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A novel metagenomic approach uncovers phage genes as markers for increased disinfectant tolerance in mixed *Listeria monocytogenes* communities

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ABSTRACT

Listeria monocytogenes is an important human pathogen with a high mortality rate. Consumption of contaminated ready-to-eat food is the main mode of transmission to humans. Disinfectant-tolerant *L. monocytogenes* have emerged, which are believed to have increased persistence potential. Elucidating the mechanisms of *L. monocytogenes* disinfectant tolerance has been the focus of previous studies using pure cultures. A limitation of such approach is the difficulty to identify strains with reduced susceptibility due to inter-strain variation and the need to screen large numbers of strains and genes. In this study, we applied a novel metagenomic approach to detect genes associated with disinfectant tolerance in mixed *L. monocytogenes* planktonic communities. Two communities, consisting of 71 and 80 isolates each, were treated with the food industry disinfectants benzalkonium chloride (BC, 1.75 mg/L) or peracetic acid (PAA, 38 mg/L). The communities were subjected to metagenomic sequencing and differences in individual gene abundances between biocide-free control communities and biocide-treated communities were determined. A significant increase in the abundance of *Listeria* phage-associated genes was observed in both communities after treatment, suggesting that prophage carriage could lead to an increased disinfectant tolerance in mixed *L. monocytogenes* planktonic communities. In contrast, a significant decrease in the abundance of a high-copy *emrC*-harbouring plasmid pLmN12-0935 was observed in both communities after treatment. In PAA-treated community, a putative ABC transporter previously found to be necessary for *L.*

monocytogenes resistance to antimicrobial agents and virulence, was among the genes with the highest weight for differentiating treated from control samples. The undertaken metagenomic approach in this study can be applied to identify genes associated with increased tolerance to other antimicrobials in mixed bacterial communities.

INTRODUCTION

The *Listeria* genus consists of small rod-shaped, non-sporing Gram-positive bacilli and currently comprises 26 species (Carlin et al., 2022), out of which only *Listeria monocytogenes* and *Listeria ivanovii* are considered pathogenic (Greenwood, 2018; Orsi and Wiedmann, 2016). *L. monocytogenes* is known as one of the most important foodborne human pathogens due to the high mortality rates associated with listeriosis and its persistence in food processing environments (FPEs) (Disson et al., 2021; Palma et al., 2020). Ingestion of *L. monocytogenes* can cause listeriosis in humans, which predominantly affects three well-defined risk groups, namely pregnant women, unborn or newly delivered infants and immunocompromised adults such as the elderly or individuals with predisposing conditions (Greenwood, 2018; Skandamis N., 2021).

The main mode of infection with *L. monocytogenes* is the consumption of contaminated ready-to-eat (RTE) foods. RTE foods can support growth and, when consumed by susceptible groups, can contribute to an increased burden of listeriosis, (both as sporadic cases or in (multi)national outbreaks (Ricci et al., 2018). Contamination of final food products can occur at any stage of food production, but contamination within food processing environments (FPEs) is considered the most important (Greenwood, 2018). Because *L. monocytogenes* can survive well on both dry and moist surfaces, it can persist in different sites within FPEs. Its persistence can be favoured by e.g., improper cleaning and disinfection (C&D) procedures, poor equipment design (Belias et al., 2022), reintroduction of genotypes that efficiently persist in raw materials or external habitats, among others. Certain *L. monocytogenes* genotypes have increased tolerance to environmental stressors, which provides them with competitive advantage to persist in the FPEs (e.g. reduced sensitivity to disinfectants, genetic determinants for increased stress tolerance, ability to attach to abiotic surfaces and form biofilms) (Cherifi et al., 2018; Magalhães et al., 2017). Therefore,

implementing efficient C&D programs to control contamination of *L. monocytogenes* in FPEs is critical. Commercial disinfectants used in the food industry commonly contain biocide substances from the quaternary ammonium compounds (QACs), peroxides and chlorine antimicrobial classes (Palma et al., 2022a). QACs such as benzalkonium chloride (BC) and didecyl dimethyl ammonium chloride (DDAC) are membrane-active agents which mainly interfere with the cytoplasmic membrane in bacteria, but also bind intracellularly to DNA (Gerba, 2015), whereas peroxides such as hydrogen peroxide (HP) and peracetic acid (PAA) are broad-spectrum biocides that work as oxidants by producing free hydroxyl radicals. They can oxidise thiol groups of enzymes and proteins and are believed to interact with the cellular phospholipid bilayers and cytosolic material (McDonnell and Russell, 1999; Palma et al., 2022a). Few specific genetic determinants associated with an increased *L. monocytogenes* tolerance to oxidative agents such as PAA and sodium hypochlorite have been described, possibly due to their multiple modes of action (Manso et al., 2020; McDonnell and Russell, 1999; Palma et al., 2022b). However, genes involved in increased tolerance to low concentrations of QACs in *L. monocytogenes* have previously been reported and involve the plasmid-mediated efflux pumps genes *bcrABC* and *emrC* (Elhanafi et al., 2010a; Kremer et al., 2017) and the chromosomally-integrated *qacH* gene on Tn6188 (Müller et al., 2013) and *emrE* gene on genomic island LGI1 (Kovacevic et al., 2016a) Other efflux pumps genes (e.g., *lde* and *mdrL*) have also been described to be involved in increased QACs tolerance in *L. monocytogenes*, however, previously they have been detected in both QACs tolerant and sensitive isolates (Ivanova et al., 2023).

Previous studies aiming to elucidate the genetic basis of the increased tolerance to food industry disinfectants have been conducted on single *L. monocytogenes* isolates using genomic and transcriptomic analysis (Assisi et al., 2021; Casey et al., 2014; Jiang et al., 2020; Manso et al.,

2020). These studies focused on whole genome sequencing or gene expression of pure cultures, which makes it difficult to identify tolerant strains and mechanisms of increased tolerance, mainly because of the need to screen large number of strains and the potential complex mechanisms of reduced susceptibility. In this study, we apply metagenomics as a novel approach to identify the genes associated with increased tolerance in mixed planktonic *L. monocytogenes* communities to two of the commonly used disinfectants in the food industry, namely BC and PAA as representatives of QACs and peroxides, respectively. Additionally, we investigated the distribution of plasmids, known disinfectant tolerance genes and virulence genes in the communities before and after biocide treatment.

MATERIAL AND METHODS

Listeria monocytogenes isolates and design of mixed communities

Two mixed *L. monocytogenes* communities were designed from a collection of 330 isolates. The isolates were selected to represent different isolation sources: RTE food (n=176), FPE (n=117), animal clinical (n=22), human clinical (n=11), natural environment (n=3) and a clinical reference strain (n=1), and origins: Norway (n=98), Denmark (n=96), Switzerland (n=65), Slovenia (n=45), Canada (n=11), Germany (n=7), Italy (n=4), USA (n=1), England (n=1), Finland (n=1) and an unknown country of origin (n=1). The isolates had previously undergone whole genome sequencing using Illumina (Fagerlund et al., 2020, 2022; Gilmour et al., 2010; Palma et al., 2022a; Papić et al., 2019, 2020; Wambui et al., 2020), and their minimum inhibitory concentrations (MICs) has been determined by an in house developed broth microdilution assay to BC (Ivanova et al., 2023) and PAA (Supplementary Table 1). While the BC community (n=71) was designed to include QAC-tolerant *L. monocytogenes* isolates (MIC ≥ 1.25 mg/L), the

approach for the PAA community (n=80) was to select isolates with diverse genotypes (MLST types, serotypes and lineages) and metadata (isolation sources and countries of origin) because all 330 *L. monocytogenes* isolates in the strain collection had identical MIC values for PAA (60 mg/L) as determined by the in-house broth microdilution assay (Supplementary Table 2). Isolate metadata and accession numbers of the raw sequencing data can be obtained from Supplementary Table 1.

In silico characterisation of Listeria monocytogenes

The raw sequencing data were quality checked using FastQC v.0.11.5 (Andrews, 2010), trimmed with bbtools v.36.49 (Bushnell et al., 2017), assembled by SPAdes v.3.15.3 (Bankevich et al., 2012) and quality of assemblies were evaluated with Quast v.5.0.2 (Gurevich et al., 2013). Serotypes and seven gene MLST profiles were determined using lissero v.0.4.1 and the scheme of Doumith et al (Doumith et al., 2004) and mlst v.2.23.0 (Seemann, n.d.) and the scheme of Ragon et al (2008), respectively, while lineages were defined in the BIGSdb-Lm database at Institute Pasteur (Moura et al., 2016). SNP analysis was performed for the total *L. monocytogenes* collection using CSI phylogeny with the *L. monocytogenes* EGDe (GenBank accession no. AL591824.1) as a reference genome (Kaas et al., 2014). IQ-TREE v.2.1.4-beta was used to infer a maximum likelihood (ML) phylogenetic tree from the concatenated and aligned SNPs produced by CSI phylogeny under the GTR+G substitution model with ultrafast bootstrapping (Nguyen et al., 2015). The ML tree was visualised with iTOL (Letunic and Bork, 2019). To verify that all isolates belonged to the *L. monocytogenes* species, blastn was carried out using the *L. monocytogenes*-specific *hly* gene encoding listeriolysin O. Furthermore, abricate v.0.5 (Seemann, n.d.) was used to screen the isolates for known QAC resistance genes (*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 et al., 2016b)) and *bcrABC* (accession no. JX023284.1 (Elhanafi et al., 2010b)).

Design of BC and PAA communities

The PAA community was designed to be genetically diverse and consisted of 80 isolates selected based on MLST characteristics and source of isolation. Since the BC community was to be treated with a BC concentration higher than the MIC determined for sensitive isolates, only the 71 BC tolerant isolates were selected. Of these, six *L. monocytogenes* strains exhibited BC tolerant phenotype, but harboured no BC genes in their genomes (Ivanova et al 2023). The metadata of the isolates selected for the BC and PAA communities is provided in Supplementary Figures 1 and 2, respectively, and in Supplementary Table S1.

