocides of *L. monocytogenes* biofilms formed on polyvinyl chloride (PVC) conveyor belt coupons with biocide treatments on days 4, 5, 6, and 7, but the authors emphasized that they could not rule out biofilm age as being responsible for the effects as the biofilms became tolerant to all biocides tested. Similar observations, however, less pronounced and not statistically tested tolerance build-up were observed by Lake et al. (2024) using multi-species biofilms on PVC conveyor belt coupons with cleaning and disinfection every second day during an 8-day period. However, as in the present study, Lake et al. (2024) did not see a significant tolerance development following repeated disinfection of biofilms formed on stainless steel coupons, despite the presence of a surviving microbiota throughout the experiment. The observation of biocide tolerance development on PVC, but not on stainless steel clearly points to the impact of the material upon which the biofilm forms, with PVC having more irregularities allowing for better biofilm establishment (Fagerlund et al., 2017). Comparisons of the efficacy of biocide rotation schemes on biofilms were not included in the aforementioned studies, but as shown in the present study, the daily use of PAA or SH without rotation was equally efficient as biocide rotation schemes in terms of microbial inactivation but caused differences in the surviving and regrowing microbiomes (Table 6, Figure 15 and 16). The latter indicates that biocide usage schemes should be designed to better control the most biofilm-forming genera in an FPE which may depend on the type of production, raw materials, surfaces of equipment, etc. Thus, the use of biocides and potential daily or weekly rotations of biocides could be supported by environmental monitoring programs of the surviving microbiome and pathogens of concern as also proposed by the EFSA BIOHAZ Panel (Koutsoumanis et al., 2024).

Rotation of biocides could, therefore, serve the specific purpose of limiting or interfering with the dominance of certain genera, such as *Pseudomonas* and *Serratia*, rather than the purpose of preventing development of tolerance to biocides caused by biocide tolerance genes spreading or developing. In addition, we were not able to detect HGT of the efflux pump *oqxB* carried by *S. marcescens*, *S. rhizophila, P. anguilliseptic*a and *P. chengduensis* or *qacH* carried by *L. monocytogenes* (Table S7-8) in our floor drain biofilm model system over the 8-day experimental protocol. Based on this analysis we cannot conclude if biocide rotation can prevent HGT of biocide tolerance genes in biofilms that survive for extended periods of time in hard-to-reach niches in the food industry. It was, however, clear that while the *S. marcescens* isolate benefits from a higher tolerance to BC, its dominance in BC-treated biofilms is likely due to the regrowth in the presence of low levels of BC residuals and less likely due to increase tolerance to industrial concentrations (i.e., MRC) of the biocide (Kragh et al., 2025). We and others have similarly observed how *L. monocytogenes* isolates with and without BC efflux pumps do not have increased tolerance to higher concentrations of BC (Kragh et al., 2024; Møretrø et al., 2017). Considering this it is no surprise that despite *S. rhizophila* carrying *oqxB* similar to the one in *S. marcescens*, we did not see this isolate regrowing to high abundances after BC treatments (Figure 16). Also, *oqxB* carrying *P. anguilliseptica* and *P. chengduensis* were much less prevalent than *P. cremoris* (Figure 14). For SH and PAA, there should be even fewer concerns as specific genetic markers associated with increased biocide tolerance have so far not been identified, i.e., despite many years of researching these biocides, the screening has been unsuccessful in finding isolate-specific differences in tolerance (Ivanova et al., Preprint.; Kragh et al., 2024; Palma et al., 2022). In addition, adaptation studies focusing on developing tolerance to SH and PAA have been negative (Aarnisalo et al., 2007; Kastbjerg & Gram, 2012). One could discuss if biofilm experiments with repeated biocide treatments should be considerably longer to document HGT of biocide genes and general biocide tolerance development due to other factors than biofilm age and density, but the above-mentioned limited success of finding different tolerance profiles in factories, which have been using the same biocide over longer

periods of time means that such experiment is already been conducted continuously in the industry (Palaiodimou et al., 2021). This is reflected in the present study, which is based on floor drain samples from the industry where *qac* genes made up a significant proportion of the resistome (Kragh et al., 2025). Even so, and with multiple BC efflux pumps in several isolates, the biocide rotations that included BC on one weekly day were among the best-performing biocide schemes (Figure 13). It is possible that the low performance of biocide rotations based solely on BC would be better if newer generations and mixtures of quaternary ammonium compounds were used (Willmott et al., 2024).

Together, this implies that biocide rotation could still have a place in FPE where the continued efficacy of the cleaning and disinfection program should be assessed by an environmental monitoring program. However, as the persistence of foodborne pathogens clearly depends on multiple factors, an important aspect is to properly educate staff and cleaning personnel in good hygienic control and allocate sufficient resources for cleaning and disinfection, which are recommendations that have been repeated for 25 years (Tompkin, 1999). This includes maintaining surfaces and avoid poorly hygienically designed equipment to minimize niches where biofilm may build up as it is clear not even the best biocide scheme will be efficient if it does not reach the biofilm in the necessary concentration.

