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Phylogenetic Profiles of In-House Microflora in Drains at a Food Production Facility: Comparison and Biocontrol Implications of *Listeria*-Positive and -Negative Bacterial Populations

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Listeria **species experience complex interactions with other microorganisms, which may promote growth and colonization of the organism in local environments or negatively affect them. This study investigated the microbial community at a food production facility, examining interactions between** ***Listeria*** **and the associated microbiome.** ***Listeria*** **species can be transferred between zones in the production environment by individuals or equipment, and drains may act as a reservoir for the organism, reflecting the microbial flora potentially in the production environment. Drains that were colonized by** ***Listeria*** **species and those determined to be free of** ***Listeria*** **were examined. In each case, 16S rRNA gene analysis was performed using the PhyloChip platform. Some general similarities in bacterial population structure were observed when** ***Listeria*****-negative and -positive drain communities were compared, with some distinct differences also noted. These included increased populations of the genera** ***Prevotella*** **and** ***Janthinobacterium*** **associated with the absence of** ***Listeria*** **species, whereas** ***Enterococcus*** **and** ***Rhodococcus*** **were in higher abundance in drains colonized by** ***Listeria*** **species. Based on these results, a selection of bacterial species were grown in coculture biofilm with a** ***Listeria monocytogenes*** **strain identified as having colonized a drain at the facility. Mixed-species biofilm experiments showed that** ***Janthinobacterium*** **inhibited attachment and subsequent biofilm formation of** ***L. monocytogenes*****; however,** ***Enterococcus gallinarum*** **significantly increased it. The results of this study suggest the microbial community in food processing facilities can impact the colonization of** ***Listeria*** **species and that influencing the microbiome in favor of antilisterial species may reduce the colonization of** ***Listeria*** **species and limit the likelihood of product/process contamination.**

Listeria monocytogenes can cause sporadic cases and outbreaks of severe disease among humans by exposure through contaminated foodstuffs (1, 2). Increasingly definitive molecular subtyping is linking sporadic cases and identifying outbreaks often dispersed over wide geographical areas (2). The pathogen presents a major public health problem in vulnerable groups (2, 3). This places a large responsibility on food business operators (FBOs) to ensure the production of safe food that is free from *L. monocytogenes*. Contaminated food products released to the market not only may have an impact on public health (2, 3) but also may impose a severe economic burden on the health care infrastructure, along with the associated costs to the FBO for loss of product, recalls, and damage to brand(s) and reputation (4, 5). As many processing treatments (e.g., cooking) are effective for the inactivation of *L. monocytogenes*, food product contamination is often associated with postprocessing events (6). Owing to the ubiquitous nature of the organism (7) and the frequency of ingredient contamination (8, 9), prevention of entry into food production environments can be challenging. This is further compounded by the capacity of the organism to colonize and persist in the production environment, a feature identified in previous studies (10). Taken together, these elements make *L. monocytogenes* a challenging food-borne pathogen to control. Regulators provide assistance to FBOs and others through their guidance documents, which are designed to assist with the implementation of a robust control strategy allied to cost-effective risk management (4–6, 10). A successful food safety program utilizes a multifactorial approach, which may be augmented with other elements (including molecular subtyping of strains, temperature control along all parts of the production chain, zoning at the production facility, the effective application of suitable biocides, and strategic surveillance with molecular subtyping of strains previously isolated). Each of these elements has associated costs, and a balance must be struck between safety and commercial viability (4, 5).

Microbial communities in ecological niches can influence a range of hitherto poorly understood complex interactions, which can include biofilm formation and interactions with other microbes sharing the environment, quorum signaling, and competitive exclusion, among others. Some of these events involve the production of antimicrobial compounds targeting other microbial species (11). Earlier reports have identified bacterial species capable of inhibiting *L. monocytogenes* strains (12, 13). Drains have been implicated as important sites for harborage of *L. mono-*

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cytogenes in food production facilities, frequently leading to cycles of continuous recontamination of the facility (14–16). Eradicating *Listeria* species from these drains can be particularly challenging, and biocontrol of *Listeria* species in these environments using natural means has been studied (16, 17). The objective of this study was to characterize the in-house microflora of drains at a meat food production facility as an important step in identifying bacterial species with the ability to influence the colonization potential of *Listeria* species. Candidate antilisterial strains identified were further evaluated for their interaction with in-house *L. monocytogenes* in biofilm.

