MINI-REVIEW

How the interaction of *Listeria monocytogenes* and *Acanthamoeba* spp. affects growth and distribution of the food borne pathogen

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Abstract *Listeria monocytogenes* is a foodborne opportunistic pathogen capable to switch from an environmental saprophyte to a potentially fatal human pathogen. The fact that the pathogen maintains the genes suitable for an elaborate infectious process indicates that these genes are required to survive in the environment. However, no environmental host reservoir for *L. monocytogenes* has been identified so far. The similarity of free-living, bacteria-scavenging amoebae to macrophages led to the hypothesis that protozoa may represent the missing link in the ecology and pathology of *L. monocytogenes*. Consequently, numerous studies have been published reporting on the potential of *Acanthamoeba* spp. to serve as host for a variety of pathogenic bacteria. However, the data on the interaction of *L. monocytogenes* with *Acanthamoeba* spp. are inconsistent and relatively little information on the impact of this interaction on growth and distribution of the foodborne pathogen is currently available. Hence, this review focuses on the interaction of *L. monocytogenes* and *Acanthamoeba* spp. affecting survival and growth of the foodborne pathogen in natural and man-made environments, in order to highlight the potential impact of this interplay on food safety and human health.

Keywords *Listeria monocytogenes* · *Acanthamoeba* · Protozoa · Grazing · Intracellular persistence

Introduction

Listeria monocytogenes are Gram-positive non-spore-forming rods with a low G+C content (Gasanov et al. 2005). The

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bacteria are facultative anaerobes growing in a wide range of temperatures, from 45 °C down to 1 °C due to their psychrotolerant character (Farber and Peterkin 1991). They are widely distributed in the environment where they exhibit a saprophytic lifestyle in substrates such as soil, vegetation, decaying plant material, water, and sewage. As an opportunistic intracellular pathogen, *L. monocytogenes* can cause life-threatening infections in animal and human populations at risk (Schlech et al. 1983). Infection occurs most likely due to ingestion of contaminated food (Schuchat et al. 1992). After invasion into gastrointestinal epithelial cells, *L. monocytogenes* adopts an intracellular lifestyle that enables the bacteria to multiply and spread to other tissues, as reviewed elsewhere (Bonazzi et al. 2009; Schuppler and Loessner 2010).

Amoebae are among the earliest eukaryotic microorganisms that have been studied since the invention of the microscope. Based on rRNA sequences, it is estimated that amoebae have diverged from the main line of eukaryotic descent, sometime between the divergence of yeast (~1.2×10^9 years ago) and the divergence of plants and animals (~1×10^9 years ago) (Siddiqui and Khan 2012). In 1930, *Acanthamoeba* was discovered as a contaminant of yeast culture and later placed in the genus *Acanthamoeba*. The term acanth (Greek "acanth" means "spikes") was added to "amoeba" in order to indicate the presence of spine-like structures on its surface, today known as acanthopodia. *Acanthamoeba* has long been studied as model eukaryotic cells, with special emphasis on the actin cytoskeleton-based motility (Pollard et al. 1970). *Acanthamoeba* cells move relatively fast compared to other eukaryotic cells, showing a locomotory rate of approximately 0.8 μm/s (Siddiqui and Khan 2012). The anterior pole (front end) of a moving amoebae consists of an optically translucent region of cytoplasm, referred to as hyaloplasm, whereas the cytoplasm is filled with granular material and various inclusions and is referred to as granuloplasm. Directional

movement of the trophozoites involves the formation of hyaline pseudopodia, followed by focal adhesion and detachment, thus leading to the directed movement of the whole cell body (Siddiqui and Khan 2012). The manner of *Acanthamoeba* movement is similar both at solid substratum and at the water–air interface. The adhesion forces developed between *Acanthamoeba* and the water–air interface are greater than gravity, and thus amoebae are also transported passively without detachment from the water surface (Preston et al. 2001). Actin microfilaments are most concentrated just beneath the plasma membrane, and are responsible for resisting tension and forming cytoplasmic protrusions. Depending on the environmental conditions, *Acanthamoeba* is able to switch between an active, vegetative trophozoite stage and a dormant cyst stage, characterized by cellular differentiation into a double-walled cyst form based on changes in environmental conditions (Siddiqui and Khan 2012). During the trophozoite stage, *Acanthamoeba* divides mitotically under optimal conditions, such as food supply, neutral pH, 20–30 °C and 50–80 mOsmol l^{-1} (Band and Mohrlok 1973). They feed on small organic particles, bacteria, fungi, algae, and even other protozoa. Grazing by heterotrophic protozoa is thought to represent a major source of bacterial mortality in soil and other natural ecosystems and is further considered to be a major trophic pathway, whereby the biomass produced by microorganisms re-enters the food web (Huws et al. 2008). Among protozoa, free-living amoebae are the dominant bacteria consumers in natural environments and are responsible for up to 60 % of total reduction in bacterial population (Sinclair et al. 1981). Phagocytosis is driven by structural rearrangements through polymerization of monomeric G-actin into filamentous F-actin (Alsam et al. 2005). Engulfed food contained within a vacuole is degraded following fusion of the phagosome with a lysosome (Bozue and Johnson 1996). Furthermore, *Acanthamoeba* contains one or more prominent contractile vacuoles, whose function is to expel water for osmotic regulation (Bowers and Korn 1973). The posterior pole (rear end) of the cell, referred to as uroid, is characterized by large amounts of folded membrane material (Arhets et al. 1995). There, debris accumulates and periodically detaches from the cell body.