In conclusion, it was observed that rotation between two or three biocides at industrial concentrations is not necessary or better to prevent a built-up of increased biocide tolerance (log CFU/SSC), as just one of 24 biocide schemes resulted in significantly ($P > 0.05$) increased tolerance. In general, biocide rotation schemes that were based on a combination of either PAA and SH every second day with BC and SH or PAA used on two other days were among the best-performing biocide schemes, however, with similar results for biocide schemes using just SH or PAA. None of the biocide schemes resulted in HGT of BC efflux pumps *oqxB* or *qacH*, despite these genes being carried by multiple isolates. Interestingly, not all isolates benefited from this carriage as only *S. marcescens* clearly dominated after a week with multiple treatments of BC. The biocide schemes had different selective impacts on the microbiome, but overall, all saw high numbers of *P. cremoris* (38%) dominating in the surviving biofilms. Strategic use of multiple biocides could possibly be implemented to make sure that all genera are targeted equally well. The great dominance of *Pseudomonas* and *Serratia* does, however, imply that specific interventions limiting these genera could be advantageous for minimizing biocide tolerance of multi-species biofilms using phages or novel compounds. Further studies should thus focus on additional ways to prevent biofilm formation and be supported by a continued focus on identifying the multiple factors that enable various genera to support the persistence of specific foodborne pathogens such as *L. monocytogenes*.

7. Discussion

In the last five to ten years there has been an increasing number of studies investigating the potential for development of increased tolerance or even resistance to biocides. The COVID-19 pandemic further sparked the concern that overuse and misuse could lead to the developing and spreading of tolerance to both biocides and antibiotics (Chen et al., 2021; Merchel Piovesan Pereira et al., 2021; Skandalis et al., 2021). However, most studies of biocide tolerance have been focused on the QACs because the clear link between the presence of efflux pumps and elevated MIC has been easy to make. This has led to numerous studies showing how isolates of *L. monocytogenes* can show higher tolerance to low concentrations of BC with tolerant isolates having 2-8× higher MIC-values (2.5-10 mg/L) compared to other isolates depending on the testing protocol (Luque-Sastre et al., 2018; Meier et al., 2017; Møretrø et al., 2017; Palma et al., 2022; Roedel et al., 2019, Ivanova et al., preprint). In a few cases, increased tolerance has been observed without the presence of the known QAC efflux pump genes, possibly related to the overexpression of intrinsic efflux pumps (Romanova et al., 2006). We were in our work (Sub-project 1) equally able to find all four known QAC tolerance genes (*qacH*, *bcrABC*, *emrC*, *emrE*) in a library of 240 *L. monocytogenes* isolates and showed that they are equally effective in increasing the QAC tolerance in *L. monocytogenes* when tested using the same MIC assay. We did, however, also show that these genes have no influence on the tolerance to just slightly higher concentrations, meaning that these genes are of little concern as long as the industry uses the recommended concentrations (Sub-project 1). This was equally confirmed by the work presented in Sub-projects 2 and 3, however, with the observation that residuals of QAC left behind after treatments might create a niche where some bacteria, such as *S. marcescens*, could benefit from genetically mediated elevated MICs towards BC. This can happen in the industry where BC residuals could be considered to both be a problem, selecting for unwanted bacteria with higher MIC levels, and an advantage since residuals will extend the duration of the effect of the biocide (He et al., 2022; Møretrø et al., 2017). However, we observed that BC treatment at industry concentrations of our floor drain biofilm model with *L. monocytogenes* ST121 did not lead to higher levels of *L. monocytogenes* despite this isolate carrying *qacH*, which should give the isolate an advantage (Sub-project 2). A similar observation could be made for three other isolates in the biofilm model, which carried QAC efflux pumps (Sub-project 3). This view on QAC tolerance genes has been shared by other researchers that points to the large gap between elevated MICs from QAC genes and the high concentrations used in the industry (Bland, Brown, et al., 2022; Willmott et al., 2024b). Taken together, these observations highlight that it surely can be a problem that some bacteria are able to withstand low concentrations of QACs better than others, but proper cleaning and correct dosing of biocides will still inactivate these bacteria. In addition, we saw that these efflux pumps are frequently present in floor drains within food processing environments (Sub-project 2), which could lead to concerns regarding the possible development of new QAC efflux pumps or HGT. However, when looking for signs of HGT in the week-long biocide challenge on the drain biofilm model composed of 31 bacteria, we found no evidence of HGT meaning that it maybe should not be a concern in the food industry (Sub-project 3).