MATERIALS AND METHODS

Sampling and isolation of bacterial strains. An extensive surveillance program, which included the microbiological monitoring of the drains at the facility, was undertaken, and drain contamination histories were recorded and assessed. Four drains were selected for study: two that were determined by bacterial culture methods to be free of *Listeria* species contamination (denoted drains A and B) and two that were associated with an increased frequency of *Listeria* species contamination (drains C and D). Over a 3-day period, 6 samples were taken from each drain. From these samples, the ISO standard method for detection of *Listeria* was applied to identify any *Listeria* species present (18).

PFGE of *Listeria* isolates. Subtyping of *Listeria* isolates was performed using pulsed-field gel electrophoresis (PFGE) as per the standard PulseNet protocol (19) with the following modifications: sodium dodecyl sulfate (SDS) was not included in the 1% SeaKem Gold plug agarose, and electrophoresis was performed on a contour-clamped homogeneous electric field (CHEF) Mapper for 21 h. The restriction enzyme AscI was used to generate the DNA macrorestriction profiles. Four isolates of each *Listeria* species identified in an individual sample were analyzed to determine strain diversity. The PFGE profiles generated were analyzed using BioNumerics V5.1 (Applied Maths, Belgium), by applying a Dice coefficient followed by unweighted-pair group method using average linkages (UPGMA) analysis, with "optimization" and "tolerance" settings of 1%.

Purification and preparation of genomic DNA and PhyloChip analysis. Genomic DNA was purified from bacterial samples recovered from the drain at the facility, using the DNeasy blood and tissue kit (Qiagen, United Kingdom). The quantity and quality of purified DNA were assessed using a NanoDrop spectrophotometer (Thermo Scientific, United Kingdom). Microbiome analysis using the PhyloChip platform was performed commercially by Second Genome, USA. To calculate the summary fluorescence intensity (FI) for each feature on each array, the central nine pixels of individual image features were ranked by intensity and the 75% percentile was used. Probe FIs were background subtracted and scaled to the PhyloChip control mix. Array FI data were collected as integer values ranging from 0 to 65,536. Fluorescence intensities observed from perfectly matching (PM) probes were compared to those from mismatching (MM) probes and were considered positive if the following ratios applied: PM/MM $\geq$ 1.5 and PM $-$ MM $\geq$ 50 $\cdot$ N and $r \geq 0.95$, where N indicates the array-specific noise (20) and r represents the response score (21). Only those PM FI signals from probes observed as positive in at least three independent experiments were exported from all experiments; these were then $\log_2$ transformed and used as input to empirical probe set discovery. Probes were clustered into probe sets based both on correlations in their FI values across all biological samples and on taxonomic relatedness. Where multiple clustering solutions were available, higher correlation coefficients were favored over lower ones. Taxonomic relatedness at the species level was favored over higher ranks, and sets composed of more probes were favored over those with fewer. All probe sets contained $\geq$5 probes, with average pairwise correlation coefficients of $\geq$0.85. The empirical operational taxonomic unit (eOTU) tracked by a probe set was taxonomically annotated from the combination of the 9-mers contained in all probes of the set. The mean $\log_2$ FI for each eOTU and each sample was calculated. These values are referred to as the hybridization score (HybScore), and this was used in abundance-based analysis. eOTUs were considered present if $\geq$80% of their probes were positive.

The purpose of annotating the OTUs was to link probe hybridization dynamics to taxonomic labels. To achieve this, probes were first compared against the January 2011 Greengenes database rereplicated at 98%. Using the Bayesian method to determine the most likely taxonomic node, eOTU matches, with 200 bootstrapped iterations, were performed. In each iteration, a subset of 9-mers within each probe were tested, and the percentage of iterations that resulted in the same node are referred to as confidence values, with an 80% confidence cutoff. In a second classification attempt to achieve the strain identification where possible, a special reference database from the October 2012 Greengenes database was created using only 16S rRNA genes that could be associated with a true isolate. In both cases, the classification confidence was presented for each taxonomic node to facilitate filtering.

