Biofilm formation and genomic features of Listeria monocytogenes strains isolated from meat and dairy industries located in Piedmont (Italy)

Biofilm formation and genomic features of *Listeria monocytogenes* strains isolated from meat and dairy industries located in Piedmont (Italy)

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Abstract

Listeria monocytogenes is considered major challenge for the food industry as it can persist for long periods in food processing plants by forming biofilms. The aims of this study were: i) to assess the biofilm producing ability of 57 *Listeria monocytogenes* isolates previously subjected to whole-genome sequencing (WGS); ii) to compare the levels of biofilm formation with the presence or absence of biofilm associated genes. To determine the presence or absence of a known set of biofilm associated genes, a comparative genomic analysis was performed on each strain. Among *Listeria monocytogenes* isolates, 58%, 38.5% and 3.5% exhibited weak, moderate or strong biofilm production, respectively. No difference in biofilm production was observed between food and environmental isolates. The percentage of *Listeria monocytogenes* strains isolated from meat products (57%) classified as moderate or strong biofilm producers was higher than the percentage obtained for strains isolated from dairy products (28%). The presence of the *Stress Survival Islet* 1, the *ars*D stress gene and the truncated *inl*A protein was significantly associated with increased levels of biofilm. Combining biofilm phenotype with molecular and genotyping data may provide the opportunity to better understand the relationship between genes linked to biofilm formation in *Listeria monocytogenes*.

Keywords: *Listeria monocytogenes*; biofilm; whole-genome sequencing (WGS); *Stress Survival Islet* 1; *inl*A protein; *arsD* stress gene.

1. Introduction

Listeria monocytogenes (*L. monocytogenes*) is a foodborne pathogen that can cause listeriosis, an infectious disease characterized by high fatality rates (20–30%) in immunocompromised people. In 2018, a total of 2,621 confirmed invasive human listeriosis were reported by 28 Member States in the European Union (EU). In the United States, 928 cases of listeriosis were reported in 2019. The EU notification rate was 0.46 cases per 100,000 inhabitants whereas the overall EU case fatality was 17.6% (CDC, 2021; EFSA and ECDC, 2021). This makes listeriosis one of the most serious food-borne diseases worldwide. *L. monocytogenes* is a ubiquitous environmental bacterium which can contaminate raw and processed food products at different production stages (Acciari et al., 2017; Gómez et al., 2015; Wijnands et al., 2014). The consumption of contaminated ready-to-eat (RTE) food products is a common source of human infection (Di Ciccio et al., 2020; Ricci et al., 2018), with various foodstuffs such as meat, cheese and fresh products being associated with listeriosis cases (Bolocan et al., 2016; Zhu et al., 2017). The ability of this ubiquitous pathogen to survive, proliferate and produce biofilms in harsh conditions helps its persistence in the environment (Pasquali et al., 2018). All these specific features of *L. monocytogenes* forced governments and food safety agencies around the globe to set criteria to reduce the presence of this food-borne pathogen in the food chain. In the EU, producers must show that *L. monocytogenes* will not exceed the limit of 100 CFU/g during its shelf life if a RTE food product allows the growth of this pathogen or the absence in 25 g before the food has left the control of the food business operator. The absence in 25 g is required also in RTE intended for infants and for medical purpose (European Commission (EC), 2005). On the other hand, the United States has adopted a zero-tolerance policy for the presence of *L. monocytogenes* in RTE foods (Archer, 2018; Hingston et al., 2017). It is known that the processing environment is often a common source of contamination of *L. monocytogenes* in foods (McCollum et al., 2013; Pérez-Rodríguez et al., 2008) and the presence of a biofilm is a potential cause of repeated bacterial food contaminations (Colagiorgi et al., 2017; Panebianco et al., 2021). The bacteria organized in a biofilm develop resistance against harsh environmental conditions such as desiccation, nutrient deprivation or antimicrobial treatment (Alavi and Hansen, 2013; Esbelin et al., 2018; Ferreira et al., 2014; Zoz et al., 2017). Biofilms, in fact, are responsible for persistence of bacteria in food industries (Di Ciccio et al., 2015). As reported by several authors, several factors such as temperature, time, surface type, origin and nutrient availability can

affect the formation of microbial biofilms (Cherifi et al., 2017; Govaert et al., 2018). In a study carried out by Bonsaglia et al. (2014), almost all *L. monocytogenes* strains isolated from from food production environment were able to produce biofilm on stainless steel and glass surfaces. Biofilm formation differed according to temperature and surface. Isolates of *L. monocytogenes* can be categorized into lineages, multilocus sequence typing (MLST) clonal complexes (CCs) and core genome MLST (cgMLST) types (Moura et al., 2016; Ragon et al., 2008). Lineage II strains are common in foods and food processing environments (Orsi et al., 2011). Among different CCs, CC1, CC2, CC4, and CC6 are highly associated with a clinical origin and the most likely to cause disease. Other CCs, such as CC9 and CC121, are frequently isolated from food and food processing environments (Maury et al., 2016). As shown by several authors, biofilm-forming ability varied widely among strains and some strains can persist better than other in the food processing environment (Koreňová et al., 2016; Wang et al., 2015). Although some studies have revealed genetic factors necessary for *L. monocytogenes* biofilm formation on abiotic surfaces (Jordan et al., 2008; Kumar et al., 2009; Longhi et al., 2008; Riedel et al., 2009; van der Veen and Abee, 2010; Zhu et al., 2017, 2008), further surveys are needed to confirm the importance of certain genetic elements and to identify new ones. In this context, the recent advances in next generation sequencing technologies (NGS) have brought several opportunities, allowing the fast identification and characterization of genetic determinants (Rubiola et al., 2020); among them, whole-genome sequencing (WGS) allows the sequencing of entire bacterial genomes in an efficient, timely and cost-effective way, enabling the characterization of *L. monocytogenes* isolates down to a resolution of a single nucleotide difference, thereby allowing accurate virulence, persistence and clonal tracing (Ferreira et al., 2014). Some *L. monocytogenes* strains may persist in food processing facilities for prolonged periods of time. This can be due to many factors, including enhanced biofilm formation, tolerance to disinfectants, the presence of prophages, resistance markers on plasmids, premature stop codons in *inl*A, stress-survival islet (SSI) 1 or SSI-2 (Muhterem-Uyar et al., 2018). In this regard, WGS analysis may represent a powerful tool for associating genotype with biofilm phenotype that has led to the apparent diversity in the ability of strains of this pathogen to produce biofilms.