Use of QAC has been suspected to lead to possible cross-resistance to antibiotics, however, conflicting results have been presented (Bland, Waite-Cusicet al., 2022; Guérin et al., 2021; Roedel et al., 2019). While new formulations of QACs are marketed as more effective (Willmott et al., 2024a), our observations of BC being the most selective and least effective biocide among those tested (Sub-projects 2-3) mean that it is worthwhile focusing on using other biocides such as SH and PAA, either alone or in rotation schemes. We did not see any difference in tolerance to these biocides among 240 *L. monocytogenes* isolates, representative of the diversity of this foodborne pathogen. More importantly, there has to the best of our knowledge

not been any scientific reports of specific tolerance genes to SH or PAA. This can likely be linked to the broad mechanism of action for these biocides (Maillard, 2018). As would be expected, we did not see QAC tolerance genes have any effect on survival when SH or PAA were used, which again confirms that QAC tolerance genes are not an issue when either SH or PAA is the biocide used. It would be reasonable to assume that this also goes for hydrogen peroxide due to its similar mode of action. As we both tested a large collection of *L. monocytogenes* (n=240) isolates from the food industry for biocide tolerance and tried to develop biocide tolerance in a subset of these it is clear that we can answer the first research question by concluding that foodborne pathogens as *L. monocytogenes* can indeed develop tolerance to low levels of QAC-based biocides, but not oxidizing agents such as SH and PAA. We and others have, in addition, shown that *L. monocytogenes* isolates that are carrying the QAC tolerance genes cannot be further adapted to BC, while those without the genes can only be adapted to withstand the same increased concentration as those carrying the tolerance genes (Bolten et al., 2022). This again highlights that there is no basis to believe that QAC tolerance should suddenly increase further.

However, as the oxidizing agents SH and PAA showed lower specific selective pressure on the surviving microbiome and regrowing biofilm whilst performing best in terms of microbial inactivation, it seems reasonable to suggest that these biocides be used more frequently than QACs (Sub-project 3). Interesting, we observed that rotation with just these two biocides (SH and PAA) had a lower effect on reducing biofilm populations than when used alone, which needs further investigation as we had expected similar or higher efficacy of the SH-PAA rotation. However, using the PAA or SH alone was very effective with no increase in tolerance (Sub-project 3). We did see that using BC once a week had a positive effect on the biofilm concentrations, which could be related to the effect of the residuals preventing strong biofilm regrowth. This could imply that BC could have a role in a biocide rotation scheme. Generally, we did, however, not see biocide tolerance develop, except for in one of the 24 biocide treatment schemes tested, which means that for the second research question, it should be concluded that rotation of different biocides is not needed to prevent the development of biocide tolerance (Sub-project 2-3). It is, however, evident from other studies that depending on the equipment material, biofilms themselves can mature to become more biocide tolerant as was seen in other studies when biofilms were formed on PVC (Fagerlund et al., 2020; Lake et al., 2024). This could, however, be more explained by the physical barrier of the biofilm that is made by the excreted extracellular polymeric substances, but also because this organic load makes the biocides less efficient, as we also saw when dirty conditions and biofilms were simulated (Sub-project 1). In fact, we saw that biofilms and dirty conditions had a greater impact on biocide efficacy than the QAC tolerance genes. This implies that the most important factor when it comes to biocide tolerance is most likely not a sophisticated biocide rotation scheme but simply making sure that biofilms are not left untouched for too long. There are many factors contributing to the formation of biofilms, but temperature and residual foods, together with inadequate cleaning, are the most obvious to point out (Maillard & Centeleghe, 2023). Preventing thick and old biofilm is, therefore, a question of setting aside sufficient resources for cleaning to remove soils and application of biocides in the right concentrations. Moreover, making sure that enough resources are allocated to the education of staff, replacing worn surfaces and equipment, as well as prioritising the proper hygienic design of the production equipment. Here, it is similarly worthwhile mentioning that the studies within this project were done without prior cleaning, and therefore, the difference in efficacy between the biocide schemes using MRC and 2×MRC reflects the worst-case situations of insufficient cleaning in the industry. In addition, we offered the biofilms a good opportunity to grow with the addition of fresh media every day, simulating insufficient removal of food residues during the daily cleaning. It must, therefore, be expected that with proper cleaning before disinfection, the MRC is sufficient. The achievement of levels of “proper cleaning” should be checked with the factories' environmental monitoring program. The environmental monitoring program, including analysis of trends in hygienic levels, is also what should guide the industry when considering if biocides

should be changed and help when a “seek and destroy” practice is used to counter persistent problems (EFSA BIOHAZ Panel, 2024). Finally, it is important to communicate that for many food products, it cannot be overstated how important it is to be aware that a perfectly clean food processing environment is very difficult to obtain and that many raw materials can contain low concentrations of pathogens of concern. This means that while a proper cleaning and disinfection program is an important barrier to limit contamination with foodborne pathogens, addition hurdles should be considered whenever possible to design food products in a way that limits microbial survival and/or growth possibilities in products that are distributed, sold and consumed.