Biofilm analysis. The influence of other bacterial species on *L. monocytogenes* LmT15 attachment and biofilm formation on stainless steel coupons was modeled with the following bacteria: *Janthinobacterium lividum* NCTC 7917, *Enterococcus gallinarum* NCTC 11428, and *Rhodococcus equi* NCTC 1620. Non-*Listeria* species were selected based on the results of the PhyloChip analysis. Species identified in higher abundance in *Listeria*-negative drains than in *Listeria*-positive drains (or *vice versa*) were selected. All experiments were carried out at 14°C, as this was the environmental temperature maintained at the processing facility. Stainless steel coupons (2 cm^2) were used to represent the drain surface. Before use, each coupon was sterilized by a detergent wash, heat sterilization by autoclaving at 121°C for 15 min, and soaking for 15 min in 100% ethanol and was subsequently flamed and then air dried for 30 min in a laminar flow cabinet prior to use. For biofilm experiments, a single colony of the strain to be tested was grown for 16 h $\pm$ 1 h at 30°C in 3 ml heart infusion broth (HIB) (Oxoid, United Kingdom). A coupon was placed in 10 ml 1:10 HIB and inoculated with the strain(s) to be tested. Inocula of approximately 100 to 500 cells were added to the 10 ml, verified by direct plate count on brain heart infusion agar (BHIA) plates. To remove biofilm, the coupons were washed 3 times with sterile water to remove loosely attached cells, and then the biofilm was detached from coupons by sonication for 5 min in 10 ml Maximum Recovery Diluent (Oxoid, United Kingdom) in a 50-Hz sonic water bath (Ultrasonics Pty. Ltd., Australia). Duplicate coupons were used and enumerated at 96 h by direct plate count, using both Brilliance *Listeria* agar plates (BLA) and BHIA plates (Oxoid, United Kingdom). All non-*Listeria* species included did not grow on BLA plates. All experiments were replicated twice, on separate occasions, with standard deviations determined on the combined results.

Statistical analysis. The Student t test was used to determine the significance of relative abundances determined by the 16S rRNA analysis methods, along with the mono- and mixed-culture biofilm experiments. Results were deemed significant at a P value of <0.05.

RESULTS

***Listeria* strains in drains.** The results of standard ISO enrichment of all drain samples are summarized in Fig. S1 in the supplemental material. No *Listeria* species were identified in drains A and B. These were recorded as the *Listeria*-negative drains. Three different species, *L. monocytogenes*, *L. welshimeri*, and *L. innocua*, were identified in *Listeria*-positive drains (C and D). Drain C was found to contain six *L. monocytogenes* strains and one *L. welshimeri* strain, as determined by PFGE subtype analysis. Five *L. monocytogenes* strains, three *L. welshimeri* strains, and one *L. innocua* strain were found in drain D. A diversity of up to two different strains for each individual *Listeria* species was identified in the samples tested. Strain PFGE subtypes were compared against the surveillance records for the facility to investigate whether or not

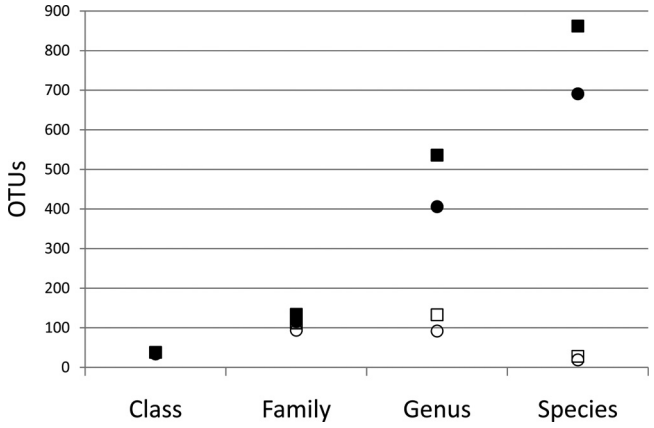

FIG 1 Diversity of drain microbiomes, as determined by PhyloChip analysis. ●, *Listeria*-negative drains, overall diversity; ○, *Listeria*-negative drains, classified OTUs only; ■, *Listeria*-positive drains, overall diversity; □, *Listeria*-positive drains, classified OTUs only.

these isolated strains showed contamination patterns characteristic of persistence at the facility. Of those that fit this criterion, 5 were repeatedly isolated from the same drain (LwT1, LinT6, LmT4, LmT8, and LmT15).

