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Microbial diversity and ecology of biofilms in food industry environments associated with *Listeria monocytogenes* persistence

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Contamination of food products with the foodborne pathogen *Listeria monocytogenes* may occur in the food processing environment. Many bacterial species co-exist in this environment and can interact in multispecies biofilms. Recent studies have shed light on the composition of microbial communities present in the same ecological habitat as *L. monocytogenes*. Others have aimed at identifying competitive or cooperative interactions between *L. monocytogenes* and other species in mixed-species biofilms. Both microbial composition and interactions may be differently influenced even by different strains belonging to the same species. Novel methodology based on recent advances in sequencing technologies promise to provide new insights into how the resident microbiota may influence the presence of *L. monocytogenes* in food industry environments.

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Introduction

A serious problem in the food industry is the presence of microbial biofilms that can harbour and transmit spoilage and pathogenic bacteria [1]. These biofilms often remain on surfaces after regular cleaning and disinfection. For the pathogen *Listeria monocytogenes*, the most common route of transfer to food products is through cross-contamination from surfaces in food processing plants [2,3]. Several recent large outbreaks of listeriosis have been traced back to *L. monocytogenes* strains persisting over extended periods of time in food processing environments [4,5], where they – like most bacteria in natural or human-made environments – are likely to reside within biofilm communities. However, perhaps unexpectedly, the capability of *L. monocytogenes* strains to form monospecies biofilms does not seem to be a key factor determining their ability to persist in food processing facilities [6••,7].

The resident microbiota in food processing plants can influence the growth of *L. monocytogenes*. In multispecies biofilms, interactions can be *competitive*, when *L. monocytogenes* is suppressed by other microorganisms; *cooperative*, when proliferation and survival of *L. monocytogenes* in biofilms are increased; or *neutral* [8–10]. Both the composition of the resident microbiota, the growth of *L. monocytogenes*, and interactions within biofilms are affected by environmental factors, such as the nature of raw materials, nutrient availability, temperature, humidity, pH, surface materials and roughness, and cleaning and disinfection (C&D) regimes [11,12]. Multispecies biofilms can provide stable niches for *L. monocytogenes*, where the encasing extracellular matrix can shelter cells and protect them from biocides and other stresses. The difficulties posed by biofilms in food industry are reflected in the large number of recent reviews concerning the use and effect of methods to control microbial biofilms in food related environments [1,10,13•,14–20]. Further knowledge of the microbial ecology of biofilms in specific food processing environments can increase our understanding of persistence of pathogens such as *L. monocytogenes*, ultimately improving our ability to manage food safety.

The current review focuses on recent advances regarding the composition and diversity of resident microbiota in food processing facilities known to harbour *L. monocytogenes*. It will also summarize the current understanding of how the resident microbiota found in food processing environments may influence *L. monocytogenes* in biofilms. We also highlight the potential of genomics technologies and other novel approaches for understanding these communities.

Microbial diversity in the food industry

In our previous review of the microbial diversity of resident microorganisms on cleaned surfaces in the food industry [12], we found that, overall, the microbiota was dominated by Gram-negative bacteria such as *Pseudomonas*, *Acinetobacter*, *Enterobacteriaceae*, *Psychrobacter*, and *Stenotrophomonas*, especially in industries with a humid production environment, such as fish, meat, and fresh produce processing plants. Gram-positive bacteria were more prevalent in dairy and dry production environments,

with lactic acid bacteria, *Staphylococcus*, and *Bacillus* as the most commonly found groups. Recent literature in general supports the major conclusions of the review [21–25,26•]. Some of the studies [23,26•] highlight the prevalence of yeast and moulds on surfaces. These eukaryotic microorganisms are reported in some studies [12] but are often not investigated, as they are not detected in analyses based on sequencing of 16S rRNA.

Microbial communities harbouring *L. monocytogenes*

L. monocytogenes is frequently isolated from the food industry, although it is always outnumbered by other types of bacteria, and selective enrichment is in most cases needed for environmental detection. In a study of *L. monocytogenes* positive surfaces in meat, fish, and dairy processing plants, sampled before C&D [24], the total psychrotrophic count was 5–9 log CFU/cm^2, while *L. monocytogenes* was present in concentrations of 2–4 log CFU/cm^2 in samples where it could be quantitatively detected (9 out of 40 positive samples); on the majority of the surfaces the concentrations were lower.