8. Conclusion

In conclusion, *L. monocytogenes* isolates showed limited variation in sensitivity to biocides in MIC assays, and it was clear that generally, biocide efficacy was more affected by residual organic matter and/or biofilm, demonstrating the need for proper cleaning prior to biocide application. Among the biocides PAA was least affected by biofilm and organic matter. It has positive important industrial and safety perspectives that the known biocide tolerance genes *qacH*, *bcrABC*, *emrC* and *emrE* did not give isolates with one of these genes any increased survival when the QAC concentrations was increased to concentrations more relevant to the ones used in the food industry. Further, these efflux pumps offered no protection to either low or higher concentrations of PAA, SH or ET. This is especially important since these genes and other efflux pumps were frequently detected in the floor drains within fish, shrimp and cheese production facilities. However, we also saw that these efflux pumps can, in certain species, lead to species dominating after the application of QAC-based biocides, while these pumps in other bacteria, such as *L. monocytogenes*, seem to not confer enough increase in tolerance to play a significant role in their competitiveness. In general, the large gap between MICs of strains with “biocide tolerant” phenotypes and the in-use industrial MRC of biocides means that there should be little concern about adaptation to biocides being a risk factor. We did not see any biocide schemes resulting in either HGT of QAC efflux pumps, increased biocide tolerance or a significant difference between biocide rotation and no rotation. Together, this means that there is no clear conclusion as to whether biocide rotation is needed. However, as seen in this project, different biocides have different selective pressures towards which bacteria survive and subsequently dominate in the regrowing biofilm, which can affect the biofilms that may form between production cycles. Applying a monitoring program, especially in factories with persistent problems, can be a solution in combination with the rotation of biocides. It is, however, crucial to highlight that we saw how soiled conditions and presence of biofilms had a greater negative impact on biocide efficacy than genetic determinants. This means that equipment and surfaces that are inadequately cleaned before biocide treatments are the biggest issues when it comes to inefficient biocide schemes. This implies that before using resources on designing and changing biocide rotation schemes or strategies it is also important to properly educate staff and cleaning personnel in good hygienic practises and allocate sufficient resources for cleaning and disinfection. This includes maintaining surfaces and replacing poorly hygienic-designed equipment to minimize niches where dirt and biofilm may build up as not even the best biocide schemes will be efficient if it does not reach the biofilm in the necessary concentration. In relation to this, it should thus be noted that increasing biocide concentrations to levels above the recommended industrial concentrations should, therefore, not be necessary to prevent biocide tolerance and should hence be avoided from an environmental, resource and health perspective. In cases with surviving biofilm, additional mechanical and chemical cleaning steps should be performed before re-application of new/increased biocide concentrations.