The objectives of this study, therefore, were: i) to test the biofilm formation of a selection of food-related *L. monocytogenes* isolates, previously subjected to WGS; ii) to compare biofilm phenotypes and genomes to identify genetic features associated with biofilm formation.

2. Materials and Methods

2.1 Selection of food-related L. monocytogenes isolates

L. monocytogenes strains (n=57) from the Bacterial Culture Collection of the Department of Veterinary Sciences, University of Turin, Italy, were included in this study. All strains were whole-genome sequenced as described by Lomonaco et al. (2018) and were chosen to represent the most prevalent CCs in local (Piedmont) meat and dairy industries. The dataset (n= 57) was built with food-related *L. monocytogenes* isolates, originated from food (n= 38) and from food-processing environment (n=19), collected in a period of 12 years (2003 – 2014). A total of 20/57 and 18/57 were isolated from meat and dairy products, respectively, whereas a total of 11/57 and 8/57 were isolated from dairy and meat environments, respectively. The genetic characteristics of the strain dataset are reported in Table 1. Routine culturing was carried out in Brain Heart Infusion (BHI, Oxoid, Milan, Italy) and each strain was activated twice in 10 ml BHI by incubation at 37°C for 24 hours. The grown cultures were used for inoculation into the wells of plastic microplates for subsequent quantification of biofilm production (see 2.2).

2.2 Biofilm formation assay

The biofilm formation of all isolates was evaluated according to a previously described protocol with slight modifications (Stepanović et al., 2007). Briefly, the turbidity of the grown cultures (overnight) in BHI (Oxoid) were adjusted to obtain a turbidity optically comparable to that of the 0.5 McFarland standard (10^8 CFU/ml) by reading the optical density (OD) at 550 nm using a spectrophotometer (Pharmacia Biotech Ultrospec-3000, Biochrom Ltd., Cambridge, UK England). Subsequent 1:100 dilutions of these bacterial suspensions (final testing inoculum of 10^6 CFU/ml) were added into each well (200 µl) of a sterile 96-well polystyrene microplate (Sarstedt, Nümbrecht, Germany). The negative control wells contained broth (BHI) only. Microplates

were incubated statically for 24 h at 37°C. The content of each plate was discarded and 200 µl of sterile phosphate-buffered saline (PBS – pH 7.3; Sigma-Aldrich S.r.l., Milan, Italy) were added to each well. This washing step was repeated three times. Then biofilms were heat-fixed by exposing the microplates to hot air at 60°C for 60 min. For staining bacterial biomass, 150 µl of 0.2% w/v crystal violet (CV; Merck, Germany) solution was added per well and plates were incubated statically for 15 min. After staining, the solution was removed by sharply tapping plates upside down, wells were washed three times with distilled water and air dried completely. In order to quantify the biofilm formation, 150 µl of 95% ethanol were added to de-stain the wells. Finally, the absorbance of the crystal violet solution present in the de-staining solution was measured at 595 nm (Abs-595 nm) using a microplate reader (iMark plate reader, Bio-Rad, Sydney, NSW, Australia). The results were interpreted based on the formula of Stepanović et al. (2007). The Abs-595 nm cut-off for the negative control was calculated by using the mean Abs-595 nm of all negative control wells (n. 9 for each microtiter plate) plus three standard deviations (Abs NC). The strains were then categorised as weak (Abs NC< Abs-595 nm ≤ 2× Abs NC), moderate (2×AbsNC < Abs-595 nm ≤ 4×AbsNC) or strong (4×AbsNC < Abs-595 nm) biofilm formers. All strains were tested in triplicate and the results were averaged.

Fisher's exact test was applied for categorical variables (presence/absence). For all tests, conducted with Graph Pad Prism 9.0 (GraphPad Software, San Diego, California, USA), $p < 0.05$ was considered significant.

2.3 Comparative Genomic Analysis

Sequences were firstly submitted to the Pasteur Institute MLST database (https://bigsdb.pasteur.fr/listeria/) and then to BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to check the presence, absence or truncation of genes and complexes (SSI-1, *inl*A, *inl*L, *ars*D, *act*A, *bcr*BC) already indicated as associated with increased biofilm formation, adhesion capacity, and persistence abilities in *L. monocytogenes* (Franciosa et al., 2009; Keeney et al., 2018; Maggio et al., 2021; Mahoney et al., 2022; Mishra et al., 2020; Popowska et al., 2017; Seneviratne et al., 2017; Travier et al., 2013). Typing and reconstruction of plasmid sequences were performed using Mob-suite software tools (Robertson and Nash, 2018). For the comparative analysis of accessory genomes, sequences were annotated with Prokka (Seemann, 2014) and the obtained .gff files were used as inputs

to construct the pan-genome of the 57 strains with Roary (Page et al., 2015). The association between genes present in the accessory genomes and the production of biofilm by the strains was carried out with Scoary (Brynildsrud et al., 2016). To overcome the possible bias of gene presence associated with specific genetic lineages, Scoary infers the population structure from the input data. Specifically, the gene_presence_absence.csv file from Roary and a list of traits file, weak, moderate, and strong biofilm producers (WP, MP and SP) groups were used as inputs. Gene sequences significantly associated (naïve p value < 0.05) with the group of the MP/SP strains by Scoary but annotated only as "hypothetical protein" by Prokka were visualized with the Integrative Genomics Viewer (IGV) (Robinson et al., 2011) and then submitted to the Pasteur Institute MLST database and BLASTn for a preliminary identification. To search for function in the hypothetical proteins, sequences were analysed with the Conserved domain database (CDD)-search web tool (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Lu et al., 2020). Presence of genomic features (SSI-1*; inl*L): significant hits were defined as those with coverage of at least 80% and a percent identity greater than or equal to 80% (BLAST analysis). Assemblies were further screened for the presence of antimicrobial resistance and stress genes by AMRFinderPlus 3.10 (Feldgarden et al., 2021). Truncations (*inl*A; *act*A; premature stop codons: PMSCs) were defined as present if a sequence was missing at least ten amino acids from the end of the sequence as compared to the EGD-e reference sequence. Sequences were translated to amino acids, aligned with MUSCLE, and manually inspected for truncations (Pirone-Davies et al., 2018).