Biocide treatment

A graphical representation of the experimental design is shown in Supplementary Figure 3. The isolates from the two communities were grown overnight on Tryptone Soya Agar (TSA, Fisher Scientific, Denmark) plates at 37°C. A single colony from each isolate was cultured individually in 1.8 mL of 1/10 strength (3 g/L) Tryptone Soya Broth (TSB, Oxoid) in 96 deep-well plates (Eppendorf AG, Germany) at 15°C for 48h. To ensure an equal ratio of each isolate in the communities, the OD_{620} values of the individual cultures were adjusted to 0.1. Fifty µL of each of the cultures belonging to BC or PAA communities were mixed, resulting in two separate mixtures consisting of 71 and 80 cultures for BC and PAA communities, respectively. From each mixture, 1 mL aliquotes were taken and stored at –20°C until DNA extraction was carried out.

These samples are further referred to as "BC_T0" and "PAA_T0". Samples were taken from each mix to determine CFU/mL following 10-fold dilution in sterile saline (0.9% w/v), spread plating on TSA plates and enumeration after 48 h at 37°C. The bacterial cocktails were exposed to a range of disinfectant concentrations at a cell concentration of 10^3 CFU/ml. To ensure treatment with sub-lethal concentrations of disinfectants, the communities were grown in three different concentrations of BC (Thermo Fisher, Kandel, Germany) (1.75, 2.0, 2.25, 2.5 and 2.75 mg/L) and PAA (Acros Organics BV, Geel, Belgium) (30, 35 and 38 mg/L) and growth was recorded by Bioscreen C (Oy Growth Curves Ab Ltd, Finland) at 15°C with measurement of OD_{540} values every 30 min. When the communities reached stationary phase, samples from multiple wells for the same biocide and concentration (technical replicates) were taken and pooled. One mL of each sample was stored at –20°C, whereas the remainder was used to determine CFU/mL. The communities were exposed to different concentrations of BC or PAA to ensure growth at sublethal concentration for each biocide. Samples with the highest biocide concentration that permitted growth of the communities underwent DNA extraction. These samples are further referred to as "BC_Treatment" and "PAA_Treatment". The corresponding unexposed communities (no biocide treatment) are further referred to as "BC_Negative_control" and "PAA_Negative_control". The biocide treatment experiment was carried out in two biological replicates for all samples. These replicates are referred to as "BR1" and "BR2".

Metagenomic sequencing

Total DNA extraction from the bacterial pellets from the 12 collected samples was performed using the DNeasy Blood and Tissue Kit (Qiagen, Denmark) following the manufacturer's instructions. DNA concentration was measured with Qubit 3.0 Fluorometer using Qubit DNA

High Sensitivity Kit (Thermo Fisher Scientific, Carlsbad, CA, USA). For the construction of the metagenomic libraries, 35 μL of input DNA was used for each sample according to the KAPA HyperPlus PCR-free Kit (Roche Diagnostics) protocol. Library concentration was measured using Qubit DNA High Sensitivity Kit and the fragment sizes were evaluated by Agilent 2100 Bioanalyzer (Agilent Technologies, Germany). Libraries with similar fragment sizes were pooled, denatured and diluted to 1.5 pM for loading in a NextSeq 500/550 Mid Output v2.5 Kit (300 cycles) (Illumina) and sequenced on a NextSeq 500 platform (Illumina). The raw metagenomics data were submitted to the European Nucleotide Archive (ENA) under the project accession number PRJEB66133 (Supplementary Table 2).

Construction of databases for read mapping

The metagenomic raw reads were mapped to several databases. The first two databases named "Bacterial community" were unique for each community based on the assembled whole genome sequences and consisted of all contigs belonging to all isolates in the respective communities. The pangenome of each community was used to create the unique "Pangenome" databases. The pangenomes of the BC and PAA communities were determined by Roary (Page et al., 2015) using .gff files as input produced by Prokka (Seemann, 2014). The "Plasmids" database was created from all complete *Listeria* spp. plasmids collected by Galata et al. (Galata et al., 2019) and from Chmielowska et al. (Chmielowska et al., 2021). The "MLST" database consisted of all allele variants in the seven housekeeping genes for the *L. monocytogenes* MLST scheme and the "Virulence" database consisted of known *Listeria* spp. virulence genes; both were acquired from CGE (Larsen et al., 2012). The "Disinfectant" database was also acquired from CGE and consisted of known disinfectant tolerance genes (Malberg Tetzschner et al., 2020), including the

heat tolerance gene "*ClpL_1*". Furthermore, the four QAC tolerance genes that all isolates had previously been screened for (*emrC, emrE, bcrABC* and *qacH*), were added to this database.

Metagenomic and bioinformatics analysis

The metagenomic raw sequencing data were quality checked using FastQC v.0.11.5 (Andrews, 2010) and trimmed using bbtools v.36.49 (Bushnell et al., 2017). The trimmed reads were mapped to the relevant databases using KMA mapping with default settings (Clausen et al., 2018). Mapping results were normalized into Fragments Per Kilobase Million (FPKM) and relative abundance. FPKM was calculated by first normalising for read depth by dividing the read counts for each gene/database entry with the total number of reads in the sample and using a scaling factor of one million. Then, the read counts for each gene/database entry were normalised according to gene length by dividing with the length of the gene in kilobases (Zhao et al., 2021). The results were visualised using R (R_Core_team, 2022).

RESULTS

Construction of the mixed L. monocytogenes communities

Metadata and genomic characterisation of the 330 *L. monocytogenes* isolates, including the detection of the QAC resistance genes *qacH, emrC, emrE* and *bcrABC*, is given in Supplementary Table 1 and summarized in Supplementary Figures 1 and 2. A maximum-likelihood (ML) SNP-based tree (Figure 1), as expected, showed that most of the isolates selected for the BC community were genetically related, whereas the PAA community consisted of phylogenetically diverse isolates. Most of the isolates for both communities originated from Denmark, Switzerland or Norway and belonged to lineage II. The FPE was the most common

isolation source, although the isolation sources were generally more diverse for the PAA community. Overall, the PAA community comprised 79 different STs. Most isolates in this community did not harbour BC tolerance genes: three strains each carried *qacH*, *bcrABC*, and *emrC*, whereas two had *emrE*. One isolate was BC tolerant but with no identified BC tolerance genes. In the BC community, most isolates belonged to lineage II (90%), ST121 (45%) or ST9 (18%), harboured *qacH* (55%) and originated from FPEs (35%) or RTE meat (31%). The frequencies of the remaining QAC tolerance genes were 20% for *bcrABC*, 10% for *emrC*, and 7% for *emrE*, whereas six (8%) were BC tolerant but with no identified BC tolerance genes. No isolate carried more than one of the four BC tolerance genes.

Biocide treatment of the mixed L. monocytogenes communities

The BC and PAA communities were treated with different concentrations of their respective disinfectants. For the BC community, we selected a concentration of 1.75 mg/L for treatment, which is close to the sub-MIC of the BC tolerant isolates (Supplementary Figure 4). In the PAA community, the highest PAA concentration allowing growth (38 mg/L) was selected for sampling, DNA extraction and further analysis (Supplementary Figure 5). The CFU/mL determined at the start and end of the experiments for both communities showed an increase in the viable cell concentration of >3 log CFU/ml for both controls and treatments (Supplementary Tables 3 and 4).

Gene and plasmid abundance

Figure 2 shows the total abundance, calculated as the sum of the Fragments Per Kilobase Million (FPKM) values for each gene in each database, obtained for each sample from the BC and PAA

communities. For both communities, the total abundance was fairly similar for all samples for the mapping results against the "Pangenome" (Figures 2a and 2b), "MLST" (Figures 2e and 2f) and "Virulence" (Figures 2g and 2h) databases. For the BC community, the total abundance from the mapping against both the "Plasmids" (Figure 2c) and "Disinfectant" (Figure 2i) databases decreased after treatment with BC, indicating a potential correlation between plasmids and disinfectant genes. The same correlating trend between the "Plasmids" and "Disinfectant" database was observed for the PAA community (Figure 2d and Figure 2j), although in this case, the negative control samples had the highest total abundance, followed by the treatment samples and the T0 samples. Moreover, the total abundance of reads mapping to the "Plasmids" and "Disinfectants" databases was generally higher for the untreated (T0 and negative control) BC community compared with the PAA community.

To examine differences in relative abundance of individual genes (database entries) underlying the results presented in Figure 2, the abundance (in FPKM) of specific genes and plasmids in the BC and PAA community samples for all the above-mentioned databases was calculated and is shown in Figure 3. The identity of individual genes of interest was determined from inspection of the abundance tables. For both communities, the "Pangenome" database had more genes with high abundance (>500 FPKM) in the negative control and treatment samples compared with in the T0 samples (Figures 3a and 3b). Similarly, for both communities, several MLST alleles were more abundant in the negative control and treatment samples compared with the T0 samples (Figures 3e and 3f). These results suggest that the composition of isolates in the cultures was altered during growth, irrespective of the presence or absence of biocides (BC or PAA) in the cultures.

Regarding the results of the mapping against the "Plasmids" database, plasmid pLmN12-0935 was significantly more abundant than other plasmids in all samples from both communities except for the two BC-treated cultures the abundance (Figures 3c and 3d). Similarly, the abundance of the BC resistance gene *emrC* was significantly higher than the abundances of all other genes from the "Disinfectant" database, except in the BC-treated cultures (an outlier dot in Figures 3i and 3j). This observation can be explained by the fact that *emrC* is located on plasmid pLmN12-0935 (Kremer et al 2017, Kropac et al 2019). The high abundance further suggests that the copy number of this plasmid was much higher than that of other plasmids found in the examined communities. Indeed, pLmN12-0935 was determined to be a high-copy number plasmid based on the coverage of the contig on which it was located compared with the coverage of the other contigs in the assemblies. For the BC community, pLmN12-0935 and *emrC* appeared to be highly abundant in the T0 samples and the negative control samples, while its abundance decreased considerably in the BC-treated samples. In contrast, in the PAA community, the abundance of this plasmid and gene was highest in the negative control samples, followed by the BC-treated samples and lowest in the T0 samples.

The mapping results against the "Virulence" database revealed that one gene had a significantly higher abundance than the rest (Figures 3g and 3h). By inspecting the abundance tables, this virulence gene was identified as *lhrC*, a multicopy sRNA gene (Orsi and Wiedmann, 2016) but no notable differences in the abundance of this gene could be observed among the treatments.