PhyloChip analysis. The microbiome diversity of *Listeria*-negative and -positive drains is shown in Fig. 1. Ninety-eight percent of OTUs could be assigned a classification at the family level, with this decreasing at the genus- and species-specific levels (40% and 6%, respectively). *Listeria*-positive drains were found to contain a greater diversity through class, family, genus, and species. *Clostridia* and gammaproteobacteria were the dominant classes of bacteria present in samples, comprising over 50% of the total microbiome in both *Listeria*-negative and -positive drains (Fig. 2). There was a difference when these were compared between drains: *Clostridia*, the most abundant class in *Listeria*-positive drains, comprised approximately 35% of the drain microbiome population, and this was estimated to be 25% in *Listeria*-negative drains; conversely, gammaproteobacteria dominated in *Listeria*-negative drains at 27% of the overall population, and this was comparable to the value for *Listeria*-positive drains, at 24%. The class representation and distribution between *Listeria*-negative and -positive drains were similar. The top nine most abundant classes were similar in both drain categories, with some minor differences in distribution noted (e.g., actinobacteria and fusobacteria were at higher abundance in *Listeria*-positive drains). Values for the top 3 most abundant classes identified in *Listeria*-positive drains were as follows: clostridia, 34.94%; gammaproteobacteria, 24.00%; and bacteroidia, 9.87%. For *Listeria*-negative drains, this was as follows: gammaproteobacteria, 27.30%; clostridia, 25.59%; and bacteroidia, 8.87%.

Diversity by family showed a similar distribution between both drain categories, with notable exceptions (Fig. 2). The 3 most abundant families in all samples (*Listeria*-positive and *Listeria*-negative) were as follows: *Lachnospiraceae*, 23.13% and 30.69%; *Pseudomonadaceae*, 9.29% and 8.74%; and *Rikenellaceae*, 7.23% and 7.86%. The seventh and eight most abundant families in *Listeria*-negative samples were *Ruminococcaceae* (2.42%) and *Prevotellaceae* (2.13%), respectively. In *Listeria*-positive drains, however, they were *Flavobacteriaceae* (3.65%) and *Comamonadaceae* (2.17%).

Table 1 shows the families of bacteria found in only one of the two categories of drains. Three families were identified exclusively in *Listeria*-negative drains, and 21 families were found only in *Listeria*-positive drains.

The relative abundances of the microbial populations were compared at the genus level between both categories, and Fig. 3 shows representative OTUs of 18 genera. These results were used to select candidates for mixed-species biofilm experiments.

Mixed-species biofilm analysis. Based on the results of the microbiome analyses, mono- and mixed-species biofilm experi-

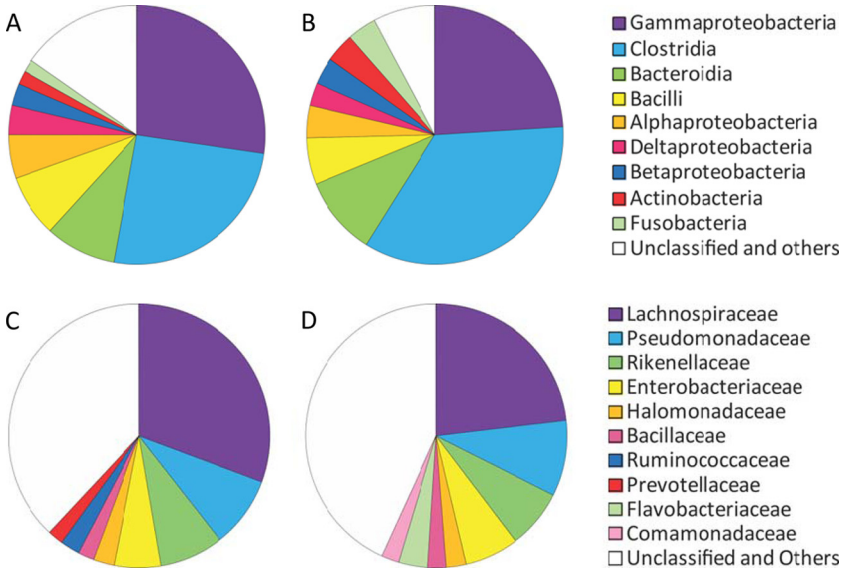

FIG 2 (A and B) The nine most abundant bacterial classes comprising the microflora of *Listeria*-negative drains (A) and *Listeria*-positive drains (B), as determined by PhyloChip analysis. (C and D) The eight most abundant bacterial families present in *Listeria*-negative drains (C) and *Listeria*-positive drains (D), as determined by PhyloChip analysis.