3. Results

3.1 Biofilm formation assay

The results of the evaluation of biofilm formation revealed that all tested *L. monocytogenes* strains are biofilm producers. The *L. monocytogenes* isolates exhibited varying levels of biofilm formation, from weak to moderate or strong production (Stepanović et al., 2007). In particular 58% (33/57), 38.5% (22/57) and 3.5% (2/57) of strains analyzed were weak, moderate and strong biofilm producers, respectively. Since only two isolates (one CC31 strain from a meat product and one CC9 strain from meat processing environment) were classified as strong biofilm producers, they were grouped together with the moderate producers for the subsequent

analyses. The percentage of isolates from meat products classified as moderate or strong biofilm producers (57%; 16/28) was higher than the percentage obtained for isolates from dairy products (28%; 8/29) - (Fisher's exact test, $p < 0,05$). Additionally, no difference in biofilm production was observed between food and environment isolates (Figure 1).

3.2 Genome Analysis

The stress survival islet (SSI-1) was present in 81% (46/57) of strains belonging to genetic lineage II; conversely it was absent in all strains (CC6) belonging to lineage I. Among Lineage II strains, the SSI-1 was absent in all isolates belonging to CC121 and CC398. After screening by AMRFinderPlus, 77% (44) of strains were found positive for the *ars*D stress gene and 17% (10 strains) for *bcr*B and *bcr*C stress genes. The presence of SSI-1 and the *ars*D gene was correlated with biofilm formation by *L. monocytogenes* ($p < 0,05$) (Figure 2). The presence of internalin L (*inl*L) seemed to be associated with CC9 and CC398, as it was shown to be present in all the isolates belonging to those groups. In contrast, it was absent in all the strains belonging to CCs 31, 121, 6 and 8. The presence of the *inl*L was not significantly associated with increased levels of biofilm. Mutations leading to PMSCs in *inl*A were identified in 45 (79%) isolates (26/45 CC9, 15/45 CC31, 4/45 CC121) and seven mutation types of PMSCs were identified. PMSCs on the *inl*A gene were found to be significantly associated with biofilm MPs and SPs. The *act*A gene was truncated in 13/57 (23%) isolates (8/13 CC9, 4/13 CC6, 1/13 CC8). The presence of a truncated *act*A protein was not significantly associated with increased levels of biofilm (Figure 2; Figure 3). Plasmid presence was not significantly associated with any of the groups. A total of 4778 genes (pangenome) were identified from all the analysed *L. monocytogenes* genomes. The core genome (i.e., genes found in at least 99% of genomes) contained 1969 genes. The accessory genome of this population was composed of 284 soft core genes (found in 95-99% of genomes), 1148 shell genes (found in 15-95% of genomes), and 1377 cloud genes (found in less than 15% of genomes). We found that 84 individual gene clusters in the genomes of the isolates studied were significantly (naïve $p < 0.05$) either positively (n=59) or negatively (n=25) associated with increased biofilm formation (Table 2 and Table 3). Of these 84 genes, 35 unique annotations were available, based on the annotations output from Prokka,

and 49 of the annotations were "hypothetical protein". Conserved domains found for hypothetical proteins are reported in Tables 1SM and 2SM. Three gene clusters were annotated as internalin precursors, internalin-J precursor (n=2), internalin-A precursor (n=1), with one being positively and 2 negatively associated with biofilm production. Several phage-like proteins were identified by manual BLAST search. The stress survival islet (SSI-1) gene cluster (lmo0444 - lmo0448) was also found to be positively associated.

4. Discussion

L. monocytogenes is one of the main causative agents for foodborne infections in Europe (Ricci et al., 2018). The ability of *L. monocytogenes* to adapt and survive under environmental stresses has been associated with the ability to form biofilms (Peng et al., 2016). Persistence of *L. monocytogenes* in the food processing environment is the major source of post-processing contamination (Ferreira et al., 2014). In the past, most research on *L. monocytogenes* persistence has focused on the role of biofilm formation (Bonsaglia et al., 2014; Wang et al., 2015). The identification of factors that enhance the biofilm formation of some strains of *L. monocytogenes* in the food processing environment would be beneficial for the development of effective disinfection strategies. In the present study, a comparative genomic analysis was performed by using WGS in addition to a phenotypic biofilm assay to correlate genotypic and phenotypic markers for biofilm formation of *L. monocytogenes* isolates collected in the meat and dairy industries located in Piedmont (Italy). The relationship between the presence, absence, or truncation of some genetic markers (such as SSI-1, *inl*A, *inl*L and *act*A) and biofilm phenotypes of different *L. monocytogenes* strains was evaluated. While all 57 isolates produced biofilms, some isolates formed significantly more biofilm than others. This result suggests that some strains may have a competitive advantage over others in the food processing environment based on their ability to form biofilms. In this context, previous research has generated contrasting results in determining whether strong or moderate biofilm formation is an indicator of persistence in processing environments (Magalhães et al., 2017; Nowak et al., 2017). Kadam et al. (2013) found that all *L. monocytogenes* strains included in their study were able to form biofilms in different experimental conditions. The relationship between lineage and/or serotype and biofilm formation has been examined by previous studies, with conflicting results. Djordjevic et al. (2002)

and Takahashi et al. (2009) observed lineage I strains to form more biofilm than lineage II, while Borucki et al. (2003) and Combrouse et al. (2013) found the opposite. Carpentier and Cerf (2011) concluded that there are no *L. monocytogenes* strains with strong persistence abilities in food processing environments. In contrast, Wang et al. (2015) showed that some *L. monocytogenes* strains persist better in food production environments than others. Other studies demonstrated that biofilm formation by *L. monocytogenes* can be affected by various genes (Chang et al., 2012; Piercey et al., 2016; Sela et al., 2006). The previous studies have focused on individual genes that are involved in biofilm formation by *L. monocytogenes*. In contrast, few studies have been performed to identify *L. monocytogenes* biofilm-relevant genes on a genome-wide scale. Up to now, there have been few systematic attempts of using WGS to identify genetic factors that contribute to biofilm formation in food-related *L. monocytogenes* strains.