Abundance of QAC tolerance genes

The abundance (in FPKM) of known BC tolerance genes obtained for the different treatments and communities upon mapping of metagenomic reads against the "Disinfectant" database is shown in Figure 4. In correspondence with the results described above, for the T0 samples (Figure 4a) and negative control samples (Figure 4c) from the BC community, the abundance of *emrC* was notably higher compared with the rest of the genes, whereas it drastically decreased in the BC-treated samples (Figure 4e). For the PAA community, the highest abundance of *emrC* was observed in the negative control samples (Figure 4d), whereas it was slightly lower in the treatment samples (Figure 4e) and lowest in the T0 samples (Figure 4b). For the remaining "Disinfectant" database genes, no marked changes in the abundance were seen when comparing the T0 samples to the Negative control samples in both communities.

Differences between Negative control and Treatment

We further investigated the effect of the biocide treatment on plasmids and pangenome genes with PCA excluding the T0 samples (Figure 5) to observe differences between the negative control samples and the treatment samples. In all the PCA plots shown in Figure 5, the two treatment biological replicates were closely located on the PC1 (*x* axis) and are further apart on the PC2 (*y* axis) (except in Figure 5c). PC1 accounted for more than 80% of the variance, whereas PC2 only accounted for 0.1–11% of the variance. Because the two replicate treatment samples in the PCA plots were close on the *x* axis, they were considered as being clustered. In addition, a clear separation between negative control samples and treatment samples were observed in the PCA plots.

Function of genes differentiating the Negative control from the Treatment

The genes and plasmids responsible for differentiating the negative control samples from the treatment samples for the "Pangenome" and "Plasmids" PCA plots (Figure 5) were further investigated. For the "Pangenome" databases, the top 50 genes with the highest weight on PC1 were assigned an overall gene function and the change in their abundance in the treatment samples compared with the negative control samples was estimated (Supplementary Tables 5 and 6 for the BC community and Supplementary Tables 7 and 8 for the PAA community). For the BC community, 40 out of the top 50 genes belonged to the "*Listeria* phage" group (Figure 6a), whereas 32 genes belonged to this group for the PAA community (Figure 6b). For both communities, the abundance of all top 50 genes belonging to the "*Listeria* phage" group was increased compared with the negative control and T0 samples. The second highest group for the BC community was the "DNA binding or replication" group, out of which *tet*(R), *pre*, *copG*, *repB* decreased in abundance, whereas *ssbA* increased in the treatment samples. The only gene that belonged to the "Cell membrane" group and which decreased in relative abundance the treatment samples was the previously discussed BC tolerance gene *emrC*. For the PAA community, the second highest gene function group was the "Cell membrane" group, which consisted of eight genes with increased (*lmo2224*, *lmo1746*, *lmo0590*), decreased (*emrC*, *lmo224*) and inconclusive (*lmo0883*) abundance. When comparing these results, it is noticeable that the PAA community had significantly more genes with high weight on PC1 belonging to the "Cell membrane" gene function group compared with the BC community. In addition, plasmid pLmN12-0935 had the highest weight on PC1 for both communities (Supplementary Tables 6 and 8).

DISCUSSION

Changes in abundance of plasmids and QAC tolerance genes after biocide treatment

The total abundance of the samples mapped against the "Disinfectant" database was proportionally very similar to that of the samples mapped against the "Plasmids" database, suggesting that one or more plasmids carried a gene belonging to the "Disinfectant" database responsible for the variance in gene abundance between samples. This hypothesis was further investigated by examining the specific abundance in FPKM of individual plasmids and disinfectant genes (Figure 3). For both communities, we observed that the small plasmid pLmN12-0935 had a noticeably higher abundance than the other plasmids, although its abundance significantly decreased after BC treatment. By mapping the QAC tolerance genes to the "Plasmids" database, we found that the plasmid pLmN12-0935 harboured the *emrC* gene. The result also showed that plasmid pLmN12-0935 had the highest weight by far on PC1. We therefore conclude that the *emrC*-harbouring plasmid pLmN12-0935 is the major driver for the differentiation between the negative control and biocide treated communities with regards to plasmid content. Interestingly, only seven and three strains harbouring pLmN12-0935 and the QAC tolerance gene *emrC* were present in the BC and PAA communities, respectively.

Interestingly, although the plasmid pLmN12-0935 carries *emrC*, its abundance decreased after PAA and BC treatment when comparing the treated groups with their respective negative control samples. It has previously been shown that plasmid-carrying bacteria can exhibit a delayed growth compared with plasmid-free strains, incurring a potential fitness cost due to plasmid carriage (Smith and Bidochka, 1998). Therefore, as pLmN12-0935 plasmid is mostly present in a high-copy number, its carriage could lead to a fitness reduction for isolates in the highly competitive low-nutrient and biocide stress-induced environment. Kropac et al. (Kropac et al.,

2019) have previously compared the growth of strains carrying a highly similar plasmid pMLST6 (two SNPs difference) with the growth of plasmid-free strains and found no growth differences, indicating that pLMST6 carriage does not incur a fitness cost. However, this was only tested with isolates grown under stress-free conditions. These findings correlate with the high abundance that was observed for the plasmid pLmN12-0935 in the negative control samples, where there was no biocide-induced stress. As of yet, no studies have investigated if the carriage of pLmN12-0935 or a similar plasmid has an effect on the fitness of isolates in competitive and biocide-stressed environments, and it is therefore only possible to speculate that the increased competition and stress may lead to reduced abundance of the plasmid. However, in the PAA-treated community, it has been previously shown that the EmrC efflux pump did not confer cross-resistance to other antimicrobials; it therefore seems likely that the carriage of a high-copy-number plasmid would not be beneficial for the cells (Roedel et al., 2019).

In general, we observed no increase in QAC tolerance gene abundance in the BC community except for the previously discussed decrease of the *emrC*-harbouring plasmid pLmN12-0935. A recent large-scale studies of 769 and 1671 *L. monocytogenes* genomes found that *bcrABC* and *qacH* were common in isolates from the same environment but were never present in the same isolate (Fagerlund et al., 2022, Ivanova et al., 2023). This correlates with the findings from this study, in which no isolate carried more than one BC tolerance gene. Because all strains in the BC community were selected based on their increased tolerance to BC and most of these strains harboured either *bcrABC* or *qacH*, a putative mechanism inhibiting the presence of both of these genes in the same isolate would mean that the isolates would not be able to take up an additional different QAC tolerance gene or would not achieve increased tolerance by doing so, which could

explain the unchanged QAC tolerance gene abundance. Moreover, because all BC community isolates were already BC tolerant, the decrease in *emrC* could be due to isolates carrying plasmid pLmN12-0935 merely being outperformed by isolates carrying other QAC tolerance genes. It could be speculated that the *emrC* gene confers low-level QAC tolerance and an increased number of plasmid copies are needed to achieve same level of tolerance as *qacH* or *bcrABC*, or that *L. monocytogenes* strains lose the plasmid due to fitness cost or are outcompeted by the other strains in the community. Collectively, these results highlight the need for increased research into the effect of biocide treatment on bacterial communities, including in a sessile state. As bacterial communities are ubiquitous in nature and better reflect the natural state of the bacteria, studying them can provide valuable information on how both the symbiotic and antagonistic relationships between them can affect cellular functions (McCarty and Ledesma-Amaro, 2019).

Changes in gene abundance after biocide treatment differentiating treatment and control samples

The vast majority of differentially abundant genes were derived from *Listeria* phages and an increase in these genes was observed in the treatment samples for both communities (Figures 6a and 6b). In general, the *Listeria* strains could carry multiple prophages. Most of them are lysogenic, infecting only a narrow host range and are resistant to their own phages (homoimmunity) (Hagens and Loessner, 2014). A possible explanation for the increased abundance of phage genes observed in this study is phage induction due to inter-strain competition as a result of nutrient poor conditions and biocide treatment. Although the specific effect of BC and PAA treatment on prophage induction in a bacterial community has not yet

been studied, many stressors such as antibiotics, UV radiation and temperature can induce the lytic cycle in *L. monocytogenes* pure cultures (Vu et al., 2019). This hypothesis is also supported by a previous study by Tian et al. (Tian et al., 2014), which investigated the effect of copper treatment on a natural multi-species bacterial community. Through a metagenomic comparison, they were able to show an enrichment in the phage community after copper treatment, implying that a lytic phage cycle was induced by stress caused by a high copper concentration. In contrast, in a transcriptomics study the genes encoding phage proteins were down-regulated in *L. monocytogenes* strain 6179 after exposure to QAC disinfectant benzethonium chloride (BZT) (Casey et al., 2014). However, it was suggested that the down-regulation could be attributed to the mildly acidic environment caused by BZT compared with the control (Casey et al., 2014). As Casey et al. investigated the effect of BZT on one *L. monocytogenes* strain, the increase in the abundance of phage-associated genes observed in this study could be considered as community-related mechanism. As prophages have already been shown to have the ability to increase survivability and fitness of *L. monocytogenes*, another possible explanation for the observed increase in the abundance of prophage-associated genes in this study could be that the phages increase host fitness in the presence of biocides (Verghese et al., 2011). This hypothesis correlates with the findings of a genome-wide association study on a collection of 197 *L. monocytogenes* strains, which found an association between prophage-related genes and phenotypic tolerance to both BC and PAA (Palma et al., 2022a).

Closer examination of the top 50 pangenome genes that differentiate the negative control from the treatment samples for the BC community revealed that the top six genes, including the QAC resistance gene *emrC*, can be derived from an isolate such as LIS08 (Kremer et al., 2017)

(Supplementary Table 5) and that the genes were closely located and their abundance was highly decreased after the BC treatment. The *emrC* gene has previously been reported to be located on a high-copy plasmid pLmN12-0935, having an 8- to 18-fold higher coverage than the largest contig in each assembly. However, isolate LIS08 carried the high-copy plasmid pLmN12-0935 integrated into a larger low-copy plasmid (Ivanova et al., 2023). Therefore, it can be concluded that the major difference between the negative control and the BC-treated samples is indeed the presence or absence of plasmid pLmN12-0935.

By examining the top 50 differentially abundant genes from the "PAA Pangenome" database, the previously identified pLmN12-0935-associated genes were again observed. Furthermore, eight genes were assigned function "Cell membrane", one of them being the putative ABC transporter gene *lmo1746* (also known as *virA*), described to be necessary for *L. monocytogenes* resistance to nisin and virulence (Grubaugh et al., 2018). As PAA has multiple modes of actions, one of which is known to be disruption of cell membranes and increase in cell wall permeability, changes in "Cell membrane" proteins in the PAA-treated community seem plausible (Vandekinderen et al., 2009).