The ecology of *L. monocytogenes* is somewhat elusive, and no host that may serve as a reservoir in natural environments could be identified so far (Fenlon 1999). Free-living amoebae have been recognized as important players in the evolution and transmission of many bacterial pathogens (Cirillo 1999; Greub and Raoult 2004; Molmeret et al. 2005; Anacarso et al. 2012). Due to their similarities to macrophages, free-living amoebae that graze on bacteria were considered as potential host reservoirs, where *L. monocytogenes* would benefit from the amoebae representing a training ground for their elaborated pathogenicity (Molmeret et al. 2005). Therefore, this review focuses on the interaction of *L. monocytogenes* and protozoa, such as *Acanthamoeba*. Although reports exist on

the survival and replication of *L. monocytogenes* in *Tetrahymena* and *Acanthamoeba* spp. (Ly and Muller 1990; Zhou et al. 2007), recent data support the view that in contrast to other intracellular pathogens *L. monocytogenes* is unable to survive and replicate after phagocytosis by *Acanthamoeba* species (Huws et al. 2008; Akya et al. 2009b, 2010; Doyscher et al. 2013). Interestingly, another, more peculiar type of interaction between *L. monocytogenes* and *Acanthamoeba* spp. was discovered recently, where *L. monocytogenes* forms large and densely packed aggregates on the surface of moving *Acanthamoeba* trophozoites (Doyscher et al. 2013). Another major finding from studies on the interaction between *L. monocytogenes* and *Acanthamoeba* trophozoites was the observation that although *Acanthamoeba* trophozoites graze on *Listeria,* viable counts of extracellular bacteria actually increased during co-culture (Fieseler et al. 2014). This observation suggests that *Acanthamoeba* supports growth of *L. monocytogenes,* which might have important implications on the ecology of the foodborne pathogen. This review aims to provide an overview on the available data referring to the interaction of *L. monocytogenes* and protozoa, placing emphasis on *Acanthamoeba* spp. in order to highlight the impact of this interplay on food safety and human health.

The search for environmental reservoirs of *L. monocytogenes*

The widespread presence of *L. monocytogenes* and other *Listeria* spp. in diverse environments, such as natural, agricultural, and food-associated, suggests that these environments may serve as sources or provide reservoirs for *L. monocytogenes* (Roberts and Wiedmann 2003; Sauders and Wiedmann 2007). Although most studies on the presence of *L. monocytogenes* in different environments have focused on food-associated and farm environments (Fenlon et al. 1995; Nightingale et al. 2004), several reports state that *L. monocytogenes* are also common in natural and other non-agricultural environments, and can survive for extended time periods in soil and water (Oliver et al. 2007). However, studies on the occurrence of *Listeria* in natural environments not associated with farms indicated that the prevalence of *L. monocytogenes* is lower than that of other *Listeria* spp. (MacGowan et al. 1994). Considering the high prevalence and densities for *L. monocytogenes* on ruminant farms (Nightingale et al. 2004), such environments represent hotspots of introduction for *L. monocytogenes* into the human food chain, through a variety of pathways: (1) use of contaminated manure for fertilization of human food crops, (2) consumption of animal products lacking an effective heat treatment (e.g., raw milk), and (3) transmission of the organism via fomites into food processing environments, where

L. monocytogenes may subsequently persist for extended time periods, thus enabling recontamination of processed foods (Oliver et al. 2007). In addition to ruminant species, *L. monocytogenes* also can be isolated from various non-ruminant species and different non-ruminant agricultural environments. For instance, *L. monocytogenes* has been isolated from the feces of wild birds (Fenlon 1985), horses (Weber et al. 1995b), swine (Yokoyama et al. 2005), poultry (Weber et al. 1995a), and other domestic animals (Weber et al. 1995b), as well as from eviscerated farmed fish (Miettinen and Wirtanen 2005). While *L. monocytogenes* in ruminants and on ruminant farms are more likely to contribute directly to human disease (e.g., through consumption of raw milk), the presence of *L. monocytogenes* in other food animals is more likely to contribute indirectly to food contamination and human disease, e.g., by facilitating introduction of this pathogen into food processing plants, or onto vegetables through contaminated manure (Fenlon et al. 1996; Rorvik et al. 2003).