Table 1 lists the studies (2014–2020) in which both analysis of the microbiota and detection of *L. monocytogenes* were performed for the same surface or sample [9,23–25,26•,27–31], providing insights into which types of bacteria are found with *L. monocytogenes* in the food industry. In general, these bacteria are the same as those that usually dominate in food industrial environments (Table 1). Rodríguez-López *et al.* [24] found that Actinobacteria was the most prevalent phylum (53%) found on the same surface as *L. monocytogenes* in the meat industry, while Proteobacteria dominated at such sites in fish (97%) and dairy plants (69%). In a study of three fruit processing plants [26•], the processing plant with the highest prevalence (100%) of *L. monocytogenes* positive surfaces was uniquely dominated by the bacteria *Pseudomonadaceae* and the fungi *Dipodascaceae*. This led to the conclusion that the composition and diversity of the bacteriota and mycobiota may be indication of persistent contamination with *L. monocytogenes*.

Other studies indicate that specific bacteria may be associated with low prevalence of *L. monocytogenes*. *Janthinobacterium* has been shown to be more prevalent in *Listeria*-negative than *Listeria*-positive drains, and to inhibit attachment and biofilm formation of *L. monocytogenes* in laboratory studies [9]. In studies of wooden vats used in cheese production, the presence of a resident microbiota dominated by the fungus *Geotrichum* was shown to inhibit *L. monocytogenes* [32].

Biofilm interactions involving *L. monocytogenes*

Correlative associations between bacteria are not the same as causal relationships, and need to be confirmed by experimentation. A number of studies employing laboratory tests, and in some cases *in situ* trials in food industry, have attempted to determine the nature of the interactions between *L. monocytogenes* and other bacteria within biofilms. For older studies we refer readers to previous reviews [2,10,15,20,33••], while an overview of recent papers (2018–2020) is presented in Table 2 [31,34–41,42•,43,44,45•,46•,47–49].

To study interactions relevant for the behavior of *L. monocytogenes* in food industry settings, model systems should consist of bacteria commonly found together with *L. monocytogenes* in biofilms in food industry environments [50]. *Pseudomonas* spp. bacteria match this description (Table 1), thus papers describing mixed-species biofilms containing *L. monocytogenes* and *Pseudomonas* spp. (except *Pseudomonas aeruginosa*) [42•,44,45•,46•,51] are highly relevant. The studies show that *L. monocytogenes* can be established in biofilms with *Pseudomonas*, as a minor part of the total bacterial population. Interestingly, the presence of *L. monocytogenes* may induce increased matrix production in biofilms with *Pseudomonas* [45•] and *L. monocytogenes* can be protected against desiccation and disinfection [44]. Other microorganisms from food industry environments recently studied in mixed-species biofilms with *L. monocytogenes* are *Bacillus*, lactic acid bacteria, *Escherichia coli*, *Vibrio*, *Salmonella*, *Staphylococcus*, and yeasts [31,34,35,37,38–41,47,48] (see Table 2). However, not all of these microorganisms are typically co-isolated with *L. monocytogenes* in food industry (Table 1), indicating that they may exist in other ecological niches than *L. monocytogenes* in factories. Other studies, examining interactions between *L. monocytogenes* and other pathogens such as *P. aeruginosa* or *Salmonella* Typhimurium [36,43,52], have very limited relevance for food industry, since these pathogens are rarely encountered together in food industry environments [12].

Both competitive and cooperative interspecies interactions between *L. monocytogenes* and other bacteria in biofilms have been described in previous reports. In recent laboratory studies (Table 2), the most common finding was that the numbers of *L. monocytogenes* in multispecies biofilm were lower than in *L. monocytogenes* monospecies biofilms. Inhibition of *L. monocytogenes* in dual species biofilms with *Bacillus cereus* or lactic acid bacteria has been explained by the production of antagonistic compounds [34,39–41]. Bacteriocin-producing lactic acid bacteria are known to be antagonists to *Listeria* spp., and have even been proposed to be used as a means to control biofilms in food production [15]. Whether or not these strains will thrive in niches where *L. monocytogenes* is found is another question.