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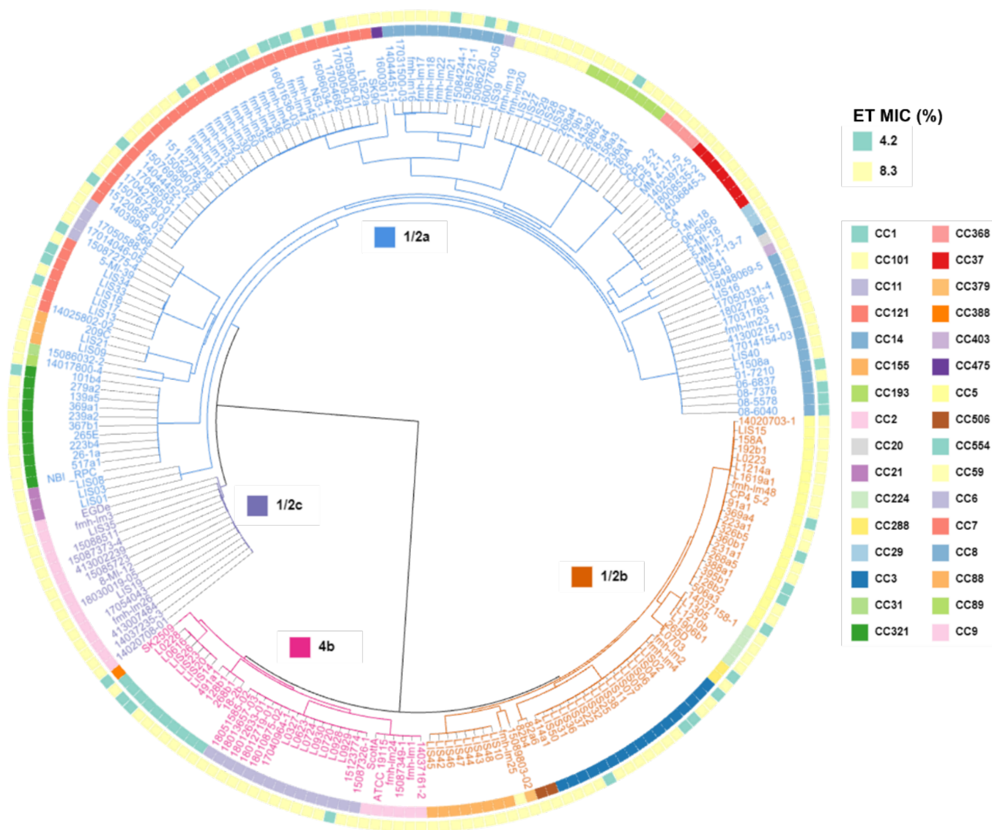
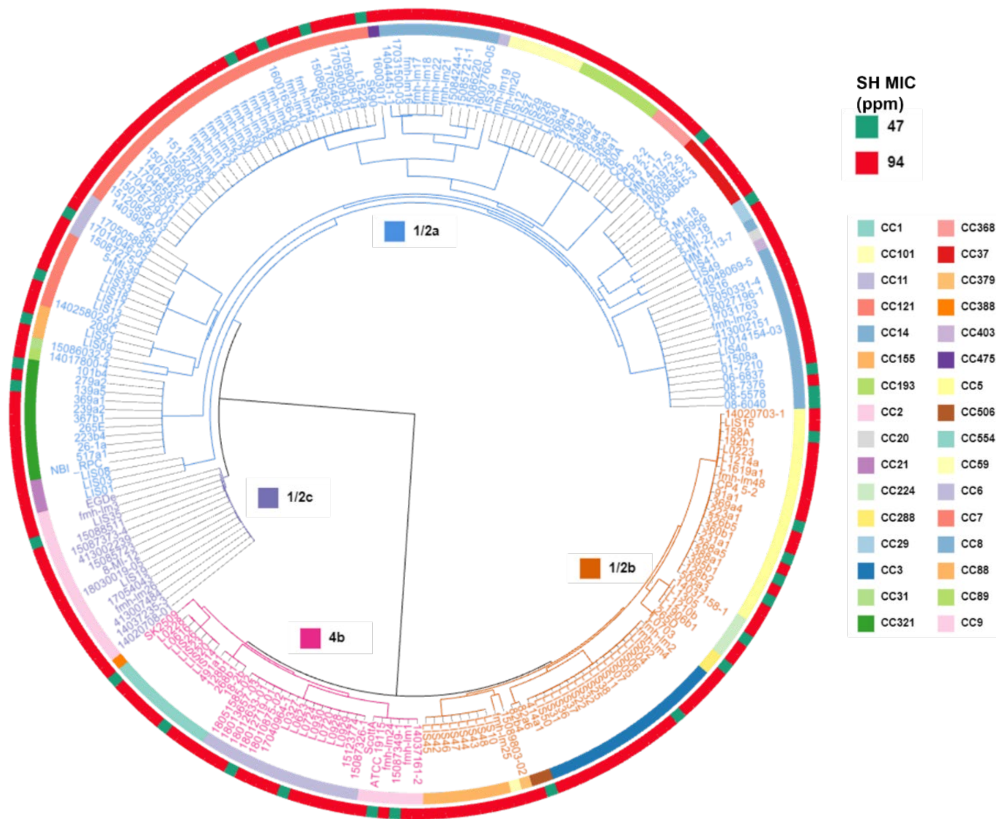
Appendix 1. Sub-project 1