Amoeba-resistant bacteria

In *L. monocytogenes*, the genes necessary for invasion into host cells, escape from the phagosome into the cytosol, and spread among host cells are harboured on a single pathogenicity island (PA1) (Roberts and Wiedmann 2003). Considering that *L. monocytogenes* has no recognized host reservoir, the question arises why an opportunistic pathogen such as *L. monocytogenes* maintains the genes needed for an elaborate infectious process, unless it requires these genes to survive in the environment, or a yet unknown host reservoir (Oliver et al. 2007). In this context, protozoa may provide such a host reservoir in environmental niches where *L. monocytogenes* might be able to draw its trumps as an intracellular pathogen, because bacteria-scavenging amoebae do resemble macrophages in many aspects (Ly and Muller 1990).

The term "amoeba-resistant bacteria" (ARB) was first coined in 2004, to define a group of bacteria evolved to become resistant to protists, since they are able to survive, grow, and exit free-living amoebae following to internalization (Greub and Raoult 2004). These amoeba-resistant microorganisms include established pathogens such as *Chlamydophila pneumoniae*, *Coxiella burnetii*, *Cryptococcus neoformans*, *Francisella tularensis*, *Legionella* spp., *Mycobacterium* spp., *Pseudomonas aeruginosa*, and *Vibrio cholerae* (Holden et al. 1984; Essig et al. 1997; La Scola and Raoult 2001; Steenbergen et al. 2001; Abd et al. 2003; Abd et al. 2007; Hagedorn et al. 2009; Sandstrom et al. 2010). Some members of the ARB group represent nonpathogenic species such as *Bradyrhizobium japonicum* or bacteria without a known association to eukaryotic cell, such as *Burkholderia cepacia* and *Pseudomonas aeruginosa*. However, most ARB members represent obligate or facultative intracellular bacteria which are long-known human pathogens (e.g., *Chlamydophila pneumoniae*, *Francisella tularensis*, *Vibrio cholerae*) or emerging pathogens (e.g., *Bosea* spp., *Simkania negevensis*, *Parachlamydia acanthamoebae*). For the latter, it was shown that they are also able to survive and multiply within macrophages, pneumocytes, and lung fibroblasts. Sero-epidemiological studies and application of molecular techniques to clinical specimens have revealed that these agents are rare causes of community- and health care-associated pneumonia, particularly among immunocompromised patients (Janda 2010). The *Legionella*-like amoebal pathogens (LLAP) were named according to their cytopathogenicity and represent the paradigm of bacteria able to lyse amoebae (Birtles et al. 1996), while others (such as *Parachlamydia acanthamoeba*) were considered endosymbionts, because a stable host/parasite ratio was maintained (Amann et al. 1997; Wagner et al. 2006). Interestingly, amoebae do not only serve as reservoirs for pathogenic bacteria in natural environments. Exposure of intracellular pathogens such as *Legionella pneumophila* to predatory protozoa has often been considered as training grounds for their intracellular survival and proliferation skills (Molmeret et al. 2005). Consequently, amoebae may act as "Trojan horses" and aid dissemination of bacterial pathogens (Brown and Barker 1999; Molmeret et al. 2005).

Interaction of *L. monocytogenes* with protozoa

Listeria monocytogenes was also mentioned as a member of the ARB group (Greub and Raoult 2004), based on the earlier observation that *L. monocytogenes* survives phagocytosis by *Acanthamoeba* (Ly and Muller 1990). The authors reported that *L. monocytogenes* survive within *Acanthamoeba* spp. and multiply, at least in *Tetrahymena pyriformis*. Co-cultures of both protozoa with *L. monocytogenes* revealed an initial decrease in numbers of bacteria, followed by resurgence to high numbers. In the case of *T. pyriformis*, the viable counts for *L. monocytogenes* reached a maximum of 10^8 cfu ml^{-1} after approximately 10 days of co-culture, thus exceeding the initial inoculum size. After 15 days *T. pyriformis* cells lysed by releasing viable *L. monocytogenes* and within 3–5 weeks the population of *T. pyriformis* broke down completely. Thus, *T. pyriformis* cells did not survive and intracellular *L. monocytogenes* also died. In co-cultures of *Acanthamoeba* and *L. monocytogenes*, the bacteria survived and after 8 days when the *Acanthamoeba* trophozoites started to encyst, some of the cells ruptured and released viable *L. monocytogenes*. After approximately 1 month, almost all *Acanthamoeba* cells were encysted and the up to then viable *L. monocytogenes* died after formation of cysts.