Effect of environmental factors

Community-intrinsic properties such as direct inhibition of one bacterium by another can explain some

Table 1

Microbial communities found in *L. monocytogenes (Lm)* positive environmental sampling points

Microbiome analysis approach	Environment	*Lm* positive sampling points analysed	Dominant microbiota found in *Lm*-positive samples or sampling points	Ref.
Culture independent; microarray analysis using PhyloChip platform	Meat production facility	2 *Lm*-positive drains; 6 samples from each drain taken over a 3-day period	*Lachnospiraceae*, *Pseudomonadaceae*, *Rikenellaceae*, *Enterobacteriaceae*. Increased abundance of *Enterococcus* and *Rhodococcus* associated with presence of *Lm*	[9]
Culture based; sequencing of 16S rRNA from randomly picked psychrotrophic colonies (grown at 15°C)	Salmon processing plant	1 *Lm*-positive conveyor belt after C&D	*Pseudomonas*, *Brochothrix*, *Stenotrophomonas*, *Serratia*	[27]
Culture based; sequencing of 16S rRNA from morphologically different colonies (grown at 25°C)	Fish and seafood processing plants	6 *Lm*-positive samples (gloves, floor, sewage channels, conveyor belt, scale lines)	*Escherichia coli*, *Staphylococcus* (*saprophyticus*, *scuri* and sp.), *Kocuria varians*, *Aerococcus viridans*, *Microbacterium* (*luteolum* and sp.), *Corynebacterium* sp., *Enterococcus aquimarinus*, *Rothia terrae*	[28]
	Meat slaughtering and processing plants	6 *Lm*-positive samples (trolley, mincer, massage drum, drain)	*Carnobacterium* (*divergens* and sp.), *Serratia* sp., *Staphylococcus* (*saprophyticus* and *vitulinus*), *Pseudomonas* sp., *Buttiauxella* sp.	
Culture independent; construction and sequencing of a 16S rRNA gene clone library	Fish sauce and hoisin/oyster sauce factories	8 *Lm*-positive floor drains	*Pseudomonas* (*psychrophila* and sp.), *Klebsiella* (*oxytoca* and sp.), *Aeromonas hydrophila*	[29]
Culture independent; pyrosequencing of 16S rRNA PCR amplicons (V1–V2 regions)	Cheese production facility	3 *Lm*-positive floor drains; samples of both drain water and biofilm, taken during production	*Pseudomonas mucidolens*, *Lactococcus lactis*, *Acetobacter tropicalis*, *Gluconobacter oxydans*, *Leuconostoc citreum*, *Chryseobacterium ureilyticum*	[30]
Culture based; sequencing of 16S rRNA from morphologically different colonies (grown at 25/30°C)	Dairy plant	1 *Lm*-positive floor drain	*Klebsiella* sp., *Escherichia coli*, *Comamonas* sp., *Acinetobacter* sp.	[31]
Culture independent; sequencing of 16S rRNA PCR amplicon using IonTorrent technology	Meat (bovine and porcine) slaughterhouse	2 locations (drain, platform/table), each sampled 7–8 times before C&D; *Lm* was not detected on all occasions	Drain: *Rhodococcus*, *Chryseobacterium*, *Microbacterium*, *Acinetobacter*, *Athrobacter*, *Sphingomonas,* *Flavobacterium*, *Rothia*, *Pseudoclavibacter*; Platform/table: *Corynebacterium*, *Facklamia*, *Jeotgalicoccus*, *Psychrobacter*	[24,25]
	Fish processing/ market	1 *Lm*-positive sump/drain, sampled 4 times before C&D; *Lm* was not detected on all occasions	*Pseudoalteromonas*, *Psychrobacter*, *Photobacterium*, *Psychromonas*, *Flavobacterium*, *Carnobacterium*	
	Cheese production facility	1 *Lm*-positive floor sample (under silo), sampled once before C&D	*Acinetobacter*, *Lactococcus*, *Pseudomonas*, *Shewanella*, *Yersinia*	
Culture based; identification of randomly picked morphologically different colonies (grown at 30°C) by biochemical (API) tests	Meat (porcine) slaughterhouse and processing plant	3 locations (tool cabinet, floor, transportation cart) each sampled 16 times over a 21-month period; *Lm* detected on 1–2 occasions in each sampling point	*Pseudomonas*, *Bacillus*, *Mannheimia haemolytica*, *Enterobacter*, *Corynebacterium*, *Leifsonia*, *Leuconostoc mesenteroides*, *Candida zeylanoides*	[23]
Culture independent; sequencing of 16S rRNA (V4 domain) and ITS2 PCR amplicons using Illumina technology	Apple and other tree fruit packing houses	3 factories, 3 sampling locations in each (floor under conveyor system; wash, dry, and wax sections), 13 samples from each sampling point; *Lm* detected in 56% (66/117) of samples	*Pseudomonadaceae*, *Flavobacteriaceae*, *Xanthomonadaceae*; Fungal families: *Dipodascaceae*, *Trichosporonaceae*, *Aureobasidiaceae*	[26•]