The biofilm formation is affected by the five-gene stress survival islet (SSI-1), which has been implicated in growth during exposure to stressful food environments (Ryan et al., 2010). Adherence and biofilm formation in *L. monocytogenes* from different serotypes is correlated to the presence of the stress survival islet SSI-1, as stated by some recent studies (Keeney et al., 2018; Maggio et al., 2021; Mahoney et al., 2022). Analysis of WGS data showed that the SSI-1 is present in most strains (46/53 - 87%) belonging to lineage II but absent in the four strains belonging to lineage I. This finding is in agreement with Painset et al. (2019). In their work, the SSI-1 was over-represented in lineage II isolates, but absent in lineage I. When we consider the CCs, SSI-1 was present in CCs 9, 31 and 8, while absent in CCs 121, 6, 398. Together, these data suggest that the presence of SSI- 1 is correlated with the biofilm-forming capacity of *L. monocytogenes* at 37°C. This finding is in agreement with Keneey et al. (2018) and in contrast to other studies where SSI-1 was not found associated with persistence (Holch et al., 2013) or to increased biofilm formation (Ebner et al., 2015). The stress gene *ars*D, involved in arsenic resistance pathways, demonstrated a similar presence- absence pattern across the strains. The positive association with biofilm formation was further confirmed by the Roary-Scoary analysis, where the gene is included in the positively associated genes list (Table 2). This gene is found in *ars* operons of different bacteria, including *L. monocytogenes* and *Enterococcus faecalis* (Lin et al., 2007). The *ars*D gene has been associated to increased biofilm formation (Mishra et al., 2020; Seneviratne et al., 2017). Internalin

proteins can affect *L. monocytogenes* biofilm formation, as well as its adhesion, virulence, internalization into eukaryotic cells and survival in different environments (Orsi et al., 2011; Piercey et al., 2016). The *inl*A gene is recognised as a major virulence factor of *L. monocytogenes*, and truncations due to PMSCs are responsible for virulence attenuation (Kim et al., 2018). Work by Franciosa et al. (2009) suggested that truncation of the *inl*A-encoding gene significantly enhances biofilm formation; however, this conclusion is not supported by the observations provided by Wang et al. (2015). Truncated *inl*A proteins are common in food-related *L. monocytogenes* strains (Jacquet et al., 2004; Kovacevic et al., 2013). Lineage II strains carry *inl*A PMSC mutations more frequently than lineage I strains (Shen et al., 2013). Many studies showed that CC9 and CC121, frequently associated with food production sectors, are hypovirulent in part due to truncations in *inl*A, a major *L. monocytogenes* virulence factor (Disson et al., 2008; Jacquet et al., 2004; Lecuit et al., 2001; Maury et al., 2016; Pasquali et al., 2018). In our study, forty-five strains, except the strains belonging to CC6, CC8, CC398 (CCs associated with listeriosis outbreaks; Chen et al., 2016) and three CC9 strains, contained a Premature stop codon (PMSC) mutation in *inl*A. Specifically, these genomes carried seven different PMSCs in *inl*A. To date, 21 mutation types of *inl*A *have* been found (Gelbíčová et al., 2016, 2015). Recently, Popowska et al. (2017) and Maggio et al. (2021) demonstrated that *inl*L contributes to the attachment of *L. monocytogenes* to abiotic surfaces, thereby playing a role in biofilm formation. However, in our study the presence of the *inl*L gene was not significantly associated with increased levels of biofilm. In addition, 22% of tested strains (13/57) harboured a truncated version of the *act*A gene, whereas 77% (44/57) harboured a full-length version of the same gene. The *act*A gene is implicated in the polymerization of actin, which is essential in the first steps of biofilm formation (Travier et al., 2013). In this study we did not observe a higher biofilm forming ability of strains harbouring a truncated version of the *act*A gene, compared to strains that harboured a full-length version of the same gene. Some *L. monocytogenes* isolates can persist in food processing facilities better than other isolates (Carpentier and Cerf, 2011). Different strains of *L. monocytogenes* may be able to survive and thrive in food processing facilities due to relevant phenotypic traits, such as attachment to surfaces, biofilm-forming ability and increased resistance to environmental stresses (Koreňová et al., 2016). Studies have previously identified genes associated with some of these abilities. Piercey, et al. (2016) found that

PMSCs in genes encoding cell wall biosynthesis, motility, metabolism, stress response, and cell surface associated proteins resulted in increased biofilm formation. In particular, interruptions of 9 genes not previously associated with biofilm formation in *L. monocytogenes* (lmo2572, lmo2488, lmo1224, lmo0434 [*inl*B], lmo0263 [*inl*H], lmo0543, lmo0057, lmo2563, and lmo0453) may affect cell surface electrostatic interactions and resulted in increased biofilm formation (Piercey et al., 2016). In our study, PMSCs in *inl*A gene were found to be associated with increased biofilm production, while *inl*A and *inl*J were among the genes that were negatively associated. Different alleles of two genes, *lmo*2003 and *lmo*2015, a Mannosyl-D-glycerate transport/metabolism system repressor and Mannosylglycerate hydrolase, respectively, were found to be positively and negatively associated with increased biofilm formation (Table 2 and Table 3). Alleles negatively associated were found to present a mutation in the aminoacidic sequence (site 133, from asparagine to histidine), in *lmo*2003 and a truncation of the first 13 nucleotides in *lmo*2015. It is worth noting that a mutation from histidine to asparagine in the sequence in a manganese superoxide dismutase gene has been demonstrated to alters biophysical properties of the gene product (Bonetta et al., 2021). In our study, the conserved domain analysis of hypothetical genes with unknown function highlighted the presence of two genes, positively associated, showing functions reported in literature as associated with increased biofilm formation (Table 1SM). The COG0745 domain, which is found in OmpR-like proteins, is involved in the regulation of the expression of surface adhesion proteins in Gram negative bacteria (Prüß, 2017; Stanley & Lazazzera, 2004; Tomaras et al., 2008; Vidal et al., 1998). The L-fucose isomerase (FucIase) and L-arabinose isomerase (AI) family is composed of FucIase, AI and similar cell surface proteins, has been found to be implicated in biofilm formation (Zhao et al., 2015). Nowak et al. (2017) evaluated persistent *L. monocytogenes* strains isolated from New Zealand mussel production facilities in order to find specific genes or genetic markers that may be linked to persistence or non-persistence. They were unable to find any markers in either the persistent or non-persistent isolate groups. In addition, they found that there was no overall clustering of persistent or sporadic isolates and that there was no association between differences in plasmids and persistence. This is consistent with our findings. Moreover, their findings indicate that persistence phenotypes, including biofilm production ability, may not be caused by single mutations, plasmids, or other genetic differences, but may be unique to distinct