The results show that the metagenomic approach in this study can be applied in high resolution screening for genetic determinants associated with tolerance to the used food industry disinfectants in mixed *L. monocytogenes* communities. Another practical application from this metagenomic approach is to study disinfectant tolerance of biofilm formed by *L. monocytogenes* or by other bacterial communities. The shotgun metagenomic approach can determine genetic determinants related to disinfectant tolerance in biofilm which is also a mixed bacterial community by sequencing entire extracted DNA from the biofilm sample. A previous study

showed that sensitivity of *L. monocytogenes* isolates to common industrial biocides is more dependent on the presence of residual organic matter or biofilm than on genetic determinants (Kragh et al., 2024). Thus, using the metagenomic approach in this study can help to gain insight to the associated markers from mixed bacterial communities such as in biofilm.

CONCLUSION

The metagenomic analysis of the two mixed *L. monocytogenes* communities revealed significant differences between the biocide-treated community and the corresponding biocide-free control community for both BC and PAA. For both communities, we found that genes associated with *Listeria* phages were especially responsible for the differentiation between the biocide-free control communities and the biocide-treated communities. Moreover, the abundance of the phage-associated genes increased after biocide treatment, suggesting prophage induction as a result of stress induced by the competitive nutrient poor environment and/or the biocide treatment. Alternatively, although not tested in our study, we speculate that prophage carriage could lead to increased biocide tolerance. In both communities, a decrease in the abundance of the small high-copy number *emrC*-harbouring plasmid pLmN12-0935 was observed after biocide treatment. Interestingly, none of the communities showed an increase in the abundance of the known QAC tolerance genes after the biocide treatment. Changes in the abundance of genes associated with the cell membrane were observed in the PAA-treated community. In conclusion, the metagenomic approach applied in this study can be practically used for identifying genetic determinants associated with increased tolerance to disinfectants in mixed bacterial communities.

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REFERENCES

Andrews, S., 2010. FastQC: A Quality Control Tool for High Throughput Sequence Data [WWW Document]. http://www.bioinformatics.babraham.ac.uk/projects/fastqc/.

Assisi, C., Forauer, E., Oliver, H.F., Etter, A.J., 2021. Genomic and Transcriptomic Analysis of Biofilm Formation in Persistent and Transient Listeria monocytogenes Isolates from the Retail Deli Environment Does Not Yield Insight into Persistence Mechanisms. Foodborne Pathog. Dis. 18, 179–188. https://doi.org/10.1089/fpd.2020.2817

Bankevich, A., Nurk, S., Antipov, D., Gurevich, A.A., Dvorkin, M., Kulikov, A.S., Lesin, V.M., Nikolenko, S.I., Pham, S., Prjibelski, A.D., Pyshkin, A. V., Sirotkin, A. V., Vyahhi, N., Tesler, G., Alekseyev, M.A., Pevzner, P.A., 2012. SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. J. Comput. Biol. 19, 455–477. https://doi.org/10.1089/cmb.2012.0021

Belias, A., Sullivan, G., Wiedmann, M., Ivanek, R., 2022. Factors that contribute to persistent Listeria in food processing facilities and relevant interventions: A rapid review. Food Control 133, 108579. https://doi.org/10.1016/j.foodcont.2021.108579

Bushnell, B., Rood, J., Singer, E., 2017. BBMerge - Accurate paired shotgun read merging via overlap. PLoS One 12, e0185056. https://doi.org/10.1371/journal.pone.0185056

Carlin, C.R., Roof, S., Wiedmann, M., 2022. Assessment of Reference Method Selective Broth and Plating Media with 19 Listeria Species Highlights the Importance of Including Diverse Species in Listeria Method Evaluations. J. Food Prot. 85, 494–510. https://doi.org/10.4315/JFP-21-293

Casey, A., Fox, E.M., Schmitz-Esser, S., Coffey, A., McAuliffe, O., Jordan, K., 2014. Transcriptome analysis of Listeria monocytogenes exposed to biocide stress reveals a multi-system response involving cell wall synthesis, sugar uptake, and motility. Front. Microbiol. 5. https://doi.org/10.3389/fmicb.2014.00068

Cherifi, T., Carrillo, C., Lambert, D., Miniaï, I., Quessy, S., Larivière-Gauthier, G., Blais, B., Fravalo, P., 2018. Genomic characterization of Listeria monocytogenes isolates reveals that their persistence in a pig slaughterhouse is linked to the presence of benzalkonium chloride resistance genes. BMC Microbiol. 18, 220. https://doi.org/10.1186/s12866-018-1363-9

Chmielowska, C., Korsak, D., Szuplewska, M., Grzelecka, M., Maćkiw, E., Stasiak, M., Macion, A., Skowron, K., Bartosik, D., 2021. Benzalkonium chloride and heavy metal resistance profiles of Listeria monocytogenes strains isolated from fish, fish products and food-producing factories in Poland. Food Microbiol. 98, 103756. https://doi.org/10.1016/j.fm.2021.103756

Clausen, P.T.L.C., Aarestrup, F.M., Lund, O., 2018. Rapid and precise alignment of raw reads against redundant databases with KMA. BMC Bioinformatics 19, 307. https://doi.org/10.1186/s12859-018-2336-6

Disson, O., Moura, A., Lecuit, M., 2021. Making Sense of the Biodiversity and Virulence of Listeria monocytogenes. Trends Microbiol. 29, 811–822. https://doi.org/10.1016/j.tim.2021.01.008

Doumith, M., Buchrieser, C., Glaser, P., Jacquet, C., Martin, P., 2004. Differentiation of the major Listeria monocytogenes serovars by multiplex PCR. J. Clin. Microbiol. 42, 3819–22. https://doi.org/10.1128/JCM.42.8.3819-3822.2004

Elhanafi, D., Dutta, V., Kathariou, S., 2010a. Genetic Characterization of Plasmid-Associated Benzalkonium Chloride Resistance Determinants in a Listeria monocytogenes Strain from the 1998-1999 Outbreak. Appl. Environ. Microbiol. 76, 8231–8238.

https://doi.org/10.1128/AEM.02056-10
Elhanafi, D., Dutta, V., Kathariou, S., 2010b. Genetic characterization of plasmid-associated benzalkonium chloride resistance determinants in a Listeria monocytogenes strain from the 1998-1999 outbreak. Appl. Environ. Microbiol. 76, 8231–8. https://doi.org/10.1128/AEM.02056-10
Fagerlund, A., Langsrud, S., Møretrø, T., 2020. In-Depth Longitudinal Study of Listeria monocytogenes ST9 Isolates from the Meat Processing Industry: Resolving Diversity and Transmission Patterns Using Whole-Genome Sequencing. Appl. Environ. Microbiol. 86. https://doi.org/10.1128/AEM.00579-20
Fagerlund, A., Wagner, E., Møretrø, T., Heir, E., Moen, B., Rychli, K., Langsrud, S., 2022. Pervasive Listeria monocytogenes Is Common in the Norwegian Food System and Is Associated with Increased Prevalence of Stress Survival and Resistance Determinants. Appl. Environ. Microbiol. 88. https://doi.org/10.1128/aem.00861-22
Galata, V., Fehlmann, T., Backes, C., Keller, A., 2019. PLSDB: a resource of complete bacterial plasmids. Nucleic Acids Res. 47, D195–D202. https://doi.org/10.1093/nar/gky1050
Gerba, C.P., 2015. Quaternary ammonium biocides: efficacy in application. Appl. Environ. Microbiol. 81, 464–9. https://doi.org/10.1128/AEM.02633-14
Gilmour, M.W., Graham, M., Van Domselaar, G., Tyler, S., Kent, H., Trout-Yakel, K.M., Larios, O., Allen, V., Lee, B., Nadon, C., 2010. High-throughput genome sequencing of two Listeria monocytogenes clinical isolates during a large foodborne outbreak. BMC Genomics 11, 120. https://doi.org/10.1186/1471-2164-11-120
Greenwood, D., 2018. Medical microbiology : a guide to microbial infections : patho- genesis, immunity, laboratory diagnosis and control. Churchill Livingstone/Elsevier.
Grubaugh, D., Regeimbal, J.M., Ghosh, P., Zhou, Y., Lauer, P., Dubensky, T.W., Higgins, D.E., 2018. The VirAB ABC Transporter Is Required for VirR Regulation of Listeria monocytogenes Virulence and Resistance to Nisin. Infect. Immun. 86. https://doi.org/10.1128/IAI.00901-17
Gurevich, A., Saveliev, V., Vyahhi, N., Tesler, G., 2013. QUAST: quality assessment tool for genome assemblies. Bioinformatics 29, 1072–5. https://doi.org/10.1093/bioinformatics/btt086
Hagens, S., Loessner, M.J., 2014. Phages of Listeria offer novel tools for diagnostics and biocontrol. Front. Microbiol. 5, 159. https://doi.org/10.3389/fmicb.2014.00159
Ivanova, M., Kragh, M.L., Szarvas, J., Tosun, E.S., Holmud, N.F., Gmeiner, A., Amar, C.F.L., Guldimann, C., Huynh, T.N., Karpíšková, R., Garcia, C.R., Gomez, D., Aboagye, E., Etter, A.J., Centorame, P., Torresi, M., Angelis, M.E. De, Pomilio, F., Okholm, A.H., Xiao, Y., Kleta, S., Lüth, S., Pietzka, A.T., Pagotto, F., Kovacevic, J., Rychli, K., Zdovc, I., Papić, B., Heir, E., Langsrud, S., Møretrø, T., Roger, S., Brown, P., Kathariou, S., Tasara, T., Aarestrup, F.M., Njage, P.M.K., Fagerlund, A., Hansen, L.T., Leekitcharoenphon, P., 2023. Large-scale phenotypic and genomic characterization of Listeria monocytogenes susceptibility to quaternary ammonium compounds. bioRxiv. https://doi.org/https://doi.org/10.1101/2023.09.07.556668
Jiang, X., Ren, S., Geng, Y., Yu, T., Li, Y., Liu, L., Liu, G., Wang, H., Shi, L., 2020. The sug operon involves in resistance to quaternary ammonium compounds in Listeria monocytogenes EGD-e. Appl. Microbiol. Biotechnol. 104, 7093–7104. https://doi.org/10.1007/s00253-020-10741-6
Kaas, R.S., Leekitcharoenphon, P., Aarestrup, F.M., Lund, O., 2014. Solving the Problem of

Comparing Whole Bacterial Genomes across Different Sequencing Platforms. PLoS One 9, e104984. https://doi.org/10.1371/journal.pone.0104984

