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# Genomic elements located in the accessory repertoire drive the adaptation to biocides in *Listeria monocytogenes* strains from different ecological niches

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## ABSTRACT

In response to the massive use of biocides for controlling *Listeria monocytogenes* (hereafter *Lm*) contaminations along the food chain, strains showing biocide tolerance emerged. Here, accessory genomic elements were associated with biocide tolerance through pangenome-wide associations performed on 197 *Lm* strains from different lineages, ecological, geographical and temporal origins. Mobile elements, including prophage-related loci, the Tn6188<sub>qacH</sub> transposon and pLMST6<sub>emrC</sub> plasmid, were widespread across lineage I and II food strains and associated with tolerance to benzalkonium-chloride (BC), a quaternary ammonium compound (QAC) widely used in food processing. The pLMST6<sub>emrC</sub> was also associated with tolerance to another QAC, the didecyldimethylammonium-chloride, displaying a pleiotropic effect. While no associations were detected for chemically reactive biocides (alcohols and chlorines), genes encoding for cell-surface proteins were associated with BC or polymeric biguanide tolerance. The latter was restricted to lineage I strains from animal and the environment. In conclusion, different genetic markers, with polygenic nature or not, appear to have driven the *Lm* adaptation to biocide, especially in food strains but also from animal and the environment. These markers could aid to monitor and predict the spread of biocide tolerant *Lm* genotypes across different ecological niches, finally reducing the risk of such strains in food industrial settings.

## 1. Introduction

Biocides used as disinfectants in the food production system play an essential role in the protection of consumers against foodborne disease such as listeriosis caused by the pathogen *Listeria monocytogenes* (*Lm*). Despite the huge investments made each year to implement sanitization protocols in food production environments (i.e. 10–11 billion euros with market growing at 4–5% per annum for the last 15 years) (SCENIHR, 2009), *Lm* contaminations in processed foodstuffs are still a major concern in Europe. *Lm* was responsible for 2549 confirmed invasive listeriosis human cases (0.47 cases per 100,000 population notification rate; mortality rate 15.8%) in 2018. The last European Union One Health Zoonoses Report reported a statistically significant increasing trend of listeriosis observed in Europe since 2009 (EFSA & ECDC, 2019). Most of the human clinical cases are linked to the consumption of ready-to-eat (RTE) foods, including fish, cheese and bovine meat, as well

as vegetable foodstuffs contaminated with *Lm* during manufacturing processing in food plants.

Cleaning and disinfection (C&D) procedures are critical for controlling *Lm* spread along the food chain and reduce the public health risk of contaminated food (Cerf et al., 2010). A variety of biocides, like quaternary ammonium compounds (QAC), biguanides, alcohols, chlorine compounds, peroxygens or acids, are commonly used during C&D in the food-processing industry, animal husbandry and healthcare settings, at levels intended to be higher than the minimal bactericidal concentration of *Lm*. However, multiple factors affect the efficacy of sanitizers used in the food industrial premises against *Lm*: the presence of high bacterial concentrations, the interference with organic matter due to insufficient cleaning prior to disinfection, as well as inadequate dosage or rinsing of biocides (Overney et al., 2017; Rodríguez-López et al., 2018). This may lead to reduced activity of biocides and to the exposure of *Lm* cell to sub-inhibitory concentrations (Overney et al., 2017;

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Rodríguez-López et al., 2018). In response, the capacity of some *Lm* strains to develop an increased tolerance to biocidal agents (e.g. higher minimum inhibitory concentrations (MIC) toward a biocide in the observed bacterial population) commonly used as disinfectants has been reported (SCENIHR, 2009). This behaviour likely derives from an evolution of phenotypes of strains linked to the acquisition of new transitory or stable properties determining the adaptation to new environmental conditions (Martín et al., 2014; Palma et al., 2020) more than to innate mechanisms.

*Lm* strains are grouped into major lineages (from I to IV), which include several multilocus sequence typing (MLST)-based clonal complexes (CC) (Haase et al., 2014; Ragon et al., 2008) shaping a structured population of phylogenetically defined groups (Cantinelli et al., 2013; Chenal-Francisque et al., 2011). Within the *Lm* population, the adaptation of some strains to constrained environmental conditions is often driven by particular genetic mechanisms, which are horizontally acquired from the bacterial dispensable genomic pool through the transfer of genetic materials (Kuenne et al., 2013; Popa and Dagan, 2011). Indeed, the horizontal gene transfer (HGT) of homologous and non-homologous genetic elements (Sheppard et al., 2018), particularly associated with mobile genetic elements, is the most likely mechanism for dissemination of strains with increased tolerance and resistance to antimicrobials. Multiple tolerance/resistance genes and overlapping genetic cascades of regulation responding to selective pressures from chemicals are harboured by these mobile genetic elements. The corresponding genetic mechanisms essentially cause a reduction of the intracellular concentration of biocide under the threshold that is harmful to the bacterium (SCENIHR, 2009). For instance, genetic mechanisms such as *emrC* efflux pump (from the small multidrug resistance (SMR) family), plasmid-encoded *bcrABC* cassette (Elhanafi et al., 2010) and the transposon *Tn6188* (Müller et al., 2013) appear to be the main genetic elements involved in the increased tolerance of *Lm* strains to benzalkonium chloride (BC), the most commonly used QAC in industrial environments (Roedel et al., 2019). These elements, often carried on mobile genetic elements, has been more frequently observed in *Lm* strains of lineage II (including genotypes frequently found in food processing) (Fox et al., 2016; Gilmour et al., 2010; Palma et al., 2020) in comparison to lineage I (including genotypes frequently involved in human listeriosis) (Maury et al., 2016).

The massive use and misuse of biocides raised several concerns. Firstly, the selection of genetic elements that can be horizontally transferred between genetically distant strains sharing the same ecological niche may reduce the options for disinfecting treatment in industrial settings (Dutta et al., 2013; Shapiro, 2015). Secondly, the occurrence of high concentrations of these compounds in the environment (i.e. surface waters, sewage treatment plants and their immediate outflows) represents a potential hazard for the selection of biocide-tolerant bacteria (SCENIHR, 2009).

Better understanding the genomic mechanisms for biocide-tolerance across *Lm* strains of diverse origins and genetic background is then necessary to improve the identification of *Lm* strains presenting high-risk properties such as enhanced tolerance to specific biocide throughout the food chain. Today, genome-wide association studies (GWAS) represent powerful tools for the identification of associations between genomic elements and phenotypic properties (e.g. biocide tolerance) (Rantsiou et al., 2018). In this study, we combined pan-genomic analyses of 197 *Lm* strains from several lineage I and II CCs, inference of their population structure, estimation of MIC data for several biocides commonly used in animal husbandry and food industrial settings in a GWAS implementing population structure correction.