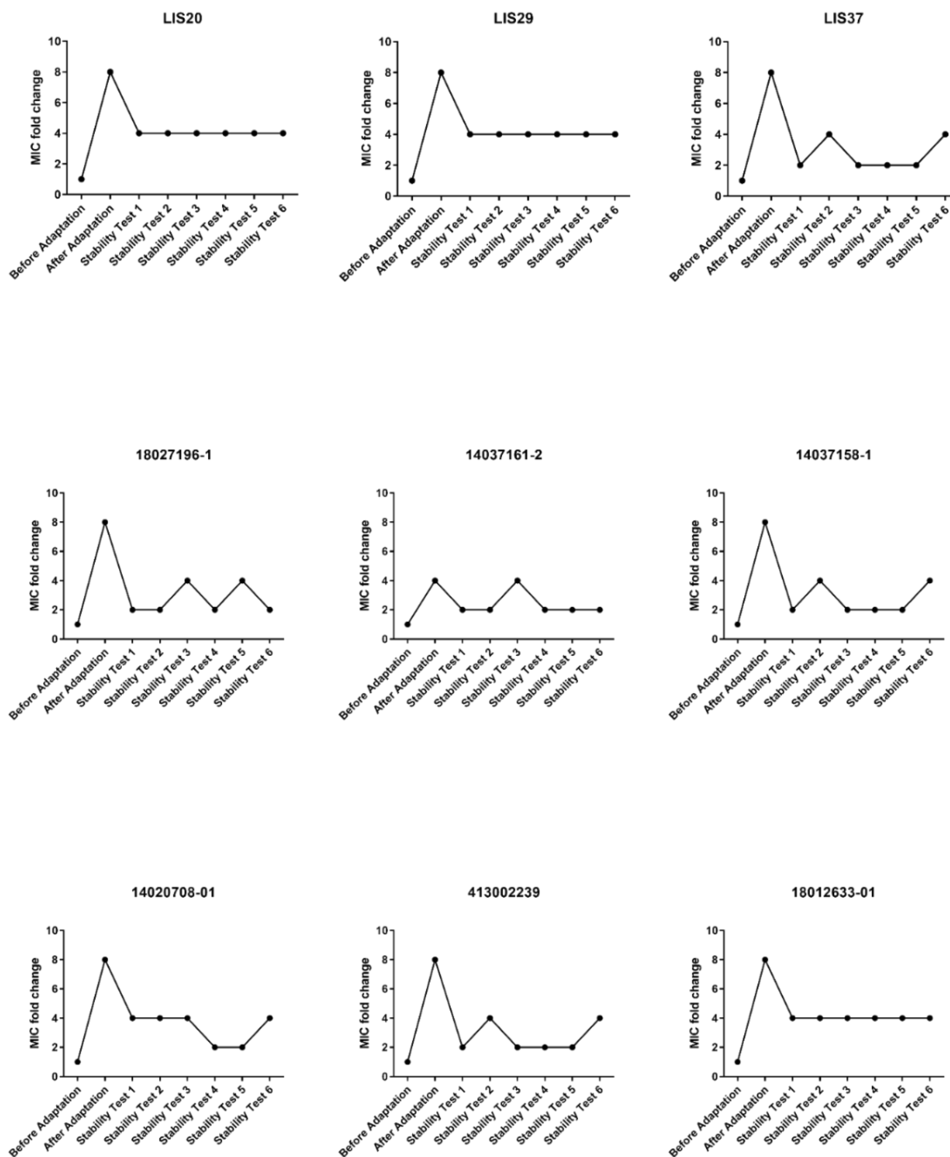
Supplementary Table S1 is an Excel data sheet that can be downloaded at:
https://ars.els-cdn.com/content/image/1-s2.0-S0956713523006448-mmc1.xlsx
The supplementary data is part of the publication published in the Open-Access journal Food Control with the doi: https://doi.org/10.1016/j.foodcont.2023.110244.



Supplementary Figure S1. Phylogenetic visualization of 221 *L. monocytogenes* isolates. Isolate nodes are colored based on serotypes (1/2a, 1/2b, 1/2c or 4b) with isolate names connected to the node. The tree is surrounded by a colored ring indicating corresponding clonal complexes (CC, n=32) for each isolate and their MIC value to ethanol (ET).