TABLE 1 Families of bacteria identified only in *Listeria*-negative or *Listeria*-positive drains, as determined by PhyloChip analysis

Bacterial community	Family (%[a])
Listeria negative only	*Clostridiales* family XI (0.15)
	Myxococcaceae (0.22)
	Synergistaceae (0.29)
Listeria positive only	A29 (0.07)
	Aerococcaceae (0.10)
	Chitinophagaceae (0.28)
	Chloroflexaceae (0.14)
	Coxiellaceae (0.10)
	Dermacoccaceae (0.24)
	Ferrimonadaceae (0.21)
	Herpetosiphonaceae (0.07)
	Hyphomicrobiaceae (0.28)
	Listeriaceae (0.10)
	Methylococcaceae (0.07)
	Mycobacteriaceae (0.10)
	Nitrosomonadaceae (0.14)
	Nocardiaceae (0.10)
	Phyllobacteriaceae (0.28)
	Piscirickettsiaceae (0.10)
	Propionibacteriaceae (0.14)
	Rhizobiaceae (0.34)
	Rhodocyclaceae (0.07)
	Thiotrichaceae (0.17)
	Trebouxiophyceae (0.03)

[a] Overall abundance in relation to total microbiome.

ments were performed to investigate the interaction if any, between *L. monocytogenes* LmT15 and the selected non-*Listeria* species identified as being associated with *Listeria*-positive or -negative drains. The results are shown in Fig. 4. When *J. lividum* was cocultured with *L. monocytogenes* LmT15, a significant reduction in attachment and biofilm formation was observed, without *J. lividum* itself being affected by the presence of LmT15. Conversely, *E. gallinarum* caused a significant increase in LmT15 attachment and biofilm formation when grown in coculture. The *E. gallinarum* cell density was not significantly altered in mixed biofilm with LmT15 compared with monoculture biofilm. The *R. equi* strain did not attach and form biofilm under the conditions tested, and it had no significant influence on LmT15 attachment and biofilm formation.

DISCUSSION

Bacteria experience complex interactions in their local environmental niche, ranging from beneficial symbiosis to inhibition and competitive exclusion of other species, and this has been previously demonstrated with *L. monocytogenes* along with other bacterial species (11–13, 22, 23). This study examined the microflora of localized communities in a food production environment. Selected drains included for study were those that were determined by conventional microbiological culture methods to lack any *Listeria* species over the course of routine microbial surveillance, along with the drains that were identified as being colonized with *Listeria* species. The microbiomes of two drains from each category were interrogated to discover the community composition profiles associated with the presence or absence of *Listeria* species. The PhyloChip platform was chosen, as this study aimed to select candidate strains based on the populations identified. To achieve this, interrogation of results was performed against the Greengenes database of strains, as well as a second database comprised of true strains (i.e., strain entries related exclusively to cultured isolates). Although microbiome analysis utilizing a high-throughput sequencing platform, such as the Roche 454 platform, may provide a more representative analysis of the true microbial diversity between samples, the PhyloChip platform, combined with the database interrogation strategy utilized in this study, allowed selection of culturable strains for mixed-biofilm analysis. Based on these data, mixed-biofilm experiments were performed to determine if specific bacterial species that were observed to exert an effect (positive or negative) on the occurrence and growth of *Listeria* at the facility could be identified. Species having negative effects could be considered to be a natural biocontrol phenomenon that could be harnessed to improve food safety.

Both of the *Listeria*-positive drains were shown to have a diverse *Listeria* species population, suggesting that *Listeria* species at the facility commonly occurred in localized, multi-*Listeria* species communities. Results of the microbiome analysis showed that the bacterial communities in *Listeria*-negative and -positive locations overlapped; nonetheless, differences in the distribution at the class and family levels were noted (Fig. 2). When examined at the genus level, it was possible to identify genera that were clearly associated with the presence of *Listeria* species, notably *Arthrobacteria*, *Bacillus*, *Clostridium*, *Dysgonomonas*, *Enterobacter*, *Microbacterium*, *Pedobacter*, *Psychrobacter*, *Rhodococcus*, *Tanerella*, and *Vibrio* (Fig. 3). *Janthinobacterium*, *Prevotella*, and *Pseudomonas* were all associated with higher abundance in *Listeria*-negative drains. Based on these observations, species from three genera, i.e., *Janthinobacterium*, *Enterococcus*, and *Rhodococcus*, were selected and studied in