## 2. Materials and methods

### 2.1. Strain collection

This study includes 197 *Lm* strains collected from different

ecological niches, i.e. food, animal and environment (metadata, including geographic location, year and source of the isolation are listed Supplementary data 1). The matrix of isolation of food strains was defined according to Painset et al. (2019) risk food matrixes: “fish and fishery product”, “meat and meat product”, “dairy product” and “fruit & vegetables” (Painset et al., 2019). The strains were selected from surveillance activities and previous studies conducted in Europe (Dreyer et al., 2016; Fritsch et al., 2019; Henri et al., 2017; Painset et al., 2019; Papić et al., 2019). Strains were collected over more than 20 years, with most of them (80%) isolated after 2010, across 16 countries in Central (n = 6), Northern (n = 5), Western (n = 3) and Southern (n = 2) Europe (Supplementary data 1). In total, 99 strains were isolated from food (dairy, fishery, meat and vegetables), 66 from (wild and farm) animals and 35 from the environment (e.g. vegetation, soil and water). We balanced our dataset with similar relative portions of *Lm* strains from nature and primary production (animal/environment), as well as secondary and tertiary production (food), for lineage I (~48% vs ~52%) and II (~53% vs ~47%).

### 2.2. Genomic data

CCs were defined as groups of 7 locus - MLSTs differing by only one gene from another member of the sequence type (ST) (Ragon et al., 2008). For part of the strains collection, the genomic sequences were already available (Dreyer et al., 2016; Fritsch et al., 2019; Henri et al., 2017; Painset et al., 2019; Papić et al., 2019). For the others, newly sequenced genomes were produced using Illumina technologies within ListAdapt project (grant #773830; <https://onehealthjeu.eu/jrp-listadapt/>). Raw reads for the whole dataset were processed using the “Assembly of reads and typing workflow” (ARTwork) open-source pipeline adopted in the Maisons-Alfort Food Safety laboratory (Anses) (Palma et al., 2020; Vila Nova et al., 2019). Briefly, the ARTwork workflow includes several tools to perform variant calling (i.e. iVar-Call2 by (Felten et al., 2017)), *de novo* assembly and annotation (Palma et al., 2020; Vila Nova et al., 2019) and assembly (Ewels et al., 2016; Gurevich et al., 2013). In parallel, the recently developed tool ConFindr was also applied for detection of inter- and intra-species contamination in raw reads based on the identification of single nucleotide variants (SNVs) from multiple alleles of core, single-copy and ribosomal-protein genes (Low et al., 2019; Pightling et al., 2019). All strains (n = 197) whose raw reads passed the quality check steps defined in ARTwork and presented less than 10 SNVs identified by Confinder according to Pightling et al. (2019), and whose draft genome presented a number of scaffolds lower than 200 and unambiguous total lengths (e.g. in the range of 2.7–3.3 kbp) were retained for downstream analyses (Supplementary data 2, <https://figshare.com/s/ae59166e78474068af7>).

### 2.3. Biocides and biocide susceptibility based on MIC

Eight biocidal compounds commonly used as disinfectants in medical, veterinary and industrial settings were selected to detect the molecular mechanisms for biocide-tolerance of *Lm*. The selected biocides along with their principal area of use, main bactericidal action and concentration are reported in Table 1.

The MICs of the studied biocides were determined using standard broth microdilution methods and results were reported in Supplementary data 3. Briefly, *Lm* strains were plated on Tryptone Soya Agar (TSA) with blood (Sigma-Aldrich). Two to three colonies were collected in 4 ml of Tryptone Soya Broth (TSB) with yeast extract (0.6%) and incubated during 24 h at 37 °C. Then, 300 µl of these precultures were added to 10 ml of Tryptone Soya Broth (TSB) with yeast extract, incubated at 37 °C during 3–4 h and adjusted to OD<sub>620nm</sub> = 0.1 (±0.02) in sterile peptone water. Finally, 20 µl of bacterial cultures at 0.1 OD<sub>620nm</sub> (diluted to 1/100 corresponding to final concentration between 3 and 6.105 CFU/ml) and 160 µl TSB with yeast extract was added to 96-wells microplates (PS, F BOTTOM, CLEAR Greiner bio one; Dutscher)

**Table 1**

Biocides category, usage and main bactericidal action of the biocide compounds tested in this study; adapted from (McDonnell and Russell, (1999); SCENIHR, 2009).

Biocide category	Biocide compound	Usage/area of application	Main bactericidal action	Possible concentration range (%)
Quaternary ammonium compounds	Benzalkonium chloride (BC) Didecyl Dimethyl Ammonium Chloride (DDAC)	Health care, household products, food industry, pharmaceutical/cosmetic (preservation)	Membrane damage, produce cytoplasmic protein aggregation (loss of tertiary structure) with disruption in the phospholipid bilayer and subsequent cellular content leakage	0.03–50
Polymeric biguanides	PolyHexaMethylene Biguanide (PHMB)	Health care, household products, water treatment, impregnation of fabrics to inhibit microbial growth	Highly adsorptive to anionic surfaces (bacterial cell walls) - damage to the cell membrane with phase separation and domain formation of membrane lipids	0.43
Alcohols	Ethanol (EtOH)	Health care, pharmaceutical/cosmetic, beverage industry (preservation)	Membrane damage and inhibition of DNA and RNA synthesis - cell wall synthesis (secondary effect)	0.1–99.9
Peroxygens	Peroxyacetic Acid (PA); Hydrogen Peroxide (HP)	Pharmaceutical/cosmetic (preservation), food and beverage industry; bleaching agent for textiles and paper	DNA strand breakage with formation of free hydroxyl radicals (OH), which oxidize thiol groups in proteins and enzymes. Interfere with cellular phospholipid bilayers and cytosolic material causing irreversible damage	0.008–0.23 (PA); 0.5–29 (HP)
Chlorine compounds	Sodium Hypochlorite (HS)	Health care, household products, industrial products, water treatment (private and industrial use)	Production of hypochlorite ion, disrupts oxidative phosphorylation and membrane associated activity	0.02–22.4
Amine acetates	N-(3-aminopropyl)-N-dodecylpropane-1 3-diamine (AMPD)	Veterinary hygiene, food and animal feeds, product preservation, hospitals (surfaces and instruments).	Biodegradable biocide with cationic properties	Not Determined

containing 20 µl of biocide dilutions (10X) to visually observe bacterial growth by turbidity changes. Plates were then incubated at 37 °C for 24 h and MIC was determined as the lowest concentration of biocide that prevents bacterial growth.

Sensitive and tolerant groups for each biocide were defined based on MICs. Strains with the lowest and highest MIC values for the tested biocide across the observed population were considered as sensitive and tolerant, respectively. Strains with intermediary MICs were included in the sensitive or tolerant group while maintaining a balanced population size between compared groups.

#### 2.4. Pangenome orthologous groups

The annotated genome feature format (GFF) files produced using Prokka v1.13.3 (Seemann, 2014) were used to identify the pangenome orthologous groups (POGs) of the studied *Lm* population through the recently developed Panaroo workflow (Tonkin-Hill et al., 2020). This tool infers pangenomes clustering coding DNA sequences (CDSs) to form ‘core’ (e.g. present in ≥99% of isolates) and non-core (‘accessory’) pangenome orthologous groups. We run Panaroo in ‘strict’ mode (i.e. stringent contamination filter) with default parameters, considering the genes present in the 95% of the strains as part of the core genome. The comparisons of pangenome repertoires were performed by with Venn diagrams produced by Venny (<http://jvenn.toulouse.inra.fr/app/index.html>) (Bardou et al., 2014).