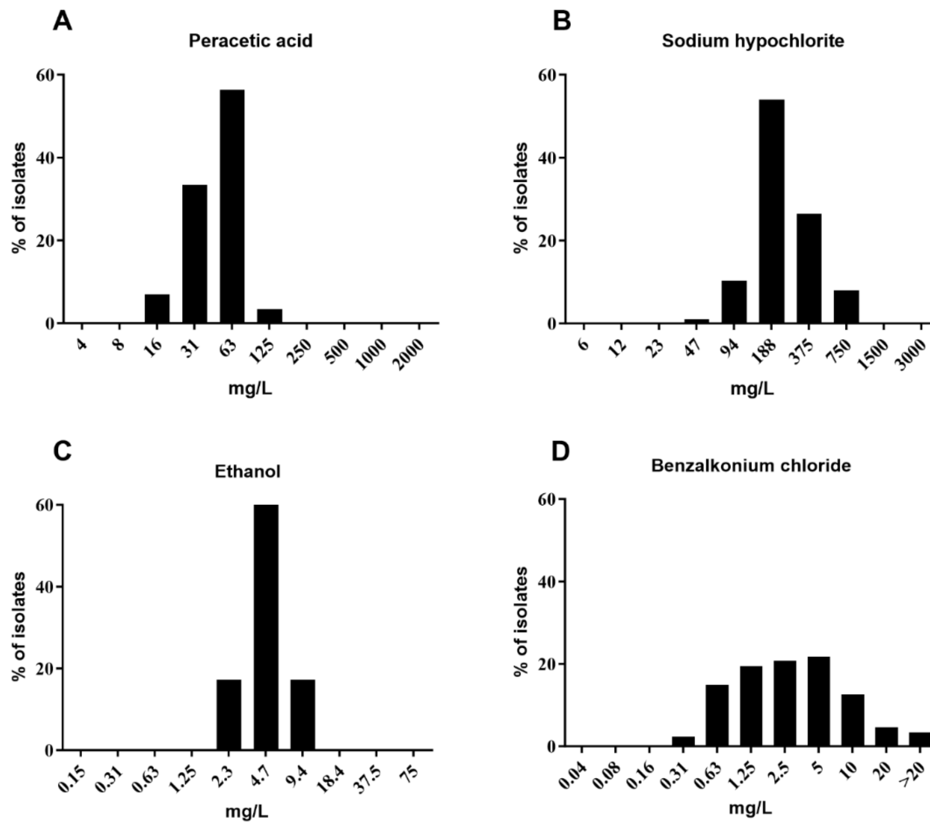
Supplementary Figure S2. Phylogenetic visualization of 221 *L. monocytogenes* isolates. Isolate nodes are coloured based on serotypes (1/2a, 1/2b, 1/2c or 4b) with isolate names connected to the node. The tree is surrounded by a coloured ring indicating corresponding clonal complexes (CC, n=32) for each isolate and their MIC value to sodium hypochlorite (SH).



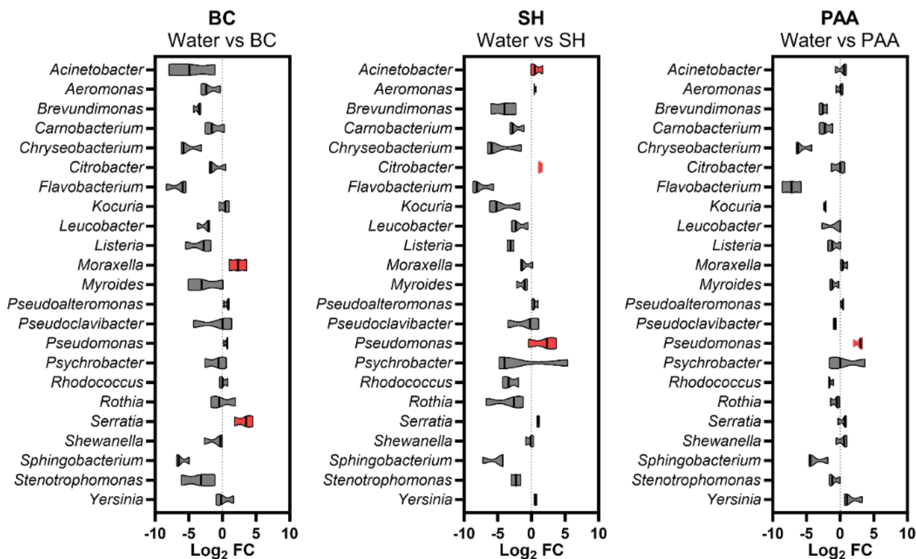
Supplementary Figure S3. Fold increase of benzalkonium chloride (BC) adapted of *L. monocytogenes* after sub-culturing in BC for 18 days. Nine originally sensitive isolates showed increased MIC (4-8x fold), but sub-culturing through six stability tests (12 days) without BC showed loss of some of the acquired tolerance.

Appendix 2. Sub-project 2

Supplementary Tables S1-S11 is an Excel data sheet that can be downloaded at: https://www.frontiersin.org/articles/10.3389/fmicb.2025.1542193/full#supplementary-material as part of the publication published in the Open-Access journal Frontiers Microbiology with the doi: https://doi.org/10.3389/fmicb.2025.1542193.