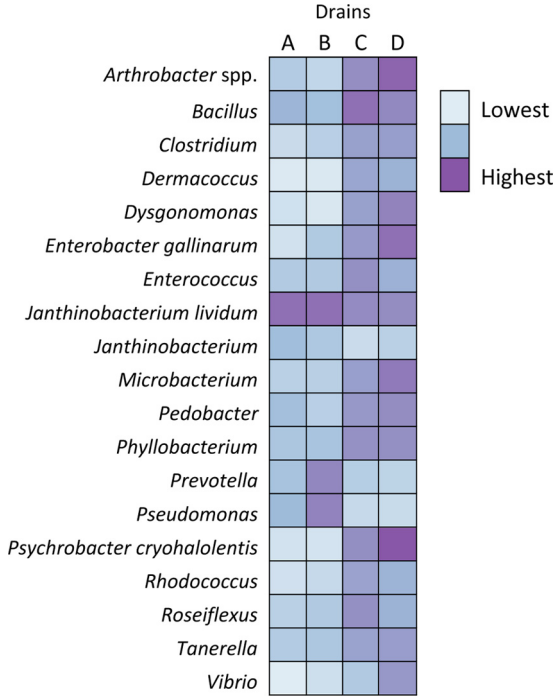

FIG 3 Heat map of relative abundances of representative OTUs from selected genera, where significant differences were seen when comparing *Listeria*-negative and *Listeria*-positive drains. All differences were calculated as significant ($P < 0.05$).

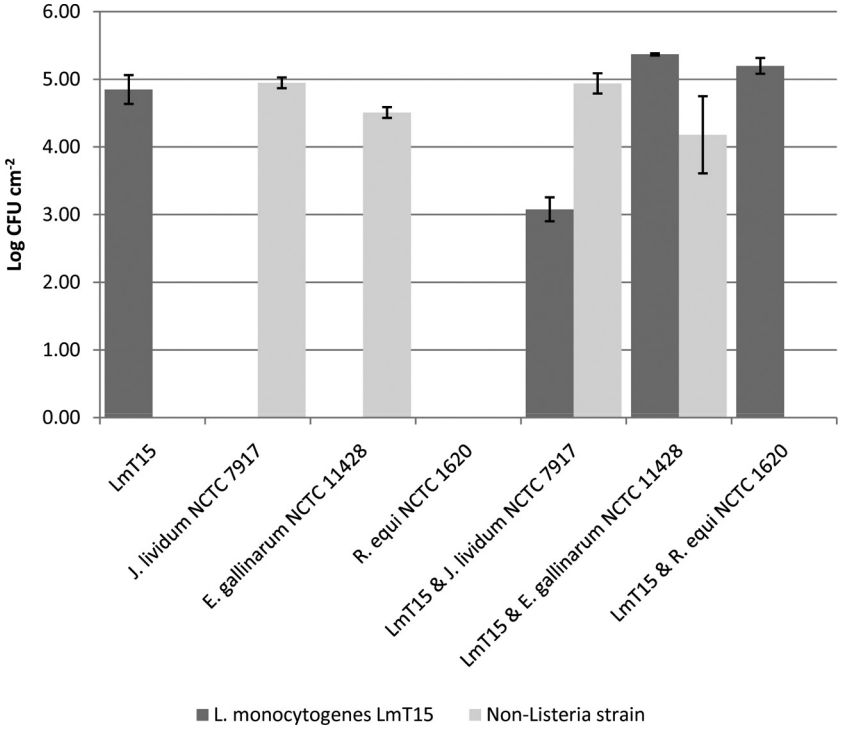

FIG 4 Mono- and mixed-culture biofilm experiments with *L. monocytogenes* strain LmT15 and selected other non-*Listeria* species. Dark gray bars, LmT15; light gray bars: non-*Listeria* strain used in coculture experiment. Error bars show calculated standard deviations.

mixed-species biofilms with *L. monocytogenes* LmT15, a naturally occurring isolate from the same positive drain.