#### 2.5. Population structure estimated by phylogenomic reconstruction and homologous recombination detection

A phylogenomic reconstruction was performed to determine the overall population structure based on the single nucleotide polymorphisms (SNPs) detected in the core genome. The alignment of pseudogenomes (including 94,004 variant sites) produced by iVarCall2 were constructed based on mapping of reads against the reference genome EGD-e (accession NC003210) and GATK variant calling analysis (McKenna et al., 2010). These pseudogenomes were used to infer trees by maximum-likelihood (ML) using the IQ-Tree software (Kropac et al., 2019). The ‘ModelFinder’ option was applied for an automated substitution model selection, with 1000 SH-aLTR (Shimodaira and Hasegawa - approximate likelihood ratio test) and ultrafast bootstrap support. The best-fit model was the transversion model with unequal base frequency TVM + F + R7 according to the Bayesian information criterion (BIC). Correction of the ML inference view regard to homologous

recombination events from the core genome was performed using ClonalFrameML (Didelot and Wilson, 2015) with “-emsim 20” (number of simulations to estimate uncertainty in the expectation maximization (EM) results). The main ClonalFrameML outputs include an inference with recombination-corrected branch lengths, an estimation of the recombination process key parameters and a genomic map of recombination location for each branch of the phylogeny (Supplementary data 4). As the overall scale of the ClonalFrameML tree reflects more the point mutation evolution than the ML tree including SNPs from homologous recombination (Didelot and Wilson, 2015), the phylogenomic tree with parsed recombination (32,240 variant sites) was retained and visualized in iTOL (Letunic and Bork, 2016) to examine specific biocide-tolerance associated POGs and phylogenomic connections between isolates metadata.

#### 2.6. Pangenome-wide detection of mutations associated with biocide-tolerance

The detection of pangenome-wide mutations associated with enhanced tolerance to each biocide under study was performed with GWAS approaches where confounding effects (i.e. clonal population structure, homologous recombination and genome wide significance) are taken into account without losing statistical power to detect associations (San et al., 2020). First, a recombination-aware phylogenetic tree-based GWAS approach was applied using the R-package treeWAS (Collins and Didelot, 2018) for testing associations between core genome SNPs and binary (tolerant vs. susceptible) or continuous (MIC values) phenotypes based on MIC data. Then, another GWAS was performed to screen POGs for associations with each studied biocide. Using the POGs presence/absence matrix produced by Panaroo as input, the Python-based sequence element enrichment analysis (pyseer) tool was run in combination with the continuous MIC data for the eight biocides described above and the phylogenomic reconstruction correcting the population structure with regard to homologous recombination events from the core genome (Lees et al., 2018). Based on the phylogeny\_distance.py script provided by the authors, we run pyseer with the linear mixed model (LMM) and the patristic distances matrix generated on the recombination-free core phylogenomic tree (based on the shared branch length between each pair’s ancestors and the root). Only POGs present in more than 1% of the total population and presenting 100% of specificity (absent in the less tolerant strains) for each biocide were retained for further analyses. To assess the genome wide significance of associated POGs, we investigated multiple testing adjustments of unique patterns of



POGs presence/absence (Bonferroni) and the quantile-quantile (Q-Q) distributions between observed likelihood-ratio test (LRT)  $p$ -values and expected  $p$ -value under a null distribution.

### 2.7. Data availability

The high-quality genomic sequences from this study are available for download at SRA/ENA database, including sequences already available at the beginning of this study. For easy access to sequences, accession numbers are provided in Supplementary data 1 available at <https://doi.org/10.6084/m9.figshare.12573968.v1>.

## 3. Results

In this study, we integrated phenotypic, pangenomic and phylogenomic characterization with advanced statistical tests to detect molecular mechanisms associated with enhanced tolerance to biocides commonly used as disinfectants in the food industry for *Lm* strains of lineages II and I.

### 3.1. Origin and molecular sub-types of *Lm* strains

The collection of strains with high-quality genomic data selected for this study encompasses a large genomic diversity of *Lm* population from lineage I and II, i.e. 29 CCs and 2 STs disassociated from CC. Each CC included 1–19 strains. The distribution of CCs from the lineage I and II for the different isolation sources is reported in Fig. 1 (see Supplementary data 1 for additional details on CCs per source of isolation).

The contiguity metrics of *de novo* assembly showed an average genome length of 2,955,717 bp, a high N50 (2,937,244), a low number of scaffolds (median of 5, 1st and 3rd quartiles corresponding to 2 and 12.5) and a mean GC% content of 37.8%. The summary data of the assembly metrics for each genome are listed in the Supplementary data 2.

### 3.2. Phylogenomic reconstruction of *Lm* strains and MICs distribution across different ecological niches

The maximum-likelihood phylogenomic reconstruction based on

coregenome SNPs identified across 197 genomes was inferred excluding homologous recombination events from the coregenome. The relative rate of recombination to mutation (R/theta) inferred by ClonalFrameML was 0.60, with a mean genetic distance between donor and recipient of recombination (nu) of 0.03 substitutions per nucleotide position. The tree excluding recombination shaped two main clades, representative of lineage I and II, and each clade consists of CC-defined clusters (Fig. 2).

Within each cluster, strains from different ecological niches (i.e. food, animal and environmental) were intermixed showing a highly conserved coregenome. However, evidences that the coregenome had undergone genetic recombination events were also observed, with lineage II strains showing higher recombination rates in comparison to lineage I after lineage divergence (Supplementary data 4). Likely due to the large use of QACs in food processing plants, the food strains showed higher MICs for this class of biocide in comparison to animal/environmental ones (Fisher's exact test;  $P < 0.00001$ ), with a higher occurrence of tolerant profiles across lineage II clade (Fig. 2). The food strains showed also the highest MIC results for peroxygens compounds (e.g. HP and PA) without correlation with the lineage they belong. On the other hand, animal/environmental strains appeared to be more tolerant to AMPD and PHMB, and tolerant profiles for these biocides prevailed across lineage I clade (Fig. 2). We did not observed associations between MIC values of EtOH or sodium hypochlorite (HS) and ecological niches or lineages. Overall, strains with susceptible and tolerant profiles with regard to the biocides under study were randomly distributed over the CC-defined phylogenetic clusters.