The former hypothesis that *L. monocytogenes* can survive predation by *Acanthamoeba castellanii* was further addressed

by Zhou and coworkers (2007). They selected LLO as target of interest because the escape from the phagosome within *Acanthamoeba* spp. may represent an important prelude to survival and replication within the cytosol. In co-culture assays of *Acanthamoeba castellanii* with *L. monocytogenes* they confirmed that the bacteria survive predation by *A. castellanii* at least for 72 h, as it was previously indicated by Ly and Muller (1990). They could also show that the presence of *L. monocytogenes* had no effect on the growth of *A. castellanii* (Zhou et al. 2007). However, the authors found no evidence for replication of *L. monocytogenes* over the period of 96 h covered in their studies. Longer time periods have been not assessed because *A. castellanii* did not remain adherent much longer than 72 h, under the conditions used in their study. Concerning the postulated role of LLO it turned out that a *hly* mutant of *L. monocytogenes* was compromised in its ability to survive predation by *A. castellanii*. However, the complemented strain did not fully recover its ability to survive predation and there was no significant difference in the ability to survive for non-pathogenic *Listeria*, lacking the *hly* gene. Consequently, there appears to be no evidence for a contribution of LLO to the survival of the bacteria after predation by free-living amoebae. This assumption is consistent with results from studies using *Salmonella* showing that its virulence factors are not required for invasion and replication within amoebae (Tezcan-Merdol et al. 2004). A more recent study explored the potential of Listeriolysin O to promote interactions between *L. monocytogenes* and the ciliate *T. pyriformis* as a free-living, bacteriovorous and ubiquitous inhabitant of natural ecosystems (Pushkareva and Ermolaeva 2010). For this purpose, co-cultures of *L. monocytogenes* and *T. pyriformis* have been investigated by microscopy, and phagocytosis was observed as soon as 15 min after mixing the microorganisms. After 1 h multiple vacuoles inside *T. pyriformis* cells harboured 5–15 bacteria each and another 3 h later undamaged *L. monocytogenes* cells were visible within the vacuoles, some of them showing division. At later stages of co-culture, *T. pyriformis* cysts together with trophozoites were present and after 14 days only cysts and cell remnants were observed. The growth of *T. pyriformis* was significantly impaired by the presence of *L. monocytogenes*, and encystment was accelerated. The authors demonstrated that LLO is responsible for the toxicity to *T. pyriformis* and induction of cyst formation by showing that the cytotoxic effect and encystment was diminished in a LLO deletion mutant of *L. monocytogenes* EGDe. In contrast, an LLO-expressing *L. innocua* strain induced mortality and encystment comparable to wild type *L. monocytogenes*. Most interestingly, *L. monocytogenes* cells entrapped in cysts remained viable and virulent as demonstrated by oral infection of guinea pigs with *T. pyriformis* cysts that contained *L. monocytogenes* cells entrapped within the cysts (Pushkareva and Ermolaeva 2010). Survival of human pathogens inside cysts of protozoa

had already been described for *Legionella pneumophila*, *Mycobacterium* spp. and *Vibrio cholerae* (Kilvington and Price 1990; Steinert et al. 1998; Abd et al. 2007). However, active stimulation of protozoan encystment by bacteria was so far only demonstrated for *L. monocytogenes* (Ly and Muller 1990; Pushkareva and Ermolaeva 2010), suggesting that *L. monocytogenes* take advantage of the ability of cysts to serve as vehicles and dormant stages for spreading in the environment. Another interesting phenomenon observed in co-cultures of *Tetrahymena* spp. with *L. monocytogenes* was the production and release of vesicles containing intact cells of the pathogen (Brandl et al. 2005). However, in comparison to co-cultures of *Tetrahymena* spp. with *Salmonella enterica* serovar Thompson, which resulted in the release of large numbers of vesicles containing intact *Salmonella* cells, grazing on *L. monocytogenes* cells primarily resulted in their digestion, and thus in an infrequent release of this pathogen in vesicles. The observation of released vesicles from the interaction of *Tetrahymena* spp. with *Salmonella enterica* was further confirmed by another study showing that vesicles containing *S. enterica* were also produced on wet leaf surfaces (Gourabathini et al. 2008). In this study, significant differences in interactions among various protist–enteric pathogen combinations were observed. Similar to *S. enterica*, vesicle production was observed during grazing of *Tetrahymena* on *Escherichia coli* O157:H7, but not during grazing on *L. monocytogenes*. Another protist, *Glaucoma* sp., showed production of vesicles with all tested bacterial species, although *L. monocytogenes* resulted in the smallest number per ciliate. This observation was further corroborated by the fact that replication of *L. monocytogenes* in the cytoplasm was not apparent under the confocal laser-scanning microscope (CLSM), whereas *E. coli* O157:H7 harboured by vesicles was shown to be able to grow and escape from the vesicles. In contrast to *Tetrahymena* sp. and *Glaucoma* sp., *Acanthamoeba palestinensis* did not produce vesicles from any of the enteric pathogens investigated, nor were cells of the pathogens trapped within their cysts, thus confirming the results of previous studies (Ly and Muller 1990; Pushkareva and Ermolaeva 2010).