L. monocytogenes lineages and/or due to more complex genetic elements, phenotypic stimuli, or gene regulation systems.

Anyway, the main limitation of this study is linked to the restricted number of strains, which is not representative of the diversity of *L. monocytogenes* found in the food industry. Indeed, it is known that biofilm forming abilities, colonisation dynamics, and survival of *L. monocytogenes* are strain-specific and related to various mechanisms besides the presence of specific genes (Gray et al., 2021a; Gray et al., 2021b; Lianou et al., 2020). Genome-wide association studies should include a wide set of strains, since they can vary significantly from survey to survey even if many belong to major clonal complexes. More studies including a larger number of strains with different genomic characteristics are needed in order to minimise variables and improve the robustness of the data derived from this kind of surveys. Finally, the variability that may occur for the same isolate on biofilm forming ability when different methods and experimental conditions are used should be taken into account when planning these studies.

5. Conclusions

We investigated the biofilm-forming ability of 57 food-related *L. monocytogenes* strains collected from local (Piedmont, Italy) food industries that were previously characterized using WGS. The majority of genes potentially linked to biofilm formation have been detected in the WGS data, further highlighting this technology as a faster and cheaper alternative to conventional typing techniques. We hypothesize that the source of isolation and the presence of some genetic markers may play a role in biofilm formation by *L. monocytogenes*. The WGS data generated represent a valuable resource to the improvement of quantitative microbial risk assessment. The characterization of CCs and the detection of genetic markers related to biofilm formation of strains circulating in local meat and dairy industries, as described in this study, provides the opportunity to improve risk assessments for *L. monocytogenes*. WGS may offer the possibility to identify the link between genome markers and phenotypic features by comparative genomics studies or genome-wide association studies. However, the main issue with these sorts of studies is related the set of strains tested, since it can vary significantly from survey to survey, highlighting the need to find a common ground. As example, we must emphasize that

the number of isolates for each CC was not homogenous in our study. Further works in this field are needed, including the detection or determination of truncation in genes across the data set to identify factors that predispose some strains of *L. monocytogenes* towards increased biofilm formation.

Author Contributions

Pierluigi Aldo Di Ciccio: conceptualization, supervision, writing –original draft. Selene Rubiola: investigation, methodology, writing – review & editing. Felice Panebianco: investigation, methodology, writing – review & editing. Sara Lomonaco: investigation, methodology, writing – review & editing. Marc Allard: writing – review & editing. Daniela Manila Bianchi: investigation, methodology. Tiziana Civera: writing – review & editing. Francesco Chiesa: formal analysis, investigation, writing – review & editing.

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Data Availability Statement

The genome assemblies investigated in this paper are available in DDBJ/EMBL/GenBank under the accession numbers listed in Table 1.

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Declaration of Interest

None

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Figure captions

Figure 1. Differences in biofilm production abilities between food and environmental isolates and meat- and dairy-related *Listeria monocytogenes* strains.

Figure 2. Correlation between biofilm production abilities of *Listeria monocytogenes* strains and presence/absence of Stress Survival Islet 1 (*SSI-1*), internalin L gene (*inlL*), arsenite efflux transporter metallochaperone (*arsD*), quaternary ammonium compound efflux SMR transporter (*bcrB-bcrC*) and premature stop codons in the internalin A (*inlA* PMSCs) or *actA* gene (*actA* PMSCs) genes.

Figure 3. Association between biofilm production abilities of the different Clonal Complexes (CC) of *Listeria monocytogenes* strains and presence/absence of Stress Survival Islet 1 (*SSI-1*), internalin L gene (*inlL*), arsenite efflux transporter metallochaperone (*arsD*), quaternary ammonium compound efflux SMR transporter (*bcrB-bcrC*) and premature stop codons in the internalin A (*inlA* PMSCs) or *actA* gene (*actA* PMSCs) genes.

Table 1. Characteristics of the 57 *Listeria monocytogenes* strains used in the present study.

ID	ID NCBI[1]	Date	Source Category	Isolation source	Lineage	Sequence Type (MLST)	Clonal Complex (MLST)
1	CFSAN046012	2011	Environment	Meat plant (food contact material)	II	9	9
2	CFSAN045995	2011	Environment	Meat plant (surfaces)	II	325	31
3	CFSAN045994	2011	Food	Meat (minced meat)	II	9	9
4	CFSAN045999	2011	Environment	Meat plant (surfaces)	II	9	9
5	CFSAN045983	2011	Food	Dairy (cheese)	II	9	9
6	CFSAN045858	2014	Environment	Meat plant (skewers)	II	9	9
7	CFSAN045850	2014	Environment	Dairy plant (surfaces)	II	325	31
8	CFSAN046037	2011	Food	Meat	II	9	9
9	CFSAN046033	2011	Food	Dairy (cheese)	II	325	31
10	CFSAN045972	2011	Food	Dairy (cheese)	II	9	9
11	CFSAN046035	2011	Food	Meat	II	9	9
12	CFSAN046031	2011	Food	Meat	II	9	9