### 3.3. Pangenomic orthologous groups of *Lm* strains from different ecological niches

The polished pangenome extracted from the Prokka-annotated genomes using Panaroo included 6324 POGs. Consistent with previous studies on the *Lm* pangenome (Bakker et al., 2013; Palma et al., 2020), 2448 POGs are present in >99% of strains, while the remaining 3876 POGs compose the accessory genome (present with a variable frequency in the observed *Lm* population). The pangenome across 197 strains showed that *Lm* genomes encode  $2887 \pm 87$  genes (average  $\pm$  standard deviation), with significant variation in the size of the accessory genome

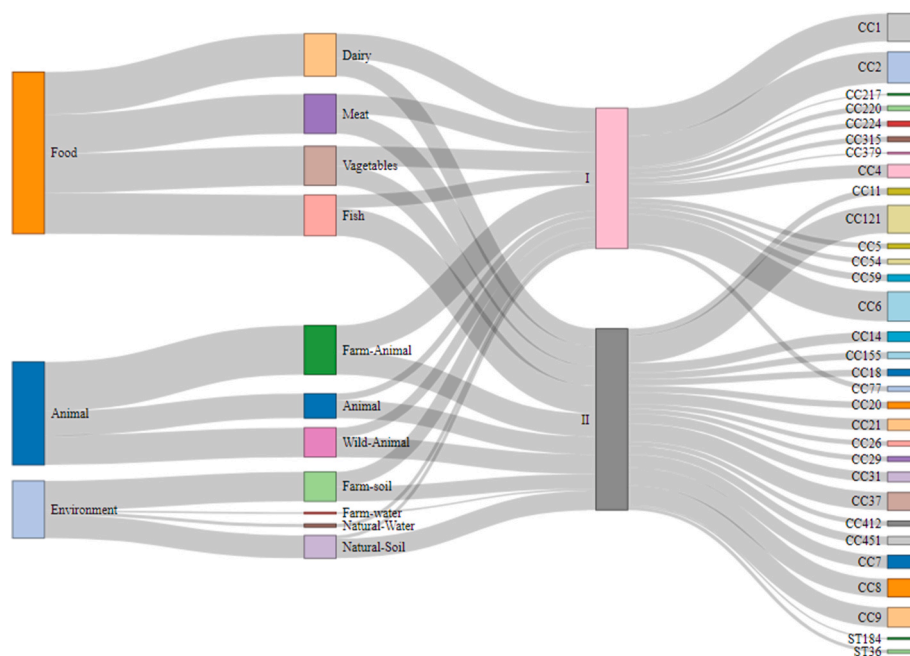
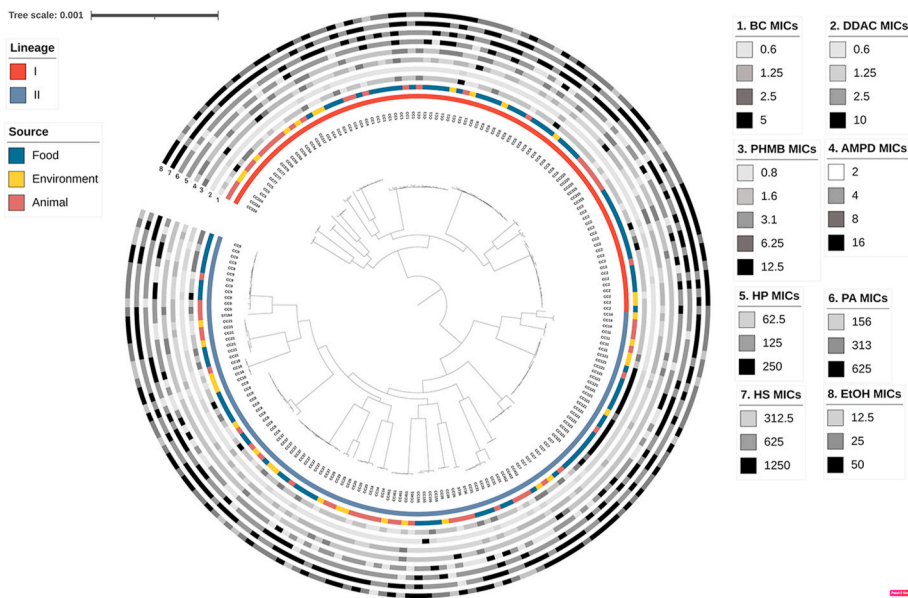


Fig. 1. Experimental dataset. Lineage I and II clonal complexes distribution per source of isolation of the *Lm* strains isolated from food, animal and environmental niches.



**Fig. 2.** Recombination-aware phylogenomic reconstruction. Coregenome SNP-based maximum likelihood phylogenomic reconstruction of 197 *Lm* isolates excluding homologous recombinant events to adjust the branch lengths. The tree is rooted on the lineage I clade. Isolates' labels were removed for clarity and leaves were annotated with the CCs to which isolates belong. For each isolate, the lineage and source (ecological niche) are reported with coloured stripes, while the MIC ranges in grey scale for the eight biocides, BC (1), DDAC (2), PHMB (3), AMPD (4), HP (5), PA (6), HS (7) and EtOH (8), from the inner to the outer ring.

ranging from 313 to 688 genes per strain. Most of the accessory genes (80%) were found in less than 15% of the population, and singletons (specific to individual strains) constituted a substantial portion (~30%) of this accessory repertoire. Although the slightly higher number of strains from food may impact the size of the accessory genome, the food strains appear to harbour more accessory genes than strains from animal and the environment (Fig. 3). The differential composition of the accessory genome may reflect the gain and loss of important accessory genomic elements like prophages, plasmids and transposons.

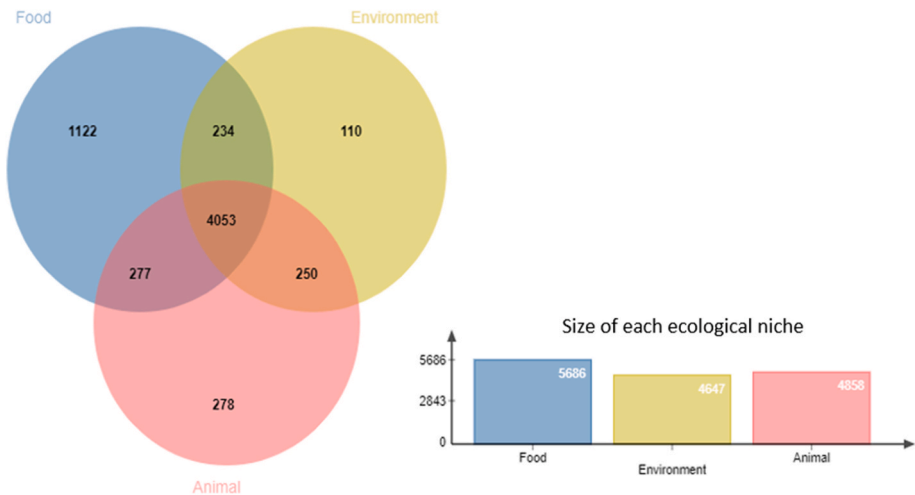
3.4. Genomic signatures of biocide-tolerance across *Lm* lineage I and II strains and their ecological niches

Molecular signals of adaptation to biocide in *Lm* strains from different ecological niches were investigated at the core and accessory scale. The recombination-aware phylogeny-based GWAS performed using treeWAS did not associate coregenome SNPs with the phenotypes of interest, using either binary or continuous traits.