phenotypical observations from studies of mixed-species biofilms. In other studies, however, extrinsic environmental factors seem to play a greater role. For example, several studies report that competition for nutrients can explain the lower counts of *L. monocytogenes* within biofilms [42•,44,46•,47–49]. Common for these studies is that biofilm formation was studied on surfaces (often horizontal) without applied shear forces and in the absence of flow. In such systems competition for nutrients and tolerance to inhibitory compounds are likely of higher importance for the prevalence of a species than the ability to attach to a surface and to build a strong matrix. An

Table 2

Studies reporting on biofilm interactions between *L. monocytogenes (Lm)* and other microorganisms; 2018–2020

Microbes co-cultured with *L. monocytogenes*	Biofilm[a]	Strains[b]	Solid surface[c]	Temp.	Culture nutrients	Duration of experiment	Conditions	Effect[d]	Ref.
Bacillus cereus	DS	3 *Lm* + 6	SS	25°C	BHI	7 days	Static	−4 to 0	[34]
Escherichia coli	DS	1 *Lm* + 1	SS	25°C	BHI, reconstituted powder milk	60 hours	Shear forces (15 rpm)	−2 to 0	[35]
Escherichia coli, *Salmonella* Typhimurium or *Salmonella* Enteritidis, *Pseudomonas aeruginosa*, *Bacillus cereus*	MS	1 *Lm* + 5	SS and PP	9, 25°C	TSB + eggyolk, TSB + meat extract, whole milk	10 days	Static	−4 to −2	[36]
Escherichia, *Klebsiella*, *Comamonas*, *Acinetobacter*	DS, MS	1 *Lm* + 4	SS	25°C	BHI	72 hours	Shear forces (90 rpm)	DS: 0 to +1; MS: <−0.5	[31]
Enterococcus, Staphylococcus, Bacillus	MS	3 *Lm* + 9	SS	25°C	BHI, whey protein, skimmed milk	10 days	Static	−2 to 0	[37]
Limosilactobacillus (Lactobacillus) fermentum, Ligilactobacillus (Lactobacillus) salivarius	DS	1 *Lm* + 2	Glass	37°C	TSB and MRS	72 hours	Static	−2 to +1	[38]
Lactobacillus delbrueckii subsp. *lactis*	DS	1 *Lm* + 1	SS	25°C	BHIYE	5 days	Static	−4	[39]
Lactobacillus, Lactiplantibacillus (Lactobacillus), Latilactobacillus (Lactobacillus), Leuconostoc	DS	1 *Lm* + 8	SS, MP, lettuce	10, 25, 30°C	TSB, water	24 hours	Static	−2 to −1	[40]
Leuconostoc	DS	2 *Lm* + 3	MP	37°C	BHI	24 hours	Static	−2 to −1	[41]
Listeria innocua, Acinetobacter, Pseudomonas, Serratia, Stenotrophomonas	MS	6 *Lm* + 11	SS	12°C	BHI	9 days	Static	−3 to −2	[42•]
Pseudomonas aeruginosa	DS	2 *Lm* + 1	MP	10, 15°C	Todd-Hewitt broth	14 days	Static	−1.5 to −0.5	[43]
Pseudomonas fluorescens	DS	1 *Lm* + 1	SS	15°C	TSB	48 hours	Static	−0.5 to 0	[44]
Pseudomonas fluorescens	DS	1 *Lm* + 1	Glass	20°C	TSB	4 days	Shear forces (80 rpm)	+1 to +2	[45•]
Pseudomonas spp. or bacteria from raw fish juice	DS, MS	6 *Lm* + 5 or unknown	SS	15°C	Fish juice (sea bream)	10 days	Static	DS: −1 to 0; MS: −3	[46•]
Vibrio parahaemolyticus	DS	2 *Lm* + 2	MP	25°C	TSB	72 hours	Static	−4 to −3	[47]
Yeasts (*Candida*, *Rhodotorula*)	DS	1 *Lm* + 4	SS	25°C	Apple juice	24 hours	Static	0 to +1	[48]
Nonidentified bacteria from salmon	MS	1 *Lm* + unknown	SS	4, 15°C	1/20 TSB or salmon broth	14 days	Static	−1 to +1	[49]