13	CFSAN045971	2011	Environment	Meat plant (surfaces)	II	9	9
14	CFSAN045970	2011	Food	Dairy (raw milk)	II	398	398
15	CFSAN045782	2012	Food	Meat (bovine fresh meat)	II	8	8
16	CFSAN045938	2012	Food	Meat	II	325	31
17	CFSAN045778	2012	Food	Dairy (bulk tank milk)	II	9	9
18	CFSAN045794	2013	Environment	Dairy plant (surfaces)	II	9	9
19	CFSAN045953	2012	Food	Meat (salami)	II	8	8
20	CFSAN045829	2013	Food	Meat	II	9	9
21	CFSAN045815	2013	Food	Dairy (cheese)	II	325	31
22	CFSAN045817	2013	Environment	Dairy plant (surfaces)	I	6	6
23	CFSAN045859	2014	Food	Dairy (cheese)	II	121	121
24	CFSAN045834	2013	Food	Dairy (cheese)	II	325	31
25	CFSAN045791	2012	Food	Dairy (cheese)	II	9	9
26	CFSAN045790	2012	Food	Dairy (cheese)	II	398	398
27	CFSAN045980	2011	Food	Dairy (cheese)	II	325	31
28	CFSAN045784	2012	Food	Meat (bovine fresh meat)	II	9	9
29	CFSAN045783	2012	Food	Meat (bovine fresh meat)	II	9	9
30	CFSAN045793	2012	Food	Meat (minced meat)	II	9	9
31	CFSAN045949	2012	Environment	Meat plant (surfaces)	II	9	9
32	CFSAN046098	2013	Environment	Dairy plant (surfaces)	I	6	6
33	CFSAN044859	2009	Environment	Dairy plant (surfaces)	I	6	6
34	CFSAN046053	2010	Environment	Meat plant (surfaces)	II	9	9
35	CFSAN044742	2003	Food	Dairy (cheese)	II	121	121
36	CFSAN044741	2003	Food	Meat (minced meat)	II	9	9
37	CFSAN046048	2010	Environment	Meat plant (surfaces)	II	9	9
38	CFSAN044748	2003	Food	Meat (swine sausage)	II	9	9
39	CFSAN044854	2008	Environment	Dairy plant (surfaces)	I	6	6
40	CFSAN044857	2009	Environment	Dairy plant (surfaces)	II	325	31
41	CFSAN044758	2003	Food	Meat (bovine minced meat)	II	9	9
64	CFSAN044767	2003	Food	Meat (bovine meat)	II	9	9
72	CFSAN044772	2003	Food	Dairy (cheese)	II	398	398
76	CFSAN044775	2003	Food	Dairy plant (surfaces)	II	9	9
83	CFSAN044778	2003	Food	Meat	II	9	9
84	CFSAN044779	2003	Food	Meat	II	121	121
85	CFSAN044780	2003	Food	Meat	II	121	121
86	CFSAN044734	2003	Food	Meat (bovine minced meat)	II	9	9
92	CFSAN044730	2003	Food	Dairy (milk)	II	9	9
99	CFSAN044747	2003	Food	Meat (minced meat)	II	9	9
G40	CFSAN044840	2005	Food	Dairy (cheese)	II	325	31
G46	CFSAN044805	2004	Environment	Dairy plant (surfaces)	II	325	31
G52	CFSAN044807	2004	Food	Dairy (cheese)	II	325	31
G67	CFSAN044812	2004	Environment	Dairy plant (surfaces)	II	325	31
G69	CFSAN044813	2004	Environment	Dairy plant (surfaces)	II	325	31
G70	CFSAN044814	2004	Food	Dairy (cheese)	II	325	31

G73	CFSAN044815	2004	Environment	Dairy plant (surfaces)	II	31	31

[1]ID of the strains in the NCBI database (https://www.ncbi.nlm.nih.gov/).

Table 2. Genes found to be positively associated with an increased biofilm formation.

Annotation[a]	**Locus name[b]**	**Function**
accB	lmo1356	Biotin carboxyl carrier protein of acetyl-CoA carboxylase
acpB	lmo0445	Capsule synthesis positive regulator AcpB
acr3	-	Arsenical-resistance protein Acr3
aroK_1	lmo1212	Shikimate kinase
arsA	-	Arsenical pump-driving ATPase
arsC_2	-	Glutaredoxin arsenate reductase
arsD	-	Arsenical resistance operon trans-acting repressor ArsD
epsJ_1	lmo0496	putative glycosyltransferase EpsJ
gadB_1	lmo0447	Glutamate decarboxylase
gadC_2	lmo0448	Glutamate/gamma-aminobutyrate antiporter
group_1049	-	hypothetical protein
group_1051	A118p40	hypothetical protein

group_1073	lmo0900	hypothetical protein
group_1191	lmo2015	Mannosylglycerate hydrolase
group_1239	lmo0988	hypothetical protein
group_1300	-	hypothetical protein
group_1321	lmo1008	hypothetical protein
group_1638	-	hypothetical protein
group_1714	lmo0586	hypothetical protein
group_1745	-	hypothetical protein
group_1757	lmo0883	hypothetical protein
group_1803	lmo1122	hypothetical protein
group_1804	lmo1125	hypothetical protein
group_1822	-	hypothetical protein
group_1857	-	tRNA-Val(gac)
group_1858	lmo1060	hypothetical protein
group_1877	-	DNA polymerase IV
group_1878	-	hypothetical protein
group_1879	-	hypothetical protein
group_1880	-	hypothetical protein
group_1881	-	hypothetical protein
group_1883	-	Tyrosine recombinase XerC
group_1895	A118p47	hypothetical protein
group_2	lmo0327	Internalin-J
group_2106	lmo0862	hypothetical protein
group_2183	lmo1006	hypothetical protein
group_2184	lmo1007	hypothetical protein
group_2199	lmo0911	hypothetical protein
group_2234	-	hypothetical protein
group_2578	A118p36	HTH-type transcriptional regulator ImmR
group_2579	A118p37	hypothetical protein
group_2580	A118p40	hypothetical protein
group_2581	A118p40	hypothetical protein
group_2582	A118p40	hypothetical protein
group_277	lmo1031	hypothetical protein
group_454	lmo1000	hypothetical protein
group_511	lmo1636	putative ABC transporter ATP-binding protein YxlF
group_63	lmo2278	Peptidoglycan L-alanyl-D-glutamate endopeptidase CwlK
group_708	-	hypothetical protein
group_910	lmo1124	hypothetical protein
mngR	lmo2003	Mannosyl-D-glycerate transport/metabolism system repressor MngR
sdpR	-	Transcriptional repressor SdpR
ssbA_2	A118ssb	Single-stranded DNA-binding protein A
truB	lmo1328	tRNA pseudouridine synthase B
xerC_2	-	Tyrosine recombinase XerC

yddE	-	putative isomerase YddE
yjjG_1	lmo0635	Pyrimidine 5'-nucleotidase YjjG
yueB_1	lmo0444	ESX secretion system protein YueB
yxeI	lmo0446	Penicillin acylase

[a]Prokka annotation.

[b]Based on *L. monocytogenes* EGDe gene list.