Kovacevic, J., Ziegler, J., Wałecka-Zacharska, E., Reimer, A., Kitts, D.D., Gilmour, M.W., 2016a. Tolerance of Listeria monocytogenes to Quaternary Ammonium Sanitizers Is Mediated by a Novel Efflux Pump Encoded by emrE. Appl. Environ. Microbiol. 82, 939–953. https://doi.org/10.1128/AEM.03741-15

Kovacevic, J., Ziegler, J., Wałecka-Zacharska, E., Reimer, A., Kitts, D.D., Gilmour, M.W., 2016b. Tolerance of Listeria monocytogenes to Quaternary Ammonium Sanitizers Is Mediated by a Novel Efflux Pump Encoded by emrE. Appl. Environ. Microbiol. 82, 939–53. https://doi.org/10.1128/AEM.03741-15

Kragh, M.L., Ivanova, M., Dalgaard, P., Leekitcharoenphon, P., Truelstrup Hansen, L., 2024. 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. Food Control 158, 110244. https://doi.org/10.1016/j.foodcont.2023.110244

Kremer, P.H.C., Lees, J.A., Koopmans, M.M., Ferwerda, B., Arends, A.W.M., Feller, M.M., Schipper, K., Valls Seron, M., van der Ende, A., Brouwer, M.C., van de Beek, D., Bentley, S.D., 2017. Benzalkonium tolerance genes and outcome in Listeria monocytogenes meningitis. Clin. Microbiol. Infect. 23, 265.e1-265.e7. https://doi.org/10.1016/j.cmi.2016.12.008

Kropac, A.C., Eshwar, A.K., Stephan, R., Tasara, T., 2019. New Insights on the Role of the pLMST6 Plasmid in Listeria monocytogenes Biocide Tolerance and Virulence. Front. Microbiol. 10, 1538. https://doi.org/10.3389/fmicb.2019.01538

Larsen, M. V., Cosentino, S., Rasmussen, S., Friis, C., Hasman, H., Marvig, R.L., Jelsbak, L., Sicheritz-Pontén, T., Ussery, D.W., Aarestrup, F.M., Lund, O., 2012. Multilocus Sequence Typing of Total-Genome-Sequenced Bacteria. J. Clin. Microbiol. 50, 1355–1361. https://doi.org/10.1128/JCM.06094-11

Letunic, I., Bork, P., 2019. Interactive Tree Of Life (iTOL) v4: recent updates and new developments. Nucleic Acids Res. 47, W256–W259. https://doi.org/10.1093/nar/gkz239

Magalhães, R., Ferreira, V., Biscottini, G., Brandão, T.R.S., Almeida, G., Teixeira, P., 2017. Biofilm formation by persistent and non-persistent Listeria monocytogenes strains on abiotic surfaces. Acta Aliment. 46, 43–50. https://doi.org/10.1556/066.2017.46.1.6

Malberg Tetzschner, A.M., Johnson, J.R., Johnston, B.D., Lund, O., Scheutz, F., 2020. In Silico Genotyping of Escherichia coli Isolates for Extraintestinal Virulence Genes by Use of Whole-Genome Sequencing Data. J. Clin. Microbiol. 58. https://doi.org/10.1128/JCM.01269-20

Manso, B., Melero, B., Stessl, B., Jaime, I., Wagner, M., Rovira, J., Rodríguez-Lázaro, D., 2020. The Response to Oxidative Stress in Listeria monocytogenes Is Temperature Dependent. Microorganisms 8. https://doi.org/10.3390/microorganisms8040521

McCarty, N.S., Ledesma-Amaro, R., 2019. Synthetic Biology Tools to Engineer Microbial Communities for Biotechnology. Trends Biotechnol. 37, 181–197. https://doi.org/10.1016/j.tibtech.2018.11.002

McDonnell, G., Russell, A.D., 1999. Antiseptics and Disinfectants: Activity, Action, and Resistance. Clin. Microbiol. Rev. 12, 147–179. https://doi.org/10.1128/CMR.12.1.147

Moura, A., Criscuolo, A., Pouseele, H., Maury, M.M., Leclercq, A., Tarr, C., Björkman, J.T., Dallman, T., Reimer, A., Enouf, V., Larsonneur, E., Carleton, H., Bracq-Dieye, H., Katz, L.S., Jones, L., Touchon, M., Tourdjman, M., Walker, M., Stroika, S., Cantinelli, T.,

Chenal-Francisque, V., Kucerova, Z., Rocha, E.P.C., Nadon, C., Grant, K., Nielsen, E.M., Pot, B., Gerner-Smidt, P., Lecuit, M., Brisse, S., 2016. Whole genome-based population biology and epidemiological surveillance of Listeria monocytogenes. Nat. Microbiol. 2, 16185. https://doi.org/10.1038/nmicrobiol.2016.185

Müller, A., Rychli, K., Muhterem-Uyar, M., Zaiser, A., Stessl, B., Guinane, C.M., Cotter, P.D., Wagner, M., Schmitz-Esser, S., 2013. Tn6188 - A Novel Transposon in Listeria monocytogenes Responsible for Tolerance to Benzalkonium Chloride. PLoS One 8, e76835. https://doi.org/10.1371/journal.pone.0076835

Nguyen, L.-T., Schmidt, H.A., von Haeseler, A., Minh, B.Q., 2015. IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. Mol. Biol. Evol. 32, 268–274. https://doi.org/10.1093/molbev/msu300

Orsi, R.H., Wiedmann, M., 2016. Characteristics and distribution of Listeria spp., including Listeria species newly described since 2009. Appl. Microbiol. Biotechnol. 100, 5273–5287. https://doi.org/10.1007/s00253-016-7552-2

Page, A.J., Cummins, C.A., Hunt, M., Wong, V.K., Reuter, S., Holden, M.T.G., Fookes, M., Falush, D., Keane, J.A., Parkhill, J., 2015. Roary: rapid large-scale prokaryote pan genome analysis. Bioinformatics 31, 3691–3. https://doi.org/10.1093/bioinformatics/btv421

Palma, F., Brauge, T., Radomski, N., Mallet, L., Felten, A., Mistou, M.-Y., Brisabois, A., Guillier, L., Midelet-Bourdin, G., 2020. Dynamics of mobile genetic elements of Listeria monocytogenes persisting in ready-to-eat seafood processing plants in France. BMC Genomics 21, 130. https://doi.org/10.1186/s12864-020-6544-x

Palma, F., Radomski, N., Guérin, A., Sévellec, Y., Félix, B., Bridier, A., Soumet, C., Roussel, S., Guillier, L., 2022a. Genomic elements located in the accessory repertoire drive the adaptation to biocides in Listeria monocytogenes strains from different ecological niches. Food Microbiol. 106, 103757. https://doi.org/10.1016/j.fm.2021.103757

Palma, F., Radomski, N., Guérin, A., Sévellec, Y., Félix, B., Bridier, A., Soumet, C., Roussel, S., Guillier, L., 2022b. Genomic elements located in the accessory repertoire drive the adaptation to biocides in Listeria monocytogenes strains from different ecological niches. Food Microbiol. 106, 103757. https://doi.org/10.1016/j.fm.2021.103757

Papić, B., Golob, M., Kušar, D., Pate, M., Zdovc, I., 2019. Source tracking on a dairy farm reveals a high occurrence of subclinical mastitis due to hypervirulent Listeria monocytogenes clonal complexes. J. Appl. Microbiol. 127, 1349–1361. https://doi.org/10.1111/jam.14418

Papić, B., Kušar, D., Zdovc, I., Golob, M., Pate, M., 2020. Retrospective investigation of listeriosis outbreaks in small ruminants using different analytical approaches for whole genome sequencing-based typing of Listeria monocytogenes. Infect. Genet. Evol. 77, 104047. https://doi.org/10.1016/j.meegid.2019.104047

R_Core_team, 2022. R: A language and environment for statistical computin. R Foundation for Statistical Computing, Vienna, Austria.

Ricci, A., Allende, A., Bolton, D., Chemaly, M., Davies, R., Fernández Escámez, P.S., Girones, R., Herman, L., Koutsoumanis, K., Nørrung, B., Robertson, L., Ru, G., Sanaa, M., Simmons, M., Skandamis, P., Snary, E., Speybroeck, N., Ter Kuile, B., Threlfall, J., Wahlström, H., Takkinen, J., Wagner, M., Arcella, D., Da Silva Felicio, M.T., Georgiadis, M., Messens, W., Lindqvist, R., 2018. Listeria monocytogenes contamination of ready-to-eat foods and the risk for human health in the EU. EFSA J. 16. https://doi.org/10.2903/j.efsa.2018.5134

Roedel, A., Dieckmann, R., Brendebach, H., Hammerl, J.A., Kleta, S., Noll, M., Al Dahouk, S., Vincze, S., 2019. Biocide-Tolerant Listeria monocytogenes Isolates from German Food Production Plants Do Not Show Cross-Resistance to Clinically Relevant Antibiotics. Appl. Environ. Microbiol. 85. https://doi.org/10.1128/AEM.01253-19

Seemann, T., 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics 30, 2068–9. https://doi.org/10.1093/bioinformatics/btu153

Seemann, T., n.d. mlst [WWW Document]. Github https://github.com/tseemann/mlst.

Seemann, T., n.d. abricate [WWW Document]. https://github.com/tseemann/abricate.

Skandamis N., P., 2021. "Listeria monocytogenes," in: McSweeney, P., McNamara, J. (Eds.), Encyclopedia of Dairy Sciences. pp. 305–312.