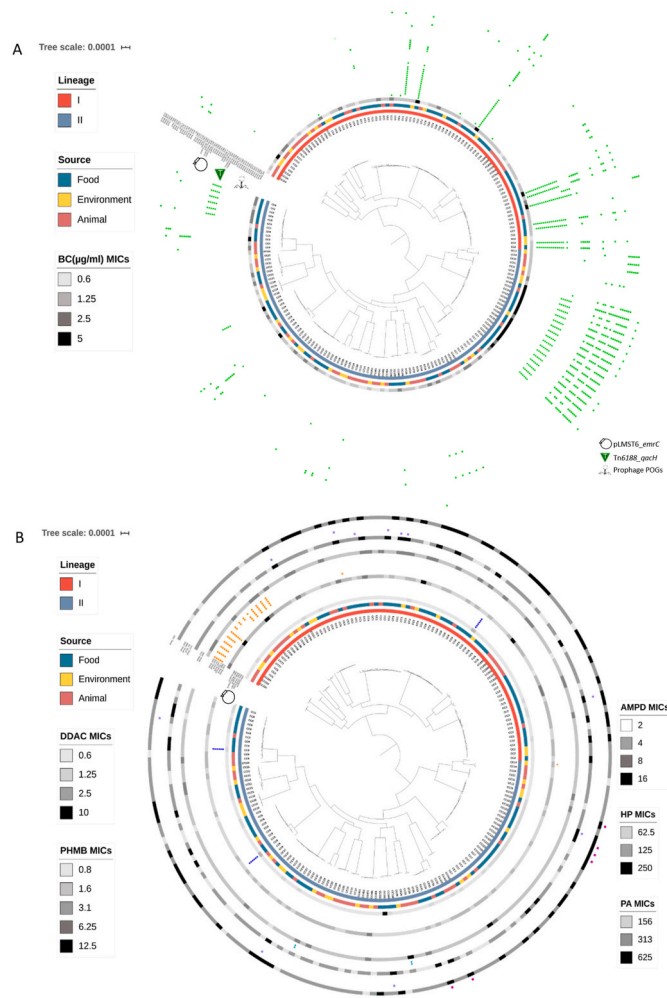
Over the 2706 accessory POGs tested for association with MICs of the eight biocides, the pyseer linear mixed model, taking into account the population structure correction, identified 699 POGs presenting

likelihood-ratio test (LRT) *p*-values lower than 0.01 (Supplementary data 5). To minimise false associations while preserving causal mutations, we excluded genomic elements identified (i) in the less tolerant strains (i.e. with lowest MICs for specific biocides), (ii) in single phylogroups (CCs) and (iii) in very low allele frequency (af) across the total population (af < 1%). In addition to frequency filtering, multiple tests using Bonferroni adjustment with  $\alpha < 0.05$  were applied on unique presence/absence patterns of POGs, following the pyseer authors' suggestions for interpreting significant (causal) associations. All the associated POGs above the LRT *p*-value  $\leq 4.42\text{E-}05$  were, hence, filtered out. At this genome-wide significance, 24 (af range: 3–13%), 2 (af: 1%) and 1 (af: 1%), POGs were retained as causally associated with tolerance to BC, AMPD and PHMB, respectively (Fig. 4A and B; use the link <https://itol.embl.de/tree/3758186129347771587974970> for interactive visualization; Supplementary data 6).

Among the causal elements strongly associated with BC tolerance, the 5-gene *Tn6188\_qacH* transposon (Müller et al., 2013) and a cluster of 14 POGs part of a prophage region were detected. The transposon *Tn6188\_qacH* was mainly harboured by strains belonging to CC121, CC9 and CC8 from lineage II, but also in two CC2 strains from lineage I. Whereas, the prophage-related POGs associated to BC tolerance were



**Fig. 3.** Pan genes composition. Venn diagram showing the pan genes composition of the *Lm* strains collected from the different ecological niches (i.e. food, animal and environment). The total size of the pan genes for each ecological niche (i.e. food, animal and environment) is reported in the bar chart.



**Fig. 4.** Biocide tolerance associations. Recombination-aware phylogenomic reconstructions of the 197 *Lm* strains and strongly associated POGs with BC (A) as well as DACC, PHMB, AMPD, HP and PA tolerance (B) by pyseer GWAS. The associations are plotted from the lowest to the highest LRT  $p$ -value from the inner to the outer circles. Dots represent the presence of associated POGs based on the strict Bonferroni genome-wide significance (LRT  $p$ -value  $\leq 4.42E-05$ ) while squares represent associated POGs based on relaxed quantile-quantile genome wide significance (LRT  $p$ -values  $\leq 6.90E-03$ ). For each biocide, the gradient of MICs value is reported in the grey scale coloured circles.

found only in lineage I strains from CC2, CC1 and CC6 phylogroups (Fig. 4B). All these strains were isolated in food, excepted for one that come from the environment. On the other hand, the few POGs associated with AMPD and PHMB tolerance, which encode for small hypothetical proteins part of prophage regions, were harboured by strains isolated from animal and environment (Fig. 4B).

The assessment of a suitable genome-wide significance is critical to differentiate true positives from false positives (i.e. type 1 errors) and false negatives (i.e. type 2 errors). Genome-wide significance by multiple testing may be overly conservative (inflate type 2 errors) and exclude POGs that may reflect the studied phenotypes. Looking at the Bonferroni genome-wide significance (blue dash line) on the Q-Q distributions, we noticed that numerous associations with inflated observed  $p$ -values (i.e. the sharp break upwards from the red line in Fig. 5) presented higher LRT  $p$ -values than the Bonferroni threshold. The true associations defined by the inflated observed  $p$ -values in the Q-Q plots included additional 34 (af range: 1–12%) and 6 (af range: 1–7%) POGs reflecting the MICs increase of BC and PHMB, respectively, with LRT  $p$ -value  $\leq 6.90E-03$ . At this less stringent genome-wide significance, few associated POGs (with af range: 1–3%) were also captured for DDAC ( $n = 6$ ), HP ( $n = 4$ ) and PA ( $n = 1$ ) enhanced tolerance (Table 2).

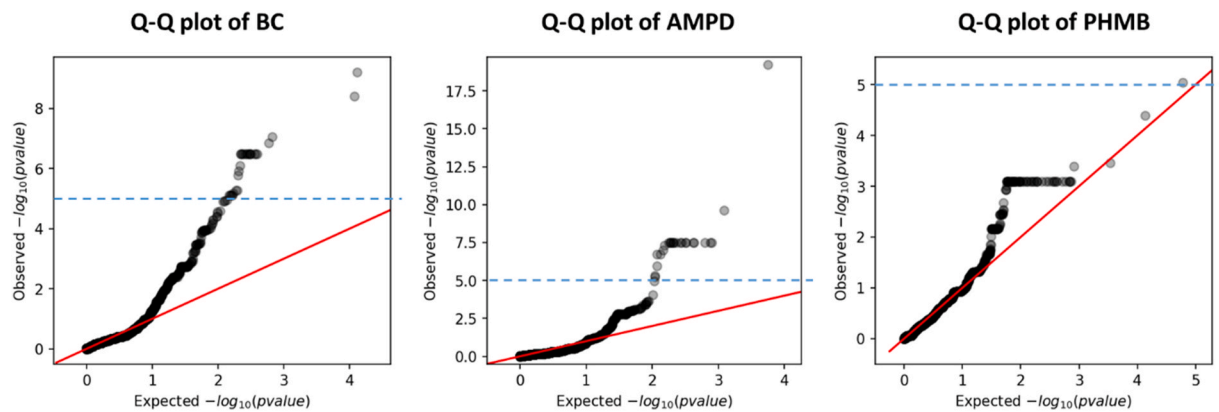
The 6-POGs associated with DDAC tolerance constituted the coding regions of the plasmid pLMST6\_ennrC (Kremer et al., 2017), whose presence appears also associated with the tolerance to BC. The pLMST6\_ennrC was previously described as responsible of increased QACs-tolerance and linked to meningitis from *Lm* ST6 strains (Kremer et al., 2017). The CC6 food strain belonging to lineage I and the CC8 and CC9 food strains from lineage II present the highest MICs for the studied QACs (BC and DDAC) and harbour the poly-specific pLMST6\_ennrC (Fig. 4). POGs encoded in the associated pLMST6\_ennrC plasmid and Tn6188\_qacH transposon appeared stratified in the Q-Q plots (Supplementary data 7), showing a polygenic effect (i.e. system of non-epistatic genes that influence a phenotypic trait) of QACs-tolerance mechanisms.