Survival and persistence of *L. monocytogenes* within *Acanthamoeba* spp.

The results from the above mentioned studies suggest that *L. monocytogenes* might at least survive, but could also be able to multiply within amoebae, as known for other bacterial pathogens (Brown and Barker 1999; Marciano-Cabral and Cabral 2003). However, these findings remained controversial. Zhou and coworkers reported that *L. monocytogenes* could survive predation by *A. castellanii* as indicated by previous work (Ly

and Muller 1990), but they found no evidence for intracellular replication of the pathogen in *A. castellanii*, and observed no killing of the amoebae (Zhou et al. 2007). Gentamicin protection assays suggested that *L. monocytogenes* can survive within amoebae cells for at least 72 h. It seemed that LLO is not necessary to survive predation by *A. castellanii*, because there was no significant difference in the ability to survive predation between different serovars and species of *Listeria*, three of them not harbouring the *hly* gene responsible for Listeriolysin O production (Zhou et al. 2007). The conclusion that *L. monocytogenes* is in fact unable to persist and replicate in *A. castellanii* or *A. polyphaga* was also confirmed by others (Huws et al. 2008; Akya et al. 2009a,b, 2010; Doyscher et al. 2013). Huws and coworkers investigated the interactions of *Bacillus cereus*, *Enterococcus faecalis*, enteropathogenic *E. coli* (EPEC), *L. monocytogenes*, *Salmonella enterica* serovar Typhimurium, and methicillin-sensitive *Staphylococcus aureus* (MSSA), with *Acanthamoeba polyphaga*. There was clear evidence of predation of all bacterial species, except for *L. monocytogenes* and *S. aureus*. Whereas the results from intracellular growth kinetic experiments and fluorescent confocal microscopy suggested that *S. aureus* is able to survive and may even multiply within *A. polyphaga*, there was no apparent evidence for intra-amoebal survival or replication of *L. monocytogenes* (Huws et al. 2008). This finding was further supported by the work of Akya et al. (2009b), who examined the viability of *L. monocytogenes* in co-cultures with different, freshly isolated *Acanthamoeba*. Co-culture assays of *L. monocytogenes* with *A. polyphaga*, *A. castellanii* and *A. lenticulata* revealed that under the experimental conditions used in their study, all amoebae were able to eliminate *L. monocytogenes*, irrespective of the *hly* gene. The mode of phagocytosis and killing of *L. monocytogenes* by amoeba trophozoites was further assessed and suggested that actin polymerisation and cytoskeletal rearrangement were involved in phagocytosis of *L. monocytogenes* cells by *A. polyphaga* trophozoites. Whereas phagosomal acidification and phagosome–lysosome fusion have been shown to be involved in killing and degradation of the *Listeria* cells, the mannose-binding protein receptor seemed not to play an important role in uptake of the bacteria by *A. polyphaga* trophozoites (Akya et al. 2009a). In a later study, short-term co-culture at 15 °C, 22 °C and 37 °C was performed to assess the fate of *L. monocytogenes* cells phagocytosed by monolayers of *Acanthamoeba* trophozoites by culture techniques and microscopy (Akya et al. 2010). Again it turned out that *A. polyphaga* trophozoites eliminated the bacteria within a few hours post-phagocytosis, irrespective of the incubation temperature used. Both, wild-type *L. monocytogenes* and a phenotypic LLO mutant were unable to either survive or multiply within *A. polyphaga* (Akya et al. 2010). Killing and degradation of *L. monocytogenes* by *Acanthamoeba* trophozoites was also obvious from a recent study by Doyscher and coworkers

(2013). Intracellular growth kinetic experiments and fluorescent confocal laser-scanning microscopy were performed to study the interaction of *L. monocytogenes* and *Acanthamoeba* spp. in co-culture. The results clearly demonstrated that phagocytosed *Listeria* cells are killed and digested in food vacuoles of *Acanthamoeba* trophozoites, suggesting that *L. monocytogenes* is incapable to infect *A. castellanii* Neff or *A. polyphaga*, and to multiply or at least survive inside *Acanthamoeba* cells (Doyscher et al. 2013). The reported period of 3–6 h for killing and digestion of *Listeria* cells correlated well with the period previously observed for elimination of intra-amoebic *L. monocytogenes* in co-cultures with *A. polyphaga* (Akya et al. 2010). In conclusion, the present data provide strong evidence for killing and digestion of *L. monocytogenes* by *Acanthamoeba* trophozoites. Thus, it seems unlikely that *Acanthamoeba* spp. harbour *L. monocytogenes*, or act as an environmental reservoir for this bacterium.