Table 3. Genes found to be negatively associated with an increased biofilm formation.

Annotation[a]	**Locus name[b]**	**Function**
esaA_2	lmo2360	ESAT-6 secretion accessory factor EsaA
group_10	lmo0264	Internalin-A
group_1445	-	Ribonuclease
group_1447		hypothetical protein
group_1449		hypothetical protein
group_1452		hypothetical protein
group_1514		hypothetical protein
group_1516	lmo1328	tRNA pseudouridine synthase B
group_1518		hypothetical protein
group_1519		hypothetical protein
group_1520		hypothetical protein
group_204	-	Internalin-J
group_2415		hypothetical protein

group_2417		hypothetical protein
group_2437		hypothetical protein
group_2668		hypothetical protein
group_2674		hypothetical protein
group_2682	lmo1356	Biotin carboxyl carrier protein of acetyl-CoA carboxylase
group_2699		hypothetical protein
group_2700		hypothetical protein
group_2702		hypothetical protein
group_297	lmo2003	Mannosyl-D-glycerate transport/metabolism system repressor MngR
group_613		hypothetical protein
group_623		hypothetical protein
mngB_3	lmo2015	Mannosylglycerate hydrolase

[a]Prokka annotation.

[b]Based on *L. monocytogenes* EGDe gene list.