The results of the mixed-species biofilm experiments are summarized in Fig. 4. Strains used included standard laboratory type strains, representative of each genera. Mixed-biofilm experiments with *J. lividum* NCTC 7917 and *L. monocytogenes* LmT15 showed that the presence of *J. lividum* reduced the attachment and biofilm growth of LmT15 on stainless steel coupons by approximately 2 log units/cm^2 under the experimental conditions applied. Biofilm formation and growth of *J. lividum* were unaffected by LmT15 under the experimental conditions used. Members of the genus *Janthinobacterium* were found to be associated with drain samples in which *Listeria* species were absent. The results of the mixed-biofilm experiments suggest that these bacteria may contribute to reducing the colonization of drains by *Listeria* species. It should be noted that the specific species and strain of *Janthinobacterium* present in the drains at the facility were not identified, and it is possible that it may elaborate a different response. Isolation and characterization of in-house representative strains will provide more information in future studies. *Janthinobacterium* produces antibacterial compounds, which have previously been shown to inhibit Gram-positive bacteria (24). Strains isolated at the facility will be screened for production of potential antimicrobial compounds which may have antilisterial properties. This study examined only simultaneous attachment and biofilm formation of the bacterial strains, and further work will also examine the effect of established biofilms on the integration of *Listeria* species into the local environment.

Enterococcus gallinarum and members of the genera *Rhodococcus* were in higher abundance in *Listeria*-positive drains. Representative strains included in mixed-species biofilm experiments for both of these were chosen: *R. equi* NCTC 1620 and *E. gallinarum* NCTC 11428. The *R. equi* strain did not form a stable biofilm when studied in mono- or mixed-species biofilm experiments, and it did not significantly affect the attachment and biofilm formation of LmT15 when cultivated in coculture, under the experimental conditions tested. This may indicate that the *Rhodococcus* strain was not a suitable choice or that additional bacterial species present in the drains in this study are required for incorporation of *Rhodococcus* into the biofilm community under the experimental conditions tested. When LmT15 was grown in coculture with *E. gallinarum*, the growth of LmT15 was significantly increased, as shown in Fig. 4. Control strategies targeted at specific microorganisms can be limited, in that they focus specifically on the microorganism of interest and not in the context of other factors that may be influencing the occurrence and colonization of that organism. In the case of other in-house microflora, for example, if other species of bacteria are promoting the growth and colonization of *Listeria* species, these bacteria should be addressed in terms of control. To this end, the results of this study suggest that in-house strains of *E. gallinarum* may promote *L. monocytogenes* colonization. *Enterobacter gallinarum* strains in *Listeria*-positive drains could be isolated and the efficacy of sanitizers in use at the facility against these strain(s) characterized. Control of bacterial species such as these, which may be facilitating the colonization of *Listeria* species, may be a key aspect in control of the organism and represent a novel approach not currently in routine use as part of food safety programs.

Species of bacteria that inhibit *Listeria* species have been identified, including *Enterococcus faecium*, *Leuconostoc mesenteroides*, and *Lactococcus lactis* (12, 13), and earlier studies have shown that application of such bacteria may be a promising tool for control of

Listeria at food production facilities (16, 17). However, food production facilities are not likely to be identical; the microbial community dynamics will vary, based on a range of factors unique to each facility (e.g., microbial species introduced into the environment through ingredients, sanitizers in use, etc.). To this end, it is important to consider that introducing bacterial species shown to have an inhibitory effect on *Listeria* species in another facility into this environment for the purposes of control of *Listeria* species may lead to problems. For example, strains themselves may be inactivated by a variety of intrinsic factors at the facility, such as sanitizers in use or the natural in-house microbial community. The use of naturally occurring in-house bacterial species, adapted to the local environment and known to have antilisterial properties, may overcome these issues. The results of this study will form the basis for further work to establish a strategy that may be applied on a case-to-case basis to help control *Listeria* species at food production facilities through the use of in-house microflora.

In conclusion, this study has characterized microbiomes of *Listeria*-colonized and *Listeria*-free drains and identified differences in community profiles between them. Species identified at higher abundance were shown to significantly increase *L. monocytogenes* attachment and biofilm formation on stainless steel coupons, and conversely, species associated with *Listeria*-free drains were shown to inhibit *L. monocytogenes* attachment and biofilm. These results suggest a novel control strategy that could be used at food processing facilities, whereby in-house microflora with the ability to inhibit *Listeria* species could be identified and used to seed localized environments and help prevent colonization of *Listeria* species.

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