L. monocytogenes form aggregates on *Acanthamoeba* cells

When Doyscher and coworkers (2013) investigated co-cultures of *L. monocytogenes* and *Acanthamoeba* spp. using confocal laser-scanning microscopy, they observed a peculiar type of interaction. It turned out that *L. monocytogenes* cells rapidly approached *Acanthamoeba* trophozoites and assembled into large aggregates of densely packed bacteria on the surface of the *Acanthamoeba* cells (Fig. 1a). The bacterial aggregates were carried along on the back of the *Acanthamoeba* cells for a time period of 10–30 min. With reference to transient storage of the bacterial cells on the posterior pole of the trophozoites as a food source, the authors designated the aggregates as backpacks. Eventually, the trophozoites changed their direction of movement and phagocytized the assembled backpacks, and the process was repeated. Once ingested by the *Acanthamoeba*, the bacteria underwent rapid digestion, and neither an escape of *L. monocytogenes* cells from the trophozoites nor survival of bacteria within cysts could be observed (Doyscher et al. 2013).

Further investigation of this phenomenon revealed that the assembly of backpacks was clearly dependent on bacterial motility. However, flagellation alone was not sufficient to initiate backpack formation, as demonstrated by co-cultures of *Acanthamoeba* spp. with a *motB* mutant of *L. monocytogenes* carrying structurally intact but non-rotating flagella. Further experiments revealed that formation of backpacks was not restricted to *L. monocytogenes* and independent of bacterial pathogenicity or virulence. As bacterial motility is known to represent a pre-ingestional adaptation strategy in order to circumvent grazing by protozoa (Matz and Jurgens 2005; Matz and Kjelleberg 2005), backpacking might
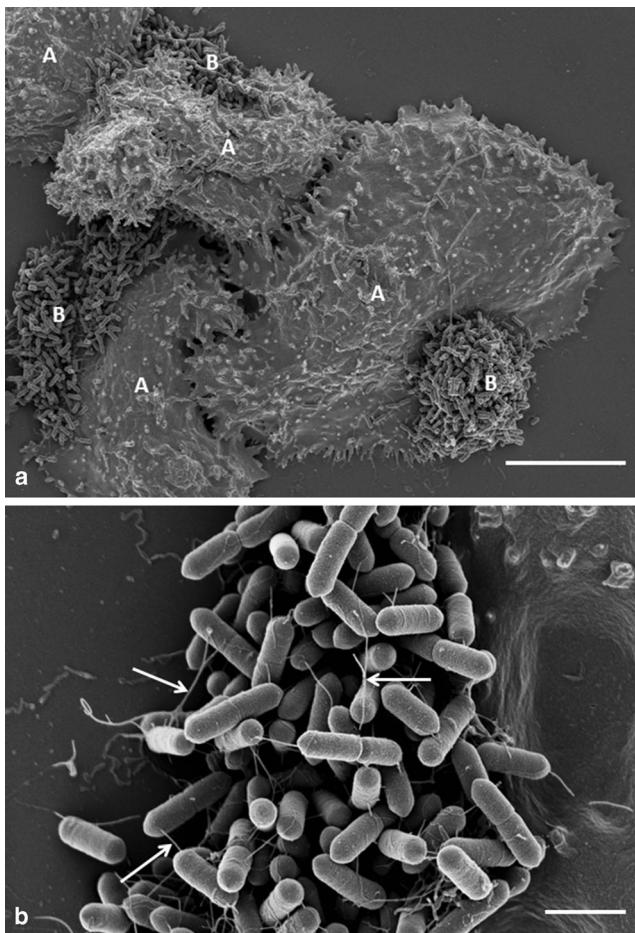
Fig. 1 Scanning electron microscopy images of *Listeria monocytogenes* aggregates on *Acanthamoeba castellanii* cells. **a** Ultrastructure of *Listeria* backpacks (*B*) formed on the surface of *Acanthamoeba castellanii* trophozoites (*A*). **b** Magnification of backpacks on *A.castellanii* demonstrates non-flagella filaments (*white arrows*) of amoebal origin in direct contact with the bacteria. Scale bars=10 μm (**a**) and 1 μm (**b**)

represent a unique and highly effective strategy of *Acanthamoeba* to counteract bacterial motility as a means to escape grazing, which evolved during long-term coexistence of the predator with their prey.