Among the additional POGs associated with BC tolerance, we also

**Table 2**

Number of pangenome orthologous groups significantly associated with biocides and related ranges of LRT  $p$ -values.

	No associated POGs	LRT $p$ -value range [min-max]	Allele frequency range [% min - % max]
BC	58	[1.43E-07-6.35E-03]	[1-13]
PHMB	7	[3.98E-05-6.96E-03]	[1-7]
DDAC	6	[1.00E-03-1.00E-03]	2
HP	4	[2.01E-03-9.16E-03]	[1-2]
AMPD	2	2.87E-08	1
PA	1	[4.87E-04-6.91E-04]	3



**Fig. 5.** Genome-wide significances. Q-Q plots reporting the probability distribution of POGs scored for association with the different biocides showing the genome-wide significance (blue dash line) based on Bonferroni correction at 5%. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

identified a cluster of 6 POGs (af 10%), including genes that encode for recombination hotspot (RHS) repeat proteins and the putative RNA 2'-phosphotransferases KptA. By blasting the sequences in public database (NCBI), we found that these POGs are included in a ~12 kbp genomic element 100% homologous to *Lm* strain 6179 (HG813249.1, from locus tag LM6179.0169 to LM6179.0174). Albeit not confirmed, this genetic element was recently observed to be conserved in ST121 strains from food processing and seems to play a significant role in the persistence of *Lm* in food production (Schmitz-Esser et al., 2015). All strains members of the lineage II CC121 and CC14 phylogroups, from either food or animal/environment, harboured the RHS-encoding insertion associated with BC tolerance. From these results we also noticed that not only a single genetic element on the accessory genome is associated with the BC phenotype, but the accumulation of genetic elements, such as the Tn6188 *qacH* along with prophage-related genes and/or the RHS-encoding insertion, on a single genome (e.g. in CC121 and CC2) appear linked to higher MICs for BC.

Among the POGs associated with the PHMB, HP and PA biocides, we mainly found genes encoding for proteins with non-characterized function. However, a cell wall surface anchor protein (and its two flanking hypothetical proteins) was detected as associated with the enhanced tolerance to PHMB in animal/environmental strains from different lineage II CCs (Fig. 4B). No association was detected for neither chemically reactive biocides (chlorines), nor biocides that cause cytoplasmic proteins denaturation and cell contents coagulation (alcohols).

#### 4. Discussion

In the framework of investigating the ecological factors driving the evolutionary dynamics of *Lm* across its ecological niches, genomic features linked to increased tolerance (in terms of MICs) to biocides used as disinfectant in industrial settings were successfully detected by means of GWAS taking into account homologous recombination events from the coregenome (Lees et al., 2018). Strong causal associations were pointed out through genome-wide significances from different approaches (Bonferroni or Q-Q), identifying several genomic elements linked to enhanced MICs for specific biocides (e.g. QACs and biguanides) across *Lm* genomes from lineage I and II. Taking into account all bacterial GWAS confounding factors (Vila Nova et al., 2019), our GWAS approach and our findings (i.e. biocide-tolerance indicator sequences) show the potential to be integrated into hazard identification approach in order to support microbiological risk assessment of *Lm* in food processing.

##### 4.1. The practical use of GWAS for discovering phenotype-associated genomic elements

The microbial GWAS performed here demonstrated significant associations between several genetic elements and tolerance to biocides (Table 2), not only identifying the presence of associated genes by GWAS (e.g. efflux pumps), but also detecting the related MGEs (e.g. plasmid and transposon). However, some technical challenges have to be addressed when performing bacterial GWAS, starting with the association model. The pyseer linear mixed model adopted in this study displayed a reliable control of false positives rate resulting in a lower type 1 errors rate in comparison to univariate model (Lees et al., 2018). The population structure and the clonal nature of bacterial reproduction are the main confounding factors leading to type 1 errors in microbial GWAS (Earle et al., 2016). As non-causal genotypic-phenotypic relationships may appear when confounding factors are not taken into account within the GWAS (Didelot and Maiden, 2010; Earle et al., 2016), it is crucial to control for the recombination rate (i.e. the frequency of genome recombination) of the observed population to successfully pinpoint truly causal associations. Other authors observed that different mutations truly associated with a specific trait can be detected including or excluding homologous recombination events from the population structure assessment (Vila Nova et al., 2019). In contrast, the same

associated mutations were observed in the present study, including or excluding homologous recombination events from the population structure assessment. Even though *Lm* is historically considered to reproduce clonally, a variable rate of horizontal gene transfer by homologous and non-homologous recombination has been observed in *Lm* (den Bakker et al., 2008), with lineage II showing higher recombination rate than lineage I. In accordance with this observation, the lineage II population from this study showed higher homologous recombination in the coregenome in comparison to lineage I. This may suggest an increased capacity of lineage II to adaptively evolve in its different ecological niches, especially in the food processing one (den Bakker et al., 2008; Zamudio et al., 2020). A major technical challenge of GWAS occurs when interpreting significant results. According to the scientific community, there is no universal genome-wide significance for microbial GWAS as well as no harmonised method to estimate it. The Bonferroni adjustment is considered a strict genome-wide significance, as it may lead to over-correction when mutations in linkage disequilibrium (LD) are correlated to some degree (Power et al., 2017). As we observed, many causal genes would not be considered using only the Bonferroni adjustment threshold. On the other hand, the graphical inspection of Q-Q distributions allowed identifying more true positive associations than the strict Bonferroni correction. Seeing the likely polygenicity of biocide-tolerance mechanisms (i.e. high number of genes affecting the phenotype due to environmental interactions with an expected low heritability), we advise to consider the less stringent genome-wide significance from Q-Q analysis to limit type 2 errors (false negatives). Whatever multiple testing or Q-Q distributions is used for obtaining the optimal genome-wide significance, filtering associations based on allele frequency should be accounted for with regard to either the genomic composition (to avoid the lineage effects) or the phenotypical behaviour (to limit false positives). Following this approach, we confidently improved the chance to identify the causal markers that universally associate with the traits of interest. Using adjusted genome-wide significance in the downstream data analysis based on the bacterial population structure (monophyletic vs panmictic) and the complexity of the phenotype under study, similar GWAS workflows can be considered as phenotype- and pathogen-independent i.e. can be universally applied to other bacterial species than *Lm* and focus on different (continuous or binary) phenotypes.