[a] DS: dual-species biofilm; MS: multispecies biofilm.
[b] Total number of strains of *L. monocytogenes* (*Lm*) + total number of strains for all other tested species (combined).
[c] SS: Stainless steel coupons; PP: polypropylene; MP: Microtiter plate.
[d] Change in numbers of *Lm* in multispecies biofilms relative to monospecies biofilms, given as change in colony forming units (cfu) for *Lm*: *log(cfu in multispecies biofilm) − log(cfu in monospecies biofilm)*. For the majority of the studies the effect varied depending on inoculation levels, strains, temperature, time and/or medium.

exception is the study by Puga *et al.* [45•] where there was 1–2 log more *L. monocytogenes* present in preformed *Pseudomonas* biofilms than in *L. monocytogenes* monospecies biofilms. The biofilms were grown on glass coverslips in a reactor with applied shear forces, and *L. monocytogenes* was found to migrate to the bottom layer of the dual species biofilm. Potentially, *L. monocytogenes* alone was unable to form thick biofilms in the presence of shear forces, while in co-culture, the strong biofilm-former *Pseudomonas* provided a protected biofilm in which *L. monocytogenes* could thrive.

When designing a model system aiming to investigate mechanisms of relevance for biofilm formation and *L. monocytogenes* prevalence in food industry, choosing the right environmental factors is equally important as choosing the right microbial consortium [50]. Typical niches where *L. monocytogenes* survives in food production environments are scratches or grooves in or between different types of (worn) materials or complex equipment, such as drains, floors, conveyors or slicers – locations which are often difficult to reach with sanitation and where nutrients and solids tend to build up – as well as locations at room temperature or colder [53]. For example, biofilm formation on open smooth stainless steel surfaces is not likely to be a significant issue in food processing facilities. Nevertheless, most reviewed studies employ stainless steel coupons as the solid surface material (Table 2).

Furthermore, some studies employ cultivation temperatures of 30°C or 37°C [38,40,41,52], conditions which are not relevant for food industrial environments where *L. monocytogenes* is a challenge.

In the current context, *in situ* investigation of interactions in multispecies biofilms in the food industry is an interesting approach, and as previously mentioned, there are some older studies showing that certain bacteria affects the prevalence of *L. monocytogenes* in drains [9,54] and cheese vats [32]. However, we are not aware of similar studies from the review period.

Strain variation in biofilm phenotypes

Certain genotypes of *L. monocytogenes* are more commonly found to colonize food processing equipment than others. A large number of studies have examined whether specific strains or variants of *L. monocytogenes* have special fitness traits that can explain persistence; however, no clear links between persistence and inherent phenotypes have been identified [3,53,55]. Lianou *et al.* [6••] recently reviewed studies examining correlations between increased ability to produce monospecies biofilm with persistence in food industry environments. The amount of biofilm formed by distinct *L. monocytogenes* strains has been found to be highly dependent on extrinsic factors such as temperature and nutrients, with inconsistent variations across different growth conditions and experimental designs [6••,7]. However, under a given set of environmental conditions, differences in biofilm formation efficiency between *L. monocytogenes* strains or genetic lineages can be seen. For example, persistent genotypes were associated with higher survival and biofilm formation capacity in the presence of sublethal concentrations of the disinfectant benzalkonium chloride [56].