Inspection of the backpacks formed on *Acanthamoeba* trophozoites by scanning electron microscopy revealed a network of thin filaments extending throughout and around the bacterial backpacks forming a mesh-like structure (Fig. 1b). Although it was not possible to definitely clarify whether the filaments emerge from the bacteria or the amoebae, these findings suggested that the filaments might be involved in attaching the bacteria to the surface of the *Acanthamoeba* trophozoites, and holding them in place until phagocytosis occurs (Doyscher et al. 2013). Similar structures have been described in the context of attachment and entry of *L. pneumophila* into *Hartmannella vermiformis*, where single bacteria were frequently observed attached to the end of small amoebal pseudopods termed filopodia (Fields et al. 1993). For HeLa cells, it was reported that *Shigella* is captured by

nanometre-thin micropodial extensions at a distance from the cellular surface prior to invasion (Romero et al. 2011). In macrophages, such filopodia are known to act as phagocytic tentacles (Kress et al. 2007). Consequently, a similar function might also be proposed for the filaments observed in the formation of *Listeria* backpacks on *Acanthamoeba*.

Acanthamoeba spp. promote extracellular growth of *L. monocytogenes*

Besides the observation of backpack formation another important finding during the investigation of co-cultures of *L. monocytogenes* and *Acanthamoeba* spp. was that extracellular bacterial counts were significantly higher in the case of co-incubation with *Acanthamoeba* in non-nutrient PAS buffer, than in the absence of amoebae. Taking into account that *L. monocytogenes* is unable to grow in PAS alone, and that the *Acanthamoeba* trophozoites graze on the bacteria, this was a surprising observation (Doyscher et al. 2013; Fieseler et al. 2014). Experiments using transwell inserts in PAS co-culture assays in order to physically separate the bacteria and the amoebae demonstrated that direct contact is not necessary for promoting growth of *Listeria* by *A. castellanii*. These results suggested that extracellular *L. monocytogenes* may be able to metabolize secreted, but so far unknown substances of *A. castellanii*. This finding is consistent with observations from an earlier study by Zhou and coworkers (Zhou et al. 2007). They investigated the survival of various species of *Listeria* as well as many serovars of *L. monocytogenes* in co-culture with *A. castellanii*, and found that the presence of *Acanthamoeba* caused numbers of *L. monocytogenes* to be higher than in the absence of amoebae. It was concluded that the bacteria were scavenging naturally dead amoebae or that metabolic waste products were sustaining the growth of the bacteria over the duration of co-culture, because they found no evidence for intracellular replication of *L. monocytogenes* (Zhou et al. 2007). Similar observations were made by Huws and coworkers (2008). They observed significantly higher extracellular numbers for *L. monocytogenes* in co-culture with *Acanthamoeba* compared with growth in the absence of amoebae. Their results from intracellular growth kinetic experiments and fluorescent confocal microscopy suggested that the higher extracellular numbers of *L. monocytogenes* in the presence of *A. polyphaga* were likely sustained on metabolic waste products released during co-culture and not due to survival or replication of *L. monocytogenes* within *A. polyphaga* (Huws et al. 2008). This hypothesis was further corroborated by Akya and coworkers who reported saprophytic growth of extra-amoebic *L. monocytogenes* cells on material released from amoebae (Akya et al. 2009b).

However, the observed ability of *Acanthamoeba* spp. to support growth of bacteria is not specific for *Listeria*, since

also other bacterial species such as *B. thermosphacta*, *C. sakazakii*, *E. coli*, and *S. aureus* were also reported to be able to utilize metabolites secreted by amoebae (Fieseler et al. 2014). Whether promoting bacterial growth by *Acanthamoeba* is exclusively due to an accidental ability of certain bacteria to dispose amoebal waste products, or represents a symbiotic strategy of *Acanthamoeba* to cultivate alimental bacteria remains to be clarified. However, the observed phenomenon is evocative to the behavior of the social amoeba *Dictyostelium discoideum*, showing a primitive farming symbiosis that includes dispersal and prudent harvesting of the crop (Brock et al. 2011). About one-third of wild-collected clones engage in husbandry of bacteria. Instead of consuming all bacteria in their patch, they stop feeding early and incorporate bacteria into their fruiting bodies. They then carry bacteria during spore dispersal and can seed a new food crop, which is a major advantage if edible bacteria are lacking at the new site.

Potential impact of the interaction of *L. monocytogenes* and protozoa on food safety

For the interaction of *L. monocytogenes* with representatives of *Tetrahymena* spp., it seems clear that the bacteria are able to survive phagocytosis by the ciliate. Later, *L. monocytogenes* cells were released from the protozoan cell, e.g., due to production and release of vesicles containing intact cells of *L. monocytogenes* by *Tetrahymena* spp. in co-culture with the pathogen (Brandl et al. 2005). Furthermore, *L. monocytogenes* cells entrapped in *Tetrahymena* cysts may remain viable and virulent within this protective structure (Pushkareva and Ermolaeva 2010). The fact that *L. monocytogenes* can take advantage of the ability of cysts to serve as vehicles and dormant stages for spreading in the environment suggests that *Tetrahymena* spp. may play a role for the ecology of *L. monocytogenes* in natural environments.