Smith, M.A., Bidochka, M.J., 1998. Bacterial fitness and plasmid loss: the importance of culture conditions and plasmid size. Can. J. Microbiol. 44, 351–355. https://doi.org/10.1139/cjm-44-4-351

Tian, R.-M., Wang, Y., Bougouffa, S., Gao, Z.-M., Cai, L., Zhang, W.-P., Bajic, V., Qian, P.-Y., 2014. Effect of copper treatment on the composition and function of the bacterial community in the sponge Haliclona cymaeformis. MBio 5, e01980. https://doi.org/10.1128/mBio.01980-14

Vandekinderen, I., Devlieghere, F., De Meulenaer, B., Ragaert, P., Van Camp, J., 2009. Optimization and evaluation of a decontamination step with peroxyacetic acid for fresh-cut produce. Food Microbiol. 26, 882–888. https://doi.org/10.1016/j.fm.2009.06.004

Verghese, B., Lok, M., Wen, J., Alessandria, V., Chen, Y., Kathariou, S., Knabel, S., 2011. comK Prophage Junction Fragments as Markers for Listeria monocytogenes Genotypes Unique to Individual Meat and Poultry Processing Plants and a Model for Rapid Niche-Specific Adaptation, Biofilm Formation, and Persistence. Appl. Environ. Microbiol. 77, 3279–3292. https://doi.org/10.1128/AEM.00546-11

Vu, H.T.K., Benjakul, S., Vongkamjan, K., 2019. Characterization of Listeria prophages in lysogenic isolates from foods and food processing environments. PLoS One 14, e0214641. https://doi.org/10.1371/journal.pone.0214641

Wambui, J., Eshwar, A.K., Aalto-Araneda, M., Pöntinen, A., Stevens, M.J.A., Njage, P.M.K., Tasara, T., 2020. The Analysis of Field Strains Isolated From Food, Animal and Clinical Sources Uncovers Natural Mutations in Listeria monocytogenes Nisin Resistance Genes. Front. Microbiol. 11. https://doi.org/10.3389/fmicb.2020.549531

Zhao, Y., Li, M.-C., Konaté, M.M., Chen, L., Das, B., Karlovich, C., Williams, P.M., Evrard, Y.A., Doroshow, J.H., McShane, L.M., 2021. TPM, FPKM, or Normalized Counts? A Comparative Study of Quantification Measures for the Analysis of RNA-seq Data from the NCI Patient-Derived Models Repository. J. Transl. Med. 19, 269. https://doi.org/10.1186/s12967-021-02936-w

FIGURES

Figure 1 Phylogenetic tree of all 330 *Listeria monocytogenes* isolates. The innermost ring of the tree shows the name of the isolate and indicates the phylogenetic lineages. The next rings show

the ST type of the isolates, their country of origin, the source from where they were isolated and the presence of BC tolerance genes. Isolates that were phenotypically tolerant to BC but did not harbour any of the four known genes were marked as “Unknown gene”. The outermost ring indicates whether the isolate belonged to the BC community (blue dot) and/or the PAA community (red star).

Figure 2 Total abundance in FPKM obtained upon mapping of the metagenomic reads from each sample against the “Pangenome”, “Plasmids”, “MLST”, “Virulence” and “Disinfectant” databases (Neg = Negative control, Treat = Treatment). Left: BC community. Right: PAA community. The x-axis depicts samples and the y-axis depicts total abundance. a,b: Pangenome, c,d: Plasmids, e,f: MLST, g,h: Virulence, i,j: Disinfectant. T0_BR1, T0_BR2: The mixed samples at the start of the experiment, Neg_BR1, Neg_BR2: The mixed samples without treatment at the end of the experiment, Treat_BR1, Treat_BR2: The mixed samples with treatment. BR1 and BR2 are biological replications.

Figure 3 FPKM of the samples mapped against the “Pangenome”, “Plasmids”, “MLST”, “Virulence” and “Disinfectant” databases. (Neg = Negative control, Treat = Treatment). Left: BC community. Right: PAA community. The x-axis shows which sample the measurement belongs to, while the y-axis shows the FPKM of a specific gene and plasmid. Left: BC community. Right: PAA community. a,b: Pangenome, c,d: Plasmids, e,f: MLST, g,h: Virulence, i,j: Disinfectant. T0_BR1, T0_BR2: The mixed samples at the start of the experiment, Neg_BR1, Neg_BR2: The mixed samples without treatment at the end of the experiment, Treat_BR1, Treat_BR2: The mixed samples with treatment. BR1 and BR2 are biological replications.

Figure 4 Abundance of known tolerance genes from the "Disinfectant" database for the T0 samples, Negative control samples and Treatment samples. The average FPKM has been taken from the two biological replicates for simplicity. Left: BC community. Right: PAA community. The x-axis shows gene abundance in FPKM, the y-axis shows the known tolerance genes. a, b: T0, c, d: Negative control, e, f: Treatment.

Figure 5 PCAs of the abundance profiles from the mapping results against the "Pangenome" and "Plasmids" databases for the Negative control and Treatment samples with FPKM abundance tables used for the PCAs. Left: BC community. Right: PAA community. a,b: Pangenome, c,d: Plasmids.

Figure 6 Distribution of gene function of top 50 pangenome genes with the highest weight on PC1 (Figure 5). The x-axis shows the gene function, while the y-axis shows the number of genes amongst the top 50 genes that belonged to the specific gene function group. a: BC community, b: PAA community

SUPPLEMENTARY TABLE

Supplementary Table 1 List of isolates used in this study including their ENA accession numbers, metadata, MIC value and plasmid information

Supplementary Table 2 ENA accession numbers for the metagenomic samples used in this study

Supplementary Table 3 Cell counts for the BC community with or without biocide treatment at T0 and end of the experiment

Supplementary Table 4 Cell counts for the PAA community with or without biocide treatment at T0 and end of the experiment

Supplementary Table 5 List of genes from pangenome of BC community and their weight on PCA analysis

Supplementary Table 6 List of plasmids present in BC community and their weight on PCA analysis

Supplementary Table 7 List of genes from pangenome of PAA community and their weight on PCA analysis

Supplementary Table 8 List of plasmids present in PAA community and their weight on PCA analysis

SUPPLEMENTARY FIGURE

Supplementary Figure 1 Distribution of metadata for isolates chosen for the BC community. Number shows the amount of isolates from the community belonging to specific group

Supplementary Figure 2 Distribution of metadata for isolates chosen for the PAA community. Number shows the amount of isolates from the community belonging to specific group

Supplementary Figure 3 Overview of the experimental work depicted as flow diagram

Supplementary Figure 4 Growth curves of the BC community for the biocide treatment experiment with BC concentrations of 1.75, 2, 2.25, 2.5 and 2.75 mg/L. A positive control consisting of clean media (without *L. monocytogenes*) and a negative control with media *L.*

monocytogenes, but without biocide was included. The x-axis depicts hours after biocide treatment and the y-axis depicts the OD540 of the community.

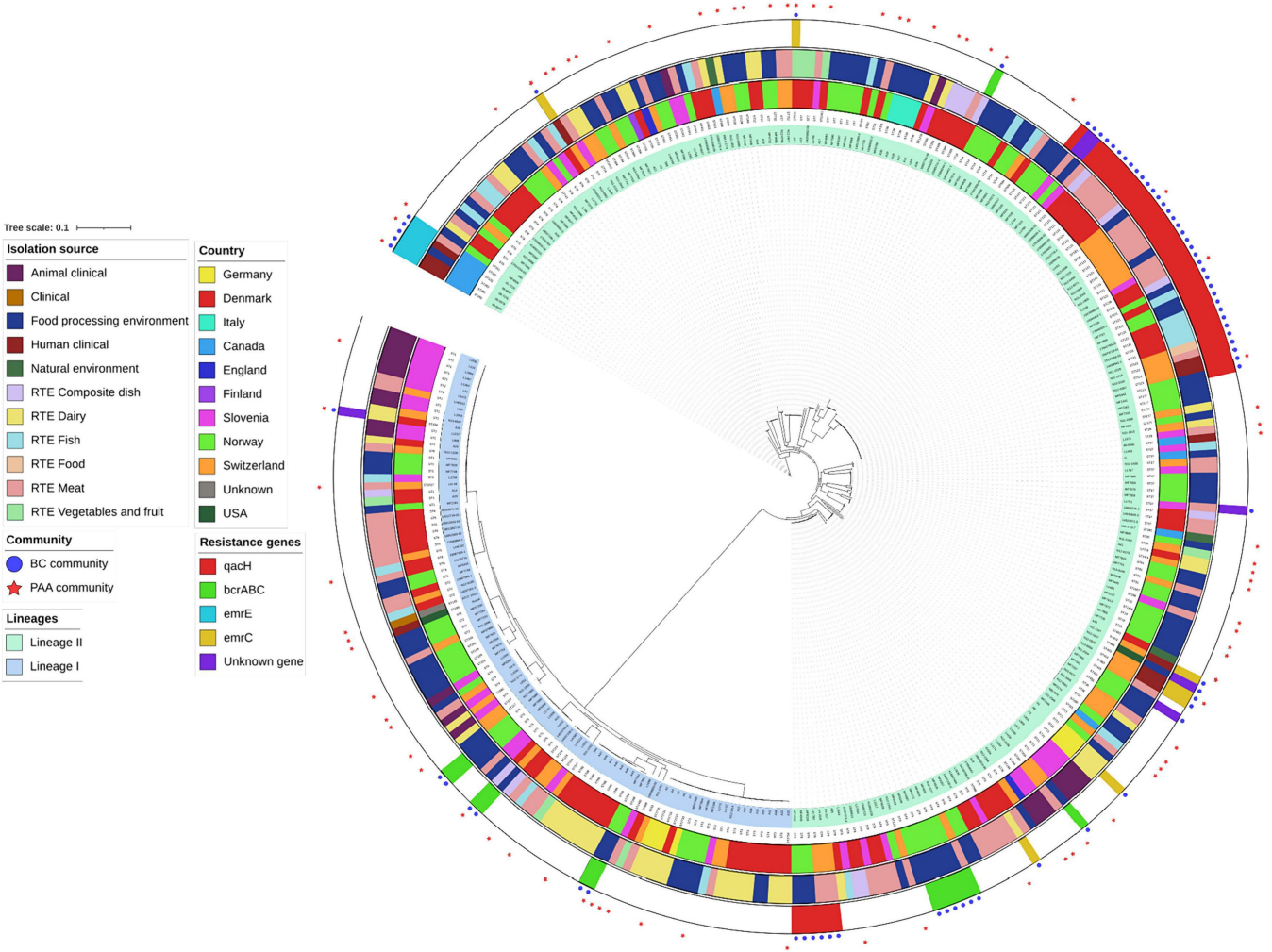
Supplementary Figure 5 Growth curves for the PAA community for the biocide treatment experiment with PAA concentrations of 30, 35 and 38 mg/L. A positive control consisting of clean media (without *L. monocytogenes*) and a negative control with media *L. monocytogenes*, but without biocide was included. The x-axis depicts hours after biocide treatment and the y-axis depicts the OD540 of the community.