Figure 1

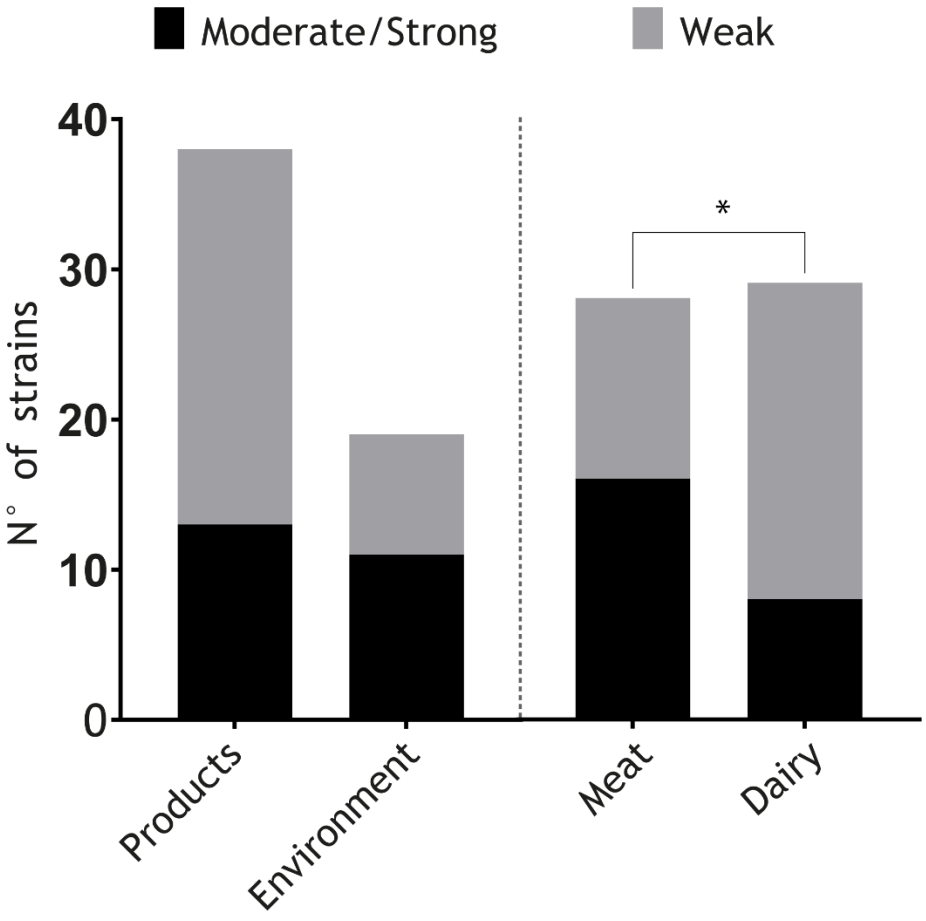

Figure 2

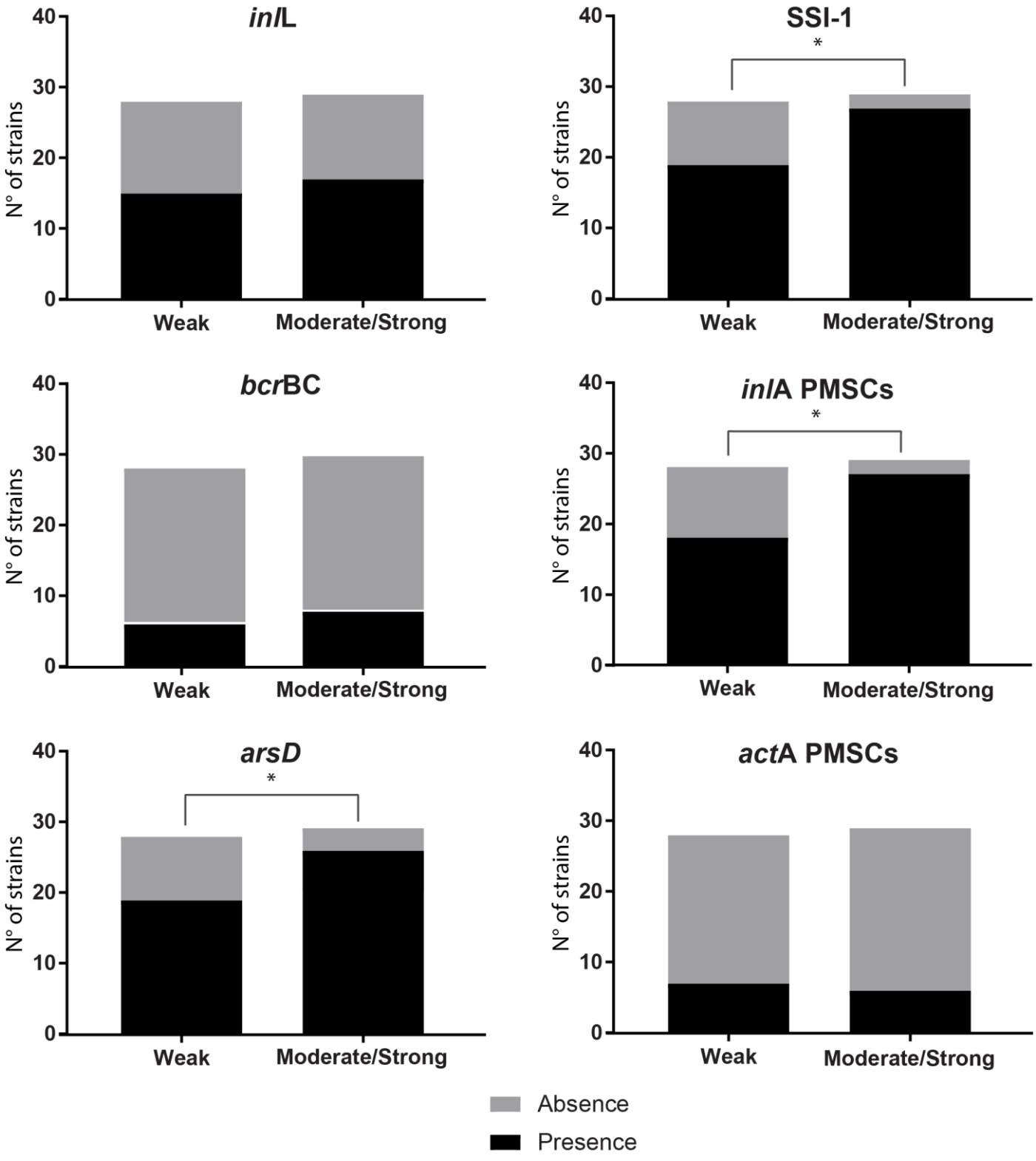

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Figure 3

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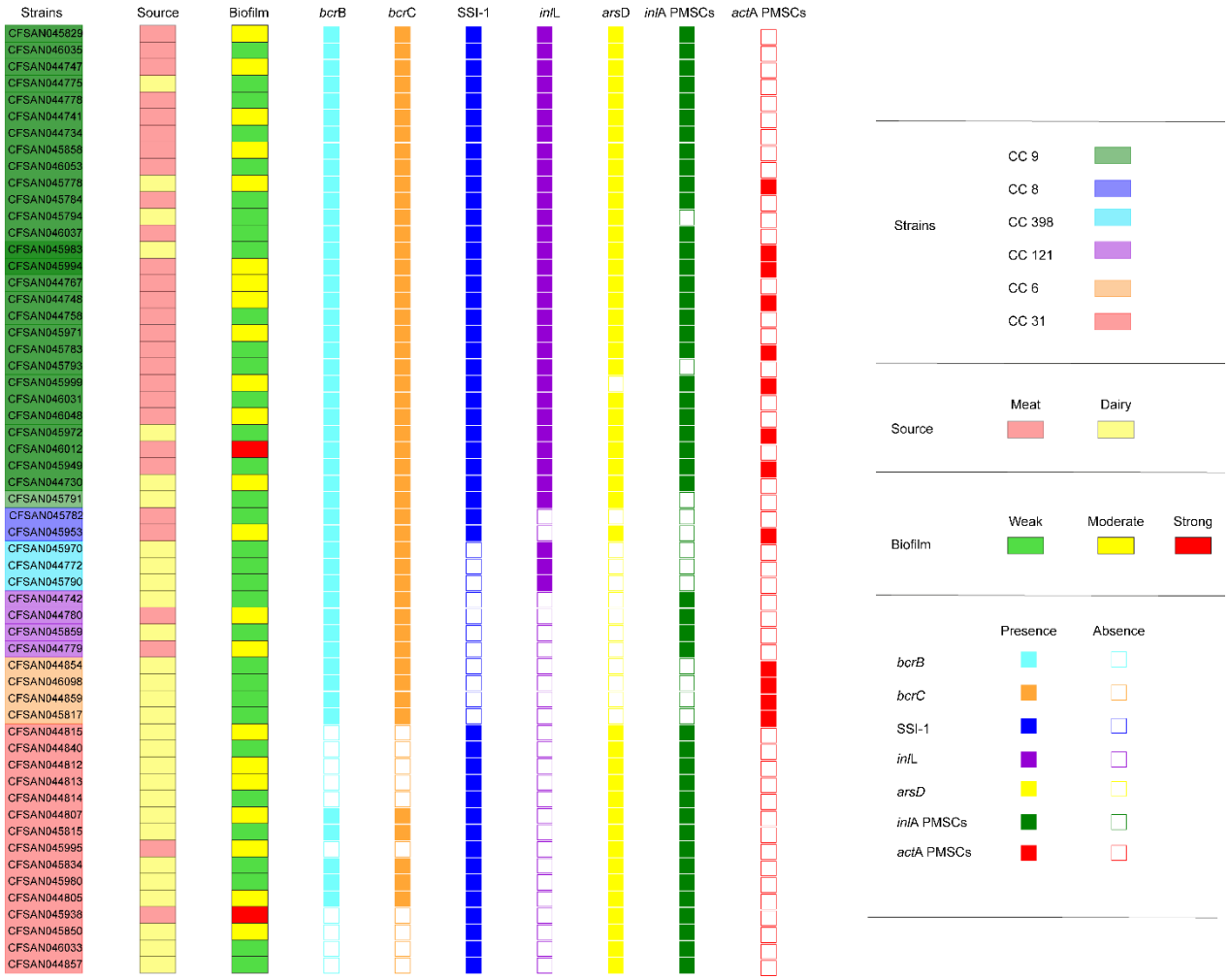
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Source
Biofilm
bcrB
bcrC
SSI-1
inlL
arsD
inlA PMSCs
actA PMSCs
CFSAN045829
CFSAN046035
CFSAN044747
CFSAN044775
CFSAN044778
CFSAN044741
CFSAN044734
CFSAN045858
CFSAN046053
CFSAN045778
CFSAN045784
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CFSAN044857
Strains
CC 9
CC 8
CC 398
CC 121
CC 6
CC 31
Source
Meat
Dairy
Biofilm
Weak
Moderate
Strong
Presence
Absence
bcrB
bcrC
SSI-1
inlL
arsD
inlA PMSCs
actA PMSCs

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