Concerning the interaction of *L. monocytogenes* with *Acanthamoeba* spp., the situation is less clear. Available data demonstrate that *L. monocytogenes* cannot survive phagocytosis by *Acanthamoeba* spp. Paradoxically, although *Acanthamoeba* trophozoites prey on *L. monocytogenes*, which results in phagocytosis and digestion of the bacteria by their predators, the pathogen seems to benefit from the presence of *Acanthamoeba* trophozoites (Fieseler et al. 2014). The observation that *Acanthamoeba* promotes growth of *L. monocytogenes* suggests that the predators may represent an important factor for the survival of the bacteria under adverse environmental conditions, with potentially major implications for food safety and human health because coexistence of *L. monocytogenes* and *Acanthamoeba* spp. is known to take place in various food-related environments.

Many food items from environmental sources such as leafy vegetables and other produce undergo only a washing step and no further processing that would help to eliminate or inactivate harmful microorganisms. In particular, the consumption of minimally processed ready-to-use fruits or vegetables and ready-to-eat salads has strongly increased over the last decade. Such products are known to frequently harbour bacterial pathogens such as *L. monocytogenes*. The investigation of fresh leafy vegetables, salads and ready-to-eat products revealed contamination with *L. monocytogenes* in seven out of 62 tomato samples, five out of ten coriander leave samples, two out of four spinach samples, and one from four cabbage samples (Pingulkar et al. 2001). The same food items are also known to carry amoebae. Consequently, amoebae have been recovered from cucumbers, cabbage, lettuce, celery, carrots, radishes, tomatoes, mushrooms, cauliflower, and spinach. *Acanthamoeba polyphaga*, *A. rhysodes*, and *A. castellanii* were the most common species recovered in this study (Rude et al. 1984). Other surveys reported *Acanthamoeba palestinensis*, *Glaucoma* sp., and *Colpoda steinii*, cultured from store-bought spinach and lettuce (Gourabathini et al. 2008) as well as other amoeba species belonging mainly to the *Vannellida* and *Tubulinida* together with *Glaucoma* sp. and *Colpoda steinii* on butterhead lettuce (Vaerewijck et al. 2011). The above mentioned surveys demonstrated that free-living protozoa on leafy greens and other vegetables are common and diverse. Thus, there is a high probability that such products are contaminated by *Acanthamoeba* spp. and *Listeria* in parallel. This situation would provide conditions where *Acanthamoeba* spp. may promote the multiplication of *L. monocytogenes* on the respective products. Beside natural ecosystems, industrial food related ecosystems exist where the presence of amoebae might have a synergistic effect on the occurrence, survival and transmission of *Listeria*, such as food processing facilities, slaughter houses, or meet-cutting plants. *L. monocytogenes* can enter processing facilities via livestock, and since microorganisms washed from surfaces end up in a drain, it is not surprising that this pathogen is frequently found in floor drains (Berrang et al. 2010). Once present in a processing facility, *L. monocytogenes* can adhere to a wide variety of surfaces (Kushwaha and Muriana 2009). A survey on the diversity of free-living protozoa in meat-cutting plants showed that there is high protozoan species richness in meat-cutting plants, including species related to known hosts of food-borne pathogens. As an important result the authors pointed out that a protozoan community consisting of amoebae, ciliates, and flagellates was present in all of the investigated plants (Vaerewijck et al. 2008). Protozoa were detected mainly in floor drains, in standing water on the floor, on soiled bars of cutting tables, on plastic pallets, and in out-of-use hot water knife sanitizers, but they were also detected on surfaces which come into direct contact with meat, such as conveyer belts, working surfaces of cutting tables, and needles of a meat tenderizer. Identification of the isolates revealed a wide variety of protozoa with representatives of *Amoebozoa*,

Chromalveolata, *Excavata*, *Opisthokonta*, and *Rhizaria* (Vaerewijck et al. 2008). Finally, household scenarios are also conceivable where *Acanthamoeba* spp. may have the potential to promote growth of *Listeria* which in turn might affect food safety at the end of the farm to fork chain. A study on the occurrence and diversity of free-living protozoa in domestic refrigerators revealed a high protozoan diversity. In particular discharge gutters turned out to be occasionally contaminated with a persistent population of flagellates and amoebae (Vaerewijck et al. 2010).

In conclusion, the results available demonstrate that *Acanthamoeba* spp. do not seem to directly act as environmental reservoirs for *L. monocytogenes*. However, the *Listeria* promoting effect of amoebae might play an important role when sharing environmental habitats but also under food storage conditions in households, which is in particular relevant due to the psychrotolerant character of *Listeria*. Thus, the interaction of *L. monocytogenes* with *Acanthamoeba* or *Tetrahymena* in natural and man-made ecosystems may have significant implications for food safety and public health.

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