Data Statement

The metagenomic raw sequence data in this study can be retrieved in the European Nucleotide Archive (http://www.ebi.ac.uk/ena) under study accession no.: PRJEB66133.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.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Isolation source
Animal clinical
Clinical
Food processing environment
Human clinical
Natural environment
RTE Composite dish
RTE Dairy
RTE Fish
RTE Food
RTE Meat
RTE Vegetables and fruit
Country
Germany
Denmark
Italy
Canada
England
Finland
Slovenia
Norway
Switzerland
Unknown
USA
Community
BC community
PAA community
Lineages
Lineage II
Lineage I
Resistance genes
qacH
bcrABC
emrE
emrC
Unknown gene

Figure 1

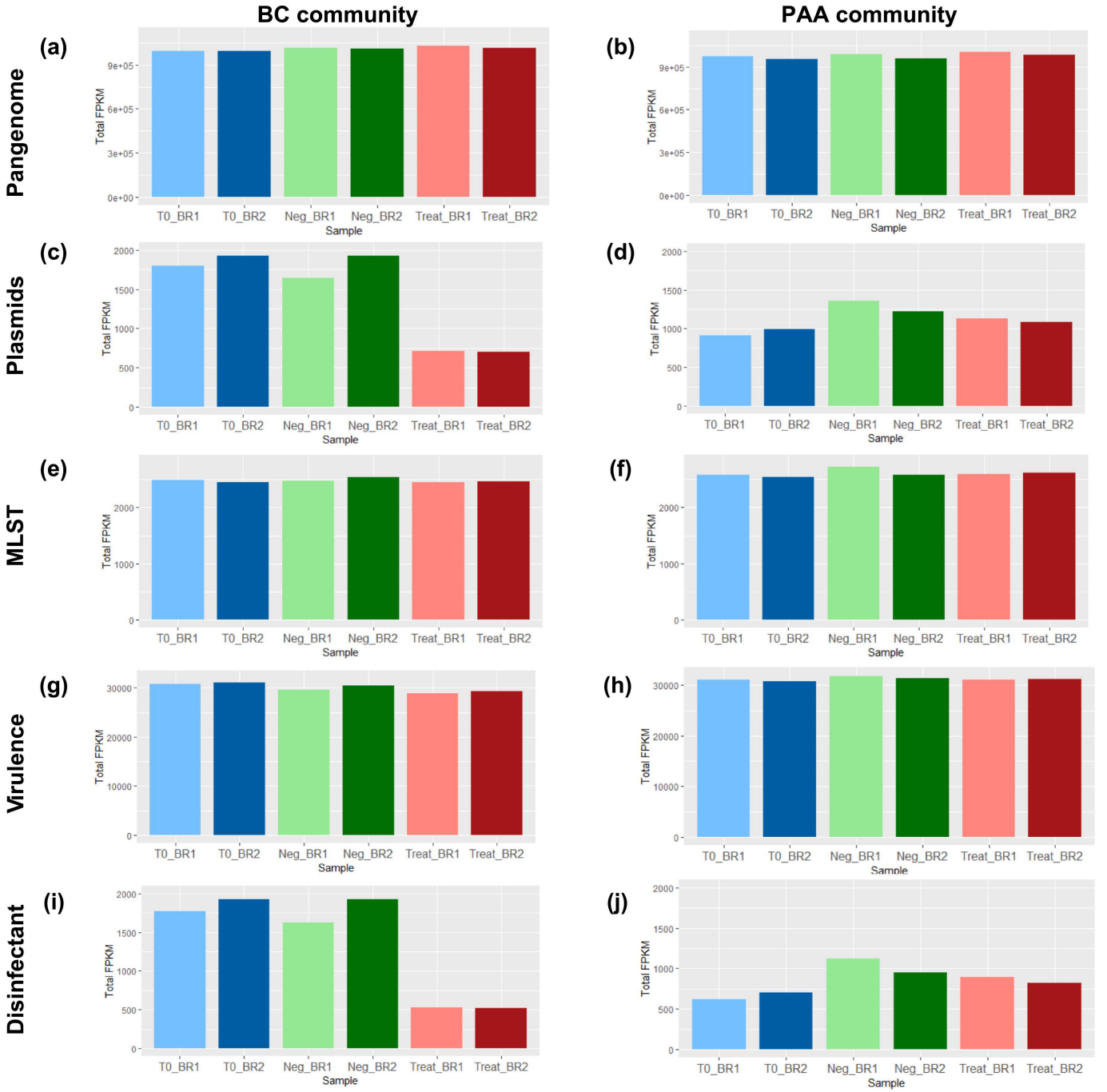
BC community
PAA community
Pangenome
Plasmids
MLST
Virulence
Disinfectant
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
Total FPKM
Sample
T0_BR1
T0_BR2
Neg_BR1
Neg_BR2
Treat_BR1
Treat_BR2

Figure 2

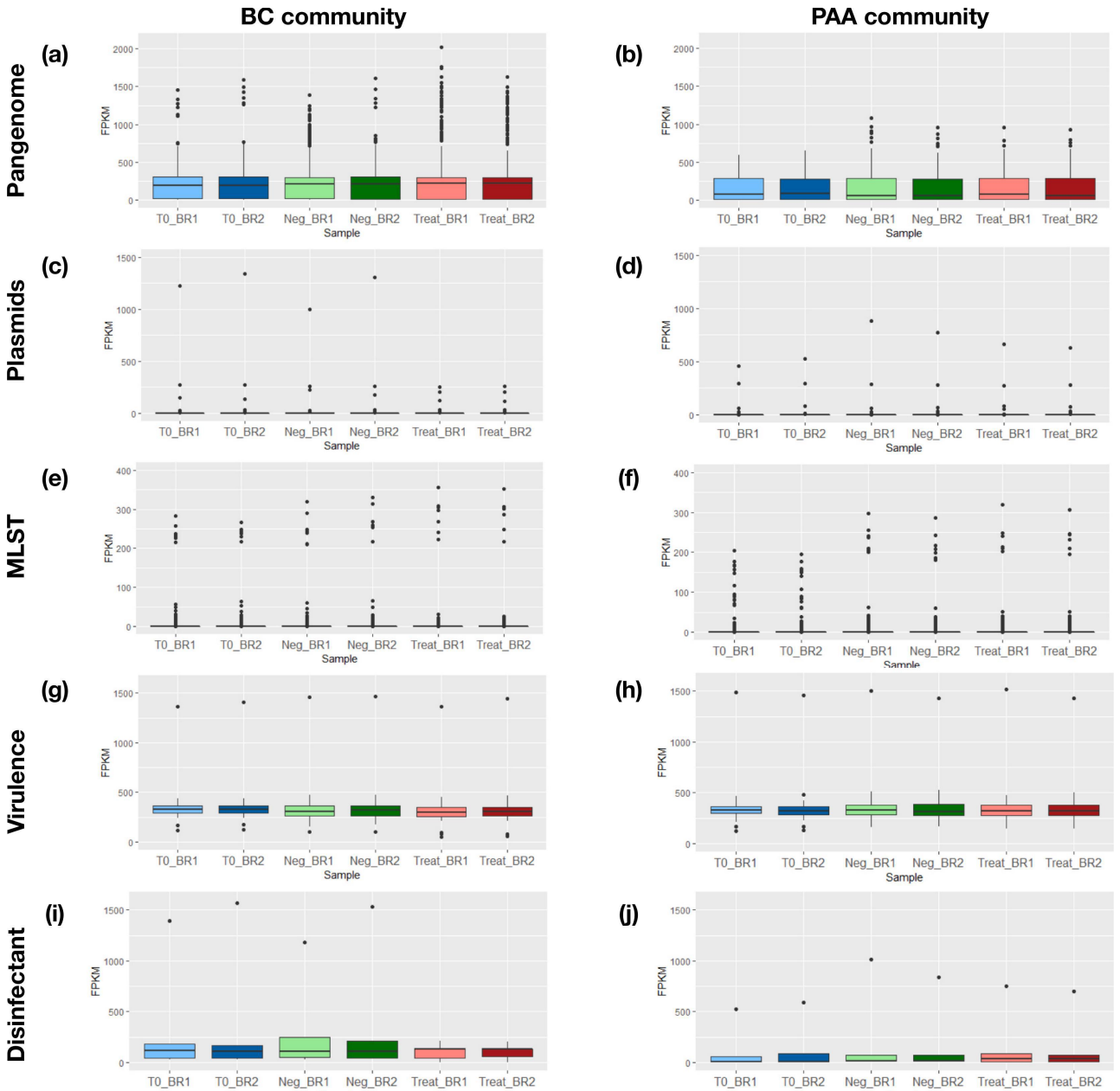
BC community
PAA community
Pangenome
Plasmids
MLST
Virulence
Disinfectant
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
(j)
FPKM
Sample
T0_BR1
T0_BR2
Neg_BR1
Neg_BR2
Treat_BR1
Treat_BR2