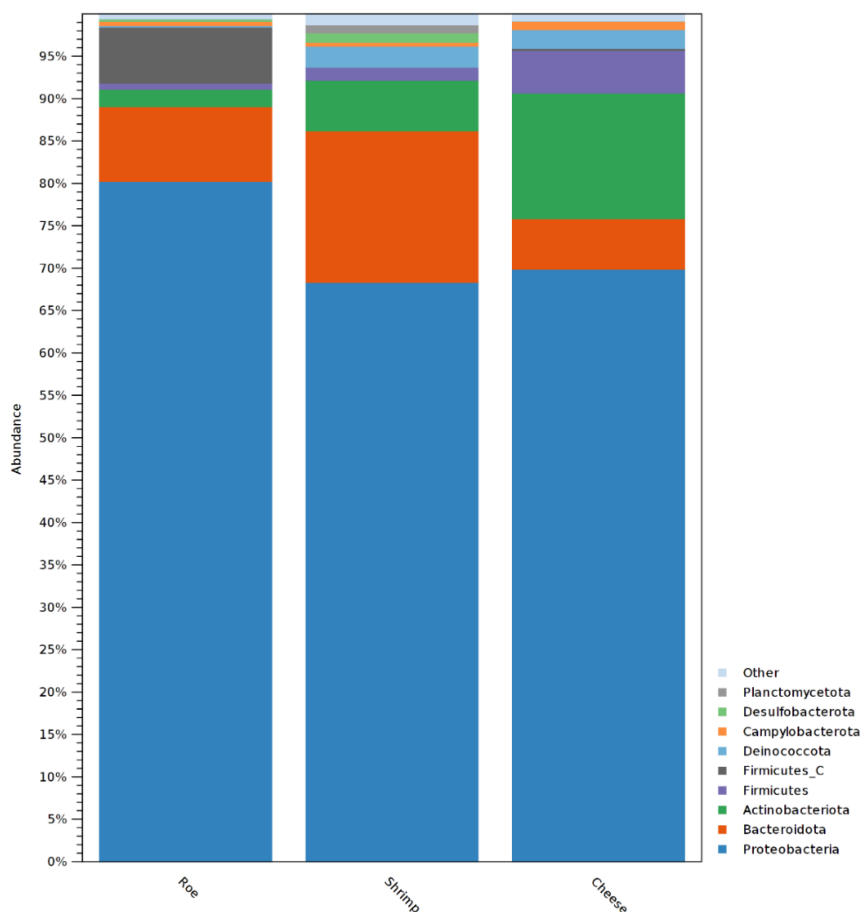


Supplementary Figure S1. Distribution of MIC-values for 87 representative drain isolates. MIC-values were determined in a minimum of two independent biological experiments with triplicates for the biocides A) peracetic acid, B) sodium hypochlorite, C), ethanol and D) benzalkonium chloride.



Supplementary Figure S2. Differential abundance analysis on changes in relative genus abundance of the regrown biofilm microbiome on day six after treatments with either benzalkonium chloride (BC), sodium hypochlorite (SH) or peracetic acid (PAA) on day three based on the comparison against the regrown control biofilm on day 6, which was treated with sterile water on day 3. The Log$_2$ fold change depicted is the average of the fold change observed in each

comparison of all three biocide concentrations (n=4), against the control (n=4). A) peracetic acid, B) sodium hypochlorite, C), ethanol and D) benzalkonium chloride. Genus boxes in red were significantly ($P_{corrected} < 0.05$) increased in relative abundance compared to controls when all biocide concentrations (n=12), were compared against the control (n=4).



Supplementary Figure S3. Taxonomic profile of all 14 drains aggregated by food production environment. Cheese n=6, roe n=4, shrimp n=4. Aggregated by phylum. Since 2021 several phylum have been renamed: Proteobacteria is officially known as Pseudomonadota, Actinobacteriota as Actinomycetota. Firmicutes as Bacillota.

Appendix 3. Sub-project 3

Supplementary Tables S1-S8 are an Excel data sheet that can be requested at malak@food.dtu.dk and online once the manuscript has been peer-reviewed.

Biocide rotation and control of microbial resistance development in the food industry

This research project aimed to investigate if foodborne pathogens, e.g., *Listeria monocytogenes*, develop tolerance to biocides and whether the rotation of different biocides can help prevent the development of biocide tolerance.

Results showed that *L. monocytogenes* could increase its tolerance to benzalkonium chloride (BC), a quaternary ammonium compound, but not to sodium hypochlorite (SH) or peracetic acid (PAA). However, BC minimum inhibitory concentrations (MIC) increased only by a few mg/mL, having no impact on the efficacy of recommended industrial concentrations (hundreds to thousands mg/ml). Also, survival of *L. monocytogenes* isolates with BC tolerance genes (*qacH*, *bcrABC*, *emrC* and *emrE*) was not improved when exposed to industrial concentrations of BC. Overall, *L. monocytogenes* isolates (n=240) showed limited variation in their sensitivity to biocides. Rather, biocide efficacy was more affected by residual organic matter and/or biofilm, demonstrating the need for proper cleaning prior to biocide application.

To investigate the effect of biocides on industrially relevant bacteria, floor drain samples from the food industry were used to develop a representative (31 species, 24 genera) biofilm model. Biocide treatments on this model showed that rotation between two or three biocides at industrial concentrations is not necessary to prevent increased biocide tolerance compared to single use of the same biocide. Individual biocides affect, which bacteria dominate in the regrowing biofilm. While rotation schemes based on PAA or SH with BC used once a week were among the best-performing biocide schemes, schemes using one biocide (SH or PAA) had similar performance, indicating rotation is not necessary.

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