##### 4.2. Genomic signals associated with tolerance to specific biocides across ecological niches

Most of the significantly associated orthologous clusters and MGEs were primarily detected in food strains. The lower prevalence of these genes in the animal and environmental niche could be due to the higher selective pressure and environmental stressors met in the food processing plants. Moreover, the presence of few QACs tolerant CC121 strains in wild animals and the farm environment might suggest a possible transmission of this subtype from the food production to these niches.

###### 4.2.1. Quaternary ammonium compounds

QACs are often the disinfectant of choice in food processing plants (Jt et al., 2002), as non-corrosive molecules with a satisfying biodegradability under aerobic conditions. However, MGEs including prophage-related genes, transposons and plasmids encoding for efflux pumps, were found to be the main actors of BC tolerance mechanisms in food strains from this study, and in several other studies and bacterial species (Bergholz et al., 2018; Nair et al., 2017). Here, we also observed that the presence of multiple associated genetic elements on genomes had a cumulative effect with the increasing of MICs for BC. Looking at the distribution of such associate elements on the phylogenomic reconstructions, we observed that the same tolerance-causing genomic element has appeared independently and several times in geographically and temporally divergent strains. This suggests that the accessory



genome of *Lm* has been impacted by independent acquisitions by HGT, with lineage II food strains showing the highest number of MGEs associated with BC tolerance. MGEs harbouring tolerance to QACs in different lineages of *Lm* may have been driven by the same selective pressure. Similar findings have been reported in pangenome-wide investigations of antimicrobials resistance mechanisms from different bacterial species (Wood et al., 2020). We found that the Tn6188 *qacH* and the pLMST6 *emrC*, previously described as linked to the enhanced capacity to tolerate BC in food-processing *Lm* strains from lineage II ST121 and lineage I ST6 respectively (Kremer et al., 2017; Kropac et al., 2019; Müller et al., 2013), were harboured by strains belonging to both lineage I and II. The fact that biocide tolerance mechanisms encoded on transposons and plasmids can be transferred between bacterial strains from phylogenetically distant lineages within the food niche poses the highest risk for the increase of tolerant genotypes of *Lm* in food industrial settings. In addition, the pLMST6 *emrC* constituted a pleiotropic marker for QACs tolerance, as it was associated with multiple phenotypic traits (i.e. BC and DDAC enhanced tolerance) that share a similar bactericidal mechanism. The presence of genetic feature such as pLMST6 in *Lm* strains results in the emergence “cross-tolerant” genotype in lineage I and II phylogroups. Cross-adaptive response to prolonged exposure to QACs-based disinfectants was previously documented *in vitro* to result in increased MICs of *Lm* strains from food processing (Lundén et al., 2000). It is then clear that the selective pressure exerted by such biocides may favour the dissemination and expression of these tolerance mechanisms.

#### 4.2.2. Polymeric biguanides

If plasmids and transposons-driven BC tolerance mechanisms represent the main risk in strains from the food processing (e.g. cutting plant), PHMB tolerance associated cell surface proteins reflect the genetic mechanism for strains at the preharvest stage (e.g. farm animals and the wild animal/environment). It is not surprising that genetic elements involved in cell wall integrity (CWI) mechanisms appear to be associated with PHMB as the biocidal action of this biocide was observed to destabilise the bacterial cell membrane altering the cell permeability (Allen et al., 2006; Broxton et al., 1984). Moreover, cell-surface located proteins were described to likely have a possible role in reducing cell exposure to PHMB by reducing the area of exposed cell surface following cellular aggregation in *Escherichia coli* (Allen et al., 2006). Polymeric biguanides found their use as general disinfectants in the food industry, but also were very successful in the disinfection of swimming pools to control algal growth (McDonnell and Russell, 1999). Indeed, their effectiveness as teat disinfectant during pre-milking applications of dairy-cows has made of them the basis for several disinfectant treatment products against mastitis-causing environmental bacterial pathogens (Gleeson et al., 2018). PHMB applications in animal husbandry and water treatment might explain the molecular adaptation to such compound in *Lm* strains from animal and environmental ecological niches.

#### 4.2.3. Peroxygens

Hydrogen peroxide and peracetic acid are commonly used broad-spectrum biocides for disinfection, with the latter showing the higher bactericidal activity. Their antibacterial action is mediated by the free radical oxidation of thiol groups in enzyme and protein. Then, the bacterial resistance mechanism has been ascribed to the expression of cellular defence systems, aiming to degrade free radicals and help repair cellular damages induced by radicals especially in bacterial populations under biofilms (Nair et al., 2017). Although the mechanisms of action of these two peroxygens have multi-targeted effects, a few POGs were detected here as associated with an enhanced tolerance of *Lm* strains towards such biocides. Despite being part of prophage regions, the associated genes presented a very low allele frequency in the observed population and uncharacterized functions. Their presence was specific to food strains, a few of which harboured additional elements associated with other biocides (e.g. BC). Even though further investigations on the

actual impact of such genotypes on peroxygens tolerance are required, the presence of associated genes on prophage regions might increase the risk of “tolerance phenotype” spreading through phage gene transfers (Popa and Dagan, 2011).

#### 4.2.4. Amine compounds

The AMPD can be found in combination with QACs, or as an alternative to them for the C&D of surfaces in contact with food and feed in the food industry (Slimani et al., 2018). A scant literature is available on this biocide and its potential tolerance mechanism in bacteria. However, susceptibility testing of bacterial suspension from different gram-negative species for such compound confirmed its effectiveness as biocide (Reichel et al., 2014). In addition, Lundén et al. (2000) observed that *Lm* strains were able to adapt to tertiary alkylamine after a 2-h sub-lethal exposure showing an increase in MICs up to 3-fold, with strains persistent in food processing presenting higher MICs than non-persistent ones. Results from our study showed a genetic signal for tolerance to AMPD depicted by a pair of small hypothetical proteins specific to animal strains with very low allele frequency in the observed population (1%). Although these sequences appeared to be strongly associated with AMPD tolerance (LRT  $p$ -value =  $2.87E-08$ ), further investigation need to confirm if additional genetic mechanisms may be disseminated from the animal niche to food.

#### 4.2.5. Alcohols and chlorine releasing compounds

Chemically reactive agents such as chlorine or with solvent molecules such as alcohols are considered multi-target reactors. Disruption of membrane structure and permeability, as well as progressive oxidations of thiol groups and deleterious effects on DNA synthesis, are known as the main effects induced by ethanol and chlorines compounds, respectively (Russell, 2003). In this study, the absence of association for the tolerance to HS and EtOH in GWAS analysis suggests that such unstable and volatile molecules, which do not persist in the environment in an active form (Gilbert and McBain, 2003), are thus likely less prone to select *Lm* tolerant phenotypes.