Figure 3

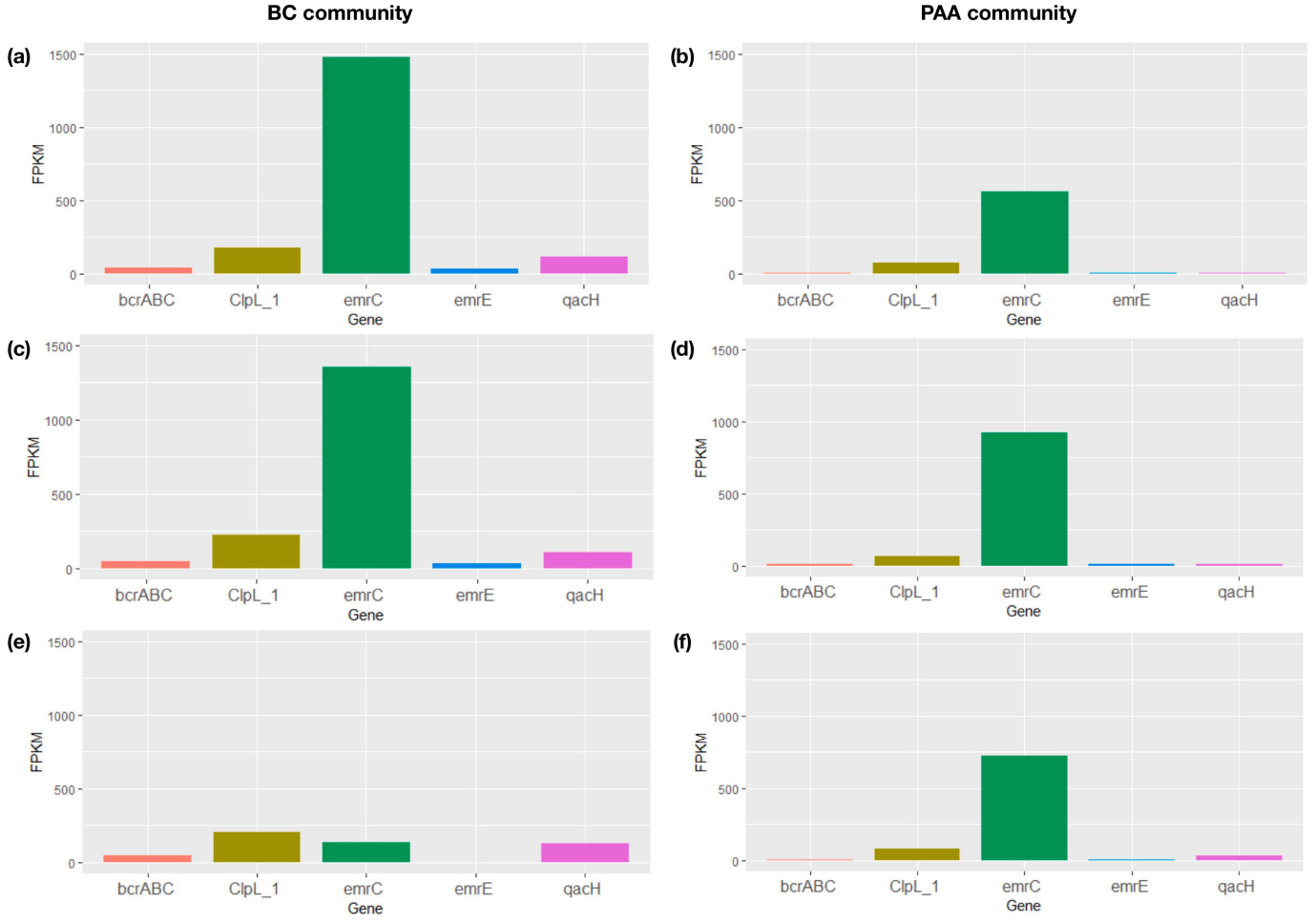
BC community
PAA community
(a)
(b)
(c)
(d)
(e)
(f)
FPKM
1500
1000
500
0
bcrABC
ClpL_1
emrC
emrE
qacH
Gene

Figure 4

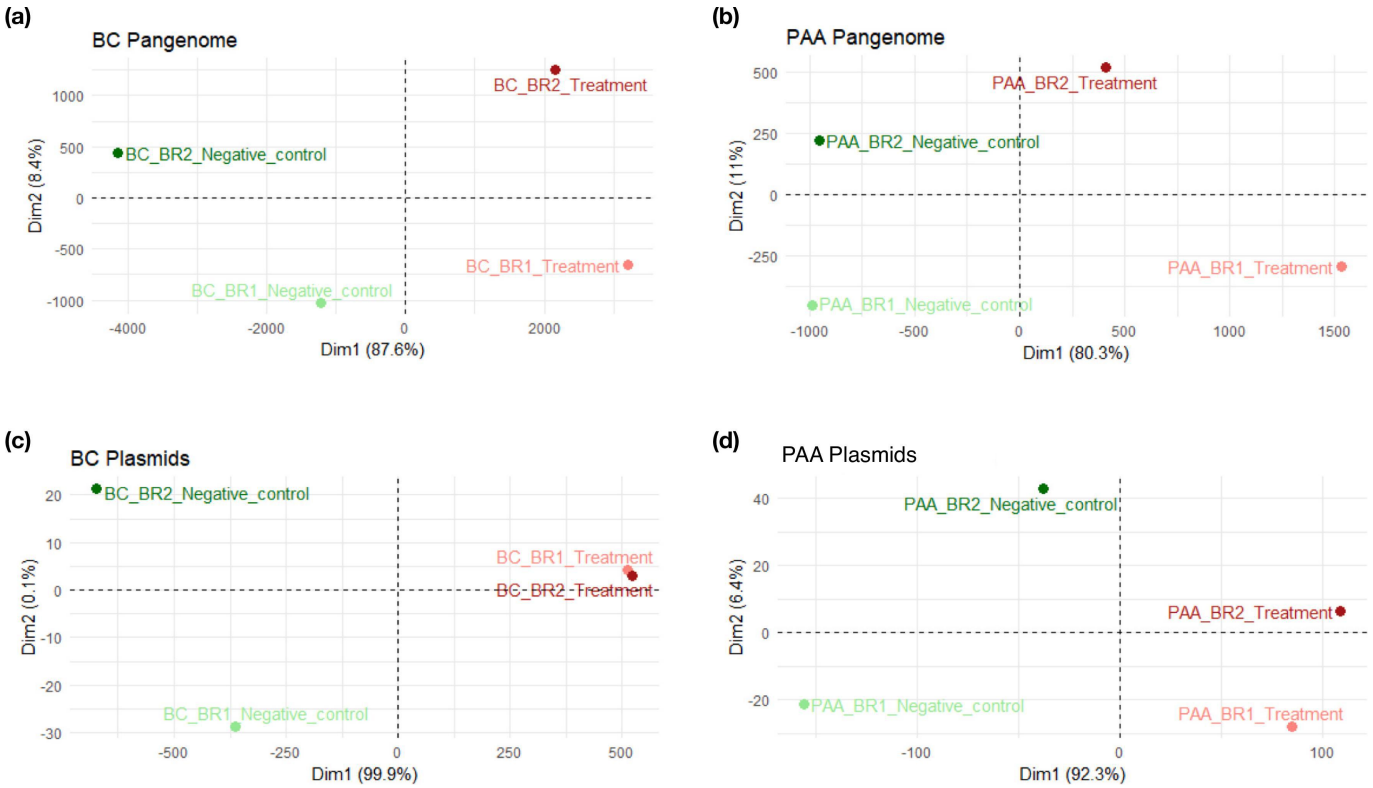
(a)
BC Pangenome
BC_BR2_Treatment
BC_BR2_Negative_control
BC_BR1_Treatment
BC_BR1_Negative_control
Dim2 (8.4%)
Dim1 (87.6%)
(b)
PAA Pangenome
PAA_BR2_Treatment
PAA_BR2_Negative_control
PAA_BR1_Treatment
PAA_BR1_Negative_control
Dim2 (11%)
Dim1 (80.3%)
(c)
BC Plasmids
BC_BR2_Negative_control
BC_BR1_Treatment
BC_BR2_Treatment
BC_BR1_Negative_control
Dim2 (0.1%)
Dim1 (99.9%)
(d)
PAA Plasmids
PAA_BR2_Negative_control
PAA_BR2_Treatment
PAA_BR1_Negative_control
PAA_BR1_Treatment
Dim2 (6.4%)
Dim1 (92.3%)

Figure 5

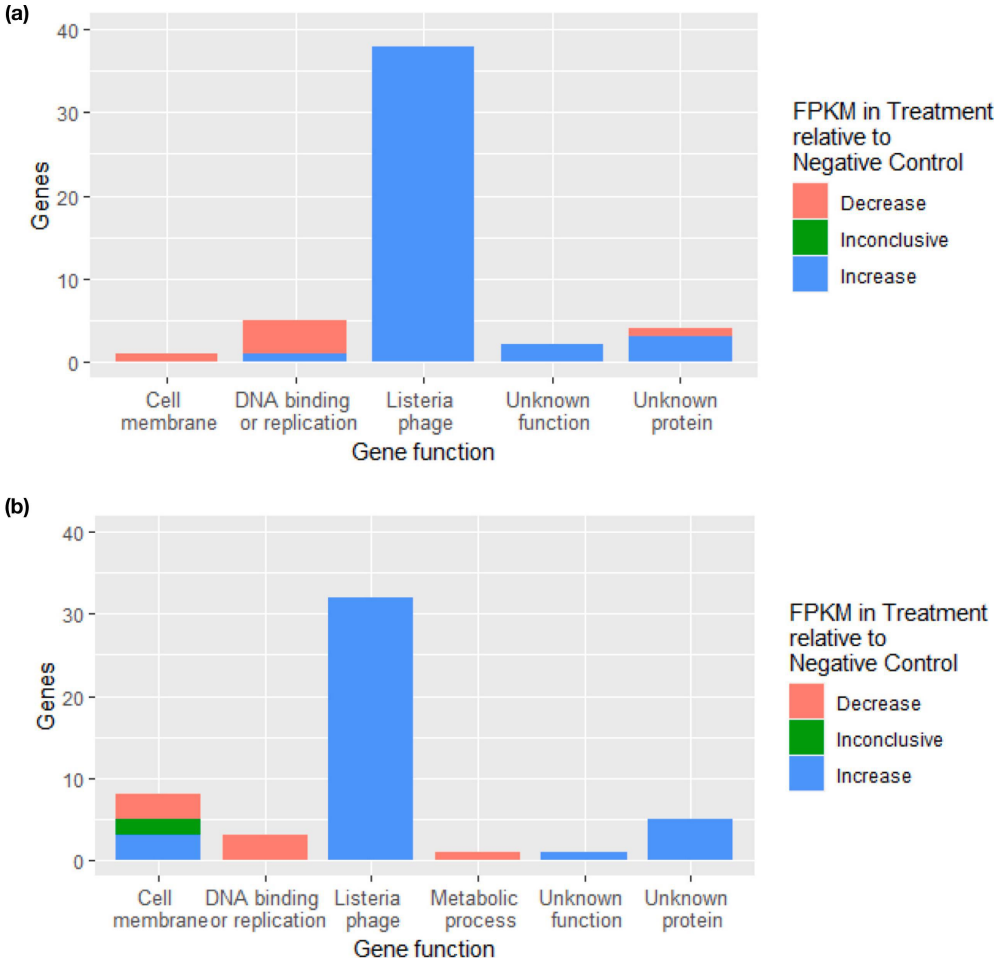
(a)
Genes
40
30
20
10
0
Cell membrane
DNA binding or replication
Listeria phage
Unknown function
Unknown protein
Gene function
FPKM in Treatment relative to Negative Control
Decrease
Inconclusive
Increase
(b)
Genes
40
30
20
10
0
Cell membrane
DNA binding or replication
Listeria phage
Metabolic process
Unknown function
Unknown protein
Gene function
FPKM in Treatment relative to Negative Control
Decrease
Inconclusive
Increase

Figure 6