Studies examining interactions between *L. monocytogenes* and other bacteria in mixed-species biofilms rarely take into account strain-to-strain variation within *L. monocytogenes* (Table 2), and *vice versa*. However, in one study where both factors were examined [42•], clear differences in the distribution of individual *L. monocytogenes* isolates was observed between monospecies and multispecies biofilms: Of six *L. monocytogenes* strains, one strain outcompeted the others, but only in the presence of both *Listeria innocua* strains and a mixed Gram-negative microbiota dominated by *Pseudomonas*. The composition of the biofilm reflected the composition of the suspension surrounding the biofilm coupons, thus the effect was not necessarily biofilm-specific [42•]. However, it may be speculated that such strain-specific variations in growth and survival within multispecies biofilms may explain why certain types of *L. monocytogenes* persists in the food industry, and highlights the significance of including more than one strain of each species in studies of interactions within microbial biofilms.

Genomics and network analysis

Further studies are needed to examine whether the presence of certain members of the resident microbiota shows a significant correlation (either positive or negative) with the occurrence of *L. monocytogenes* in food processing facilities. Recent advances in high throughput sequencing (HTS) have resulted in generation of large volumes of data on the relative composition of microbial communities, mainly through 16S rRNA gene amplicon sequencing studies [57]. The methods are sensitive enough to allow detection of nondominant members of a community which may play important roles within a given ecosystem. The technological advances and large data volumes offered by HTS methods have resulted in rapid development of more efficient data analysis methods, such as novel methods within the field of network analysis [58–60]. Microbial interaction networks have for example been used to predict that in the gut, *Barnesiella* inhibits *Clostridium difficile* infection, an interaction which was subsequently confirmed by *in vitro* co-culture experiments [61].

Another option enabled by HTS technology is the use of metatranscriptomic sequencing to study changes in gene expression profiles underlying bacterial interactions in multispecies biofilms. This approach has unraveled functionality and interactions in consortia such as biofilm communities from soil and oral biofilms, revealing for example strain-dependent effects of one species on gene expression patterns in others, as well as given insight into specific interactions between different consortium members [62,63].

Within the field of food microbiology, the majority of microbiome studies employing HTS technology have aimed to monitor fermentative processes or food spoilage, with relatively fewer studies undertaken to examine factory environments, despite the role of the processing environments as a source of both spoilage microbiota and pathogenic bacteria [57,64•]. Microbial association network analysis has been applied to the study of food microbiomes [65,66,67•] and for analysis of co-occurrence patterns between bacterial families found in the environmental microbiome of a fruit processing facility [26•]. However, this approach is still underexploited for detection of ecological correlation patterns or interactions between members of environmental biofilm communities found on surfaces in food industry. It would be interesting to see to what extent these approaches can shed light on factors responsible for *L. monocytogenes* persistence, or be used to identify niches where *L. monocytogenes* would be able to persist, if introduced to the processing environment. The elimination of potential niches would be a more proactive strategy than monitoring for the pathogen itself.

Conclusions

The problem of persistence of *L. monocytogenes* in food processing factories, as well as its association with the

formation of biofilms, has been acknowledged for many years. The microbial ecology underlying the survival of this pathogen in these man-made environments is, however, still not well understood. In recent years, researchers have started to study the microbial ecosystems associated with the presence of *L. monocytogenes* in these habitats. There is also considerable interest in examination of interactions between *L. monocytogenes* and other bacteria, in part due to the hope that biocontrol interventions may help improve the control of this pathogen in food processing environments.

The main impression from recent studies is that persistent *L. monocytogenes* share environmental niches with several other members of the resident microbiota in food factories, and that the interactions are mostly competitive in nature. For *L. monocytogenes*, attempts to find single traits that can explain persistence of certain genotypes have failed. Most probably, persistence requires a match between each specific *L. monocytogenes* strain and the microbiota and the microenvironment where it is introduced. There are few *in situ* studies on the microbiota and microenvironment where persistent *L. monocytogenes* reside, and information from such studies could guide further experimental research. With that, the focus of future studies could shift from reductionistic approaches to more complex and realistic laboratory models, enabling further investigation into causal relationships underlying interspecies or interstrain interactions and the effect of environmental factors on the composition of microbial communities in factory environments. Likewise, application of novel methodology based on recent advances in sequencing technologies and network analysis is expected to increase our understanding of pathogen persistence. The overall impact of these insights could be a shift in management of *L. monocytogenes*, where the current 'seek and destroy' strategy is replaced with a preventive approach in which environmental niches promoting pathogen growth can be removed.

Conflict of interest statement

Nothing declared.

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