### 4.3. Towards the prediction of biocide tolerance hazards for a European-wide surveillance of *Lm*

In contrast to antibiotics, whose bactericidal action targets specific cell mechanisms promoting the selection of resistance genotypes, the biocides interact with one or more generalized sites within the cell, thus, reducing their selectivity of resistant cell lines (Gilbert and McBain, 2003). However, tolerant genotypes may develop, and the acquisition of tolerance phenotype may be driven by plasmid incorporation in genetically distant *Lm* populations. Biocide tolerance does not seem to be limited to plasmid acquisition. Efflux-pumps encoding transposons on different *Lm* CCs, as well as chromosomally encoded surface proteins are linked to the tolerance for specific biocides. A clear association between these genetic elements and tolerance phenotype, in particular for QACs, polymeric biguanides and peroxygens, was demonstrated in different *Lm* CCs from specific ecological niches. It is unlikely that these genetic elements, whose biocide tolerance mechanisms result in an increased MIC, confer resistance to the recommended concentration of the active compounds. However, the plasticity of *Lm* genomes in the accumulation of multiple systems and the poly-specificity of these systems (e.g. pLMST6 *emrC*) confer a tolerant phenotype very successful in the food niche, and likely promote the acquisition of further tolerance or even resistance mechanisms through new mutations or HGT. The acquisition of novel genomic features by different *Lm* CCs, possibly under biofilm community, may trigger a mechanism of ecologically driven adaptation of tolerant genotypes in new niches.

Microbial GWAS-based analyses at the pangenomic scale are useful not only to detect tolerant genotypes across different ecological niches but also to identify the genetic element(s) responsible of the specific or poly-specific tolerance phenotype. The genetic elements here discovered

by means of hybrid tools, which implement a combination of phenotypic control data, recombination-aware phylogenomics and advanced statistical models might represent universal markers sequences for the prediction of tolerance phenotype within genomics-based epidemiological surveillance systems and databases. Cutting-edge open-source tools for GWAS, including supervised classification technique such as machine learning (Lupolova et al., 2017) or multinomial logistic regression (Guillier et al., 2020) and elastic net pangenome-spanning regressions (Lees et al., 2019) may fit the scope using such genetic elements as predictors to screen the presence of tolerant genotypes across *Lm* ecological niches. Integration of GWAS into decision-making and risk assessment is expected as technical capacity of European countries is rising and harmonizing across countries. However, tuning and consensus of such tools and workflows are still ongoing for integrating GWAS as a hazard identification approach within large-scale surveillance at European level (Koutsoumanis et al., 2019; Rantsiou et al., 2018). Indeed, the multidisciplinary of such approaches, requiring different expertise from bioinformaticians to microbiologist/epidemiologist particularly for the interpretation of results, may slow down their integration within food industry, authorities from food safety and public health (Rantsiou et al., 2018). If systematically applied within the European epidemiological context, similar workflows would eventually provide an inclusive understanding on the genomic and ecological characteristic of strains with tolerant phenotype, with an undoubted impact on the daily procedures in the food industry. For instance, this could guide in the prioritization and optimization of *Lm* contamination control measures from the animal husbandry to the food processing and likely support food industrials in the choice of the effective C&D procedures to reduce the risk of *Lm* contaminations. Despite the multiple challenges described here, the integration of GWAS-based hazard identification into quantitative microbial risk assessment (QMRA) models would also allow to develop exposure assessment models on *Lm* strains—ecological niches associations and tailor them to each strain according to its virulence potential (Fritsch et al., 2018; Koutsoumanis et al., 2019). Through these models, the risks for emerging tolerance and cross-tolerance in bacterial pathogen populations following biocide exposure could be identified and measured in all areas of biocide usage along the food chain.

#### 4.4. Raising awareness about biocide tolerance risks among food industrials

Because of the significant amounts of biocides used to control bacterial pathogens in food sector, a possible role of C&D operations in the selection of *Lm* genotypes tolerant to biocides used in the food industry has been pointed out by our study and literature data. As a consequence of the selection of tolerant genotypes, an increased ability of strains to form biofilms and to survive C&D treatments based on the same biocidal compound may appear, especially in food processing (Tezel and Pavlostathis, 2015). This highlights the importance in communicating and identifying the risk factors of disinfection treatments in food plant that might further contribute in the selection of more tolerant or even cross-tolerant strains, especially in the secondary and tertiary production. The food industry should be aware (i) of the presence of biocide tolerant genotypes in particular in the food, but also in the animal settings, (ii) of the genetic mechanism underlying such tolerance and consequently (iii) of the potential for development of specific or poly-specific biocide tolerance. Monitoring such events may be crucial for the implementation of corrective measures to ensure an effective control of the circulating pathogenic population limiting the selection, survival and dissemination of tolerant genotypes. Among the biocide under study, the highest risk for developments and dissemination of tolerance phenotype based on the observed genetic signals was linked to QACs, especially BC selective pressure in the food niche. The selective pressure that QACs exert promotes the flow of tolerance mechanisms among food strains from diverse geographical origins and their

reoccurrence along the time. When similar tolerance events appear, tailor-made control and prevention strategies, including the implementation of potential alternatives (e.g. products containing two or more compatible active compounds) to the selective disinfectant treatments, such as QACs, are scientifically sound if performed on large scale. Rotating disinfectants with different bactericidal action may represent an effective “low-cost” control strategy, already practiced in animal and food production systems, as well as clinical settings where sound biosecurity is crucial (Murtough et al., 2002). Despite this statement, researchers comparing MICs increase towards exposure to disinfecting agents with differing mechanisms of action in *Lm* strains from food processing plants concluded that rotation of disinfectants might not have the desired effect (Lundén et al., 2000). In the present study, we did not observed strains harbouring genetic elements associated with multiple classes of biocides, supporting that alternating disinfectants with different bactericidal action might contrast the spread of genotypes with acquired specific tolerance. Disinfectants rotation might also hamper the formation of biofilms, reducing the potential survival of tolerant genotypes and preventing the transmission of the MGE-encoded genes for biocide tolerance. In addition to the present study determining biocide MICs at optimal growth temperature of *Lm* (e.g. 37 °C), we also plan to investigate in a near future the influence of temperature of food processing environment (e.g. 10 °C and 20 °C) on MIC estimation in order to confirm and potentially detect other genetic elements associated with biocide tolerance. With the prospective to attain an integrated system of biocide tolerance surveillance through collaboration between food industrials, food safety authorities and research institutes, sound C&D program and data on their effectiveness in microbial pathogens contamination control should be ensured and maintained.

#### Author contributions

F.P. and L.G. conceptualized this study. F.P. performed the bioinformatics analyses and wrote the original draft. L.G. and N.R. assisted in the study development and edited the manuscript. N.R. performed reads processing. A.G., A.B. and C.S. provided phenotypic data information. Y. S. and B.F. provided raw reads data and metadata information. S.R. and L.G. acquired the funding and conducted project administration. All authors reviewed and commented the manuscript.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

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