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REVIEW

# Microbial warfare in the wild—the impact of protists on the evolution and virulence of bacterial pathogens

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## Abstract

During the long history of co-evolution with protists, bacteria have evolved defense strategies to avoid grazing and survive phagocytosis. These mechanisms allow bacteria to exploit phagocytic cells as a protective niche in which to escape from environmental stress and even replicate. Importantly, these anti-grazing mechanisms can function as virulence factors when bacteria infect humans. Here, we discuss how protozoan predation exerts a selective pressure driving bacterial virulence and shaping their genomes, and how bacteria-protist interactions might contribute to the spread of antibiotic resistance as well. We provide examples to demonstrate that besides being voracious bacterial predators, protozoa can serve as melting pots where intracellular organisms exchange genetic information, or even "training grounds" where some pathogens become hypervirulent after passing through. In this special issue, we would like to emphasize the tremendous impact of bacteria-protist interactions on human health and the potential of amoebae as model systems to study biology and evolution of a variety of pathogens. Besides, a better understanding of bacteria-protist relationships will help us expand our current understanding of bacterial virulence and, likely, how pathogens emerge.

**Keywords** Grazing resistance · Virulence · Protozoan predation · Amoebae · Ciliates · Interactions

## Introduction

Most opportunistic pathogens transit in the environment for significant periods of time instead of being directly transmitted between humans. In fact, for many pathogens (e.g., *Legionella pneumophila*, *Coxiella burnetti*, *Pseudomonas aeruginosa*, *Burkholderia cenocepacia*) humans represent accidental hosts or even evolutionary dead-ends (Levin 1996; Matz et al. 2008; Winstanley et al. 2016) which makes unlikely that virulence emerged from human selection. Therefore, it has been proposed that virulence traits might have evolved for increased environmental fitness rather for virulence *per se* (Brown et al. 2012; Martínez 2013; Erken et al. 2013). Indeed, genes encoding virulence factors (e.g., protein secretion systems, toxins) are also found in the genomes of non-pathogenic bacteria as well (Pallen and Wren 2007; Persson et al. 2009).

The environmental persistence of pathogens depends on their ability to adapt to different ecological niches and stress conditions. Among these, consumption by heterotrophic protists is a major cause of bacterial mortality in soil or aquatic environments, and even in man-made systems (Sherr and Sherr 2002; Menon et al. 2003; Pernthaler 2005; Rosenberg et al. 2009; Jousset 2012; Zhang et al. 2014). As a result, bacteria have evolved anti-predator strategies such as morphological changes, increased motility, biofilm formation, production of toxic metabolites, and resistance to lysosomal digestion (Matz and Kjelleberg 2005; Zhang et al. 2014). Anti-protozoan defenses can even involve cooperative action, such as the mobbing behavior recently reported in *P. aeruginosa* towards the amoeba *Acanthamoeba castellanii* (Shteindel and Gerchman 2020). Importantly, some of these anti-grazing mechanisms may act as virulence factors when the pathogen (incidentally) infects the human host. Hence, bacteria that have evolved strategies to defeat microbicidal mechanisms of protozoa will be better equipped (and virulent) when they encounter human immune cells. According to this hypothesis, virulence may be a coincidental by-product of grazing resistance mechanisms (Adiba et al. 2010). This is supported by the

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fact that bacterial pathogens frequently exploit conserved processes in both macrophages and protists (Fig. 1) (Segal and Shuman 1999; Pukatzki et al. 2002). Indeed, several studies have shown that deletion of virulence factors in bacteria impairs intracellular survival and growth within both amoebae and mammalian macrophages (Gao et al. 1997; Segal and Shuman 1999; Danelishvili et al. 2007; O'Connor et al. 2011; German et al. 2013; Isaac et al. 2015; Sun et al. 2018; Butler et al. 2020).

In this review, we discuss how protists exert a selective pressure for acquisition and development of virulence traits in bacteria, and how bacteria-protist relationships might contribute to the spread of antibiotic resistance as well. Protists constitute a paraphyletic and exceptionally diverse group of eukaryotic microorganisms (Adl et al. 2019). We will focus on heterotrophic protists—those traditionally known as protozoa—a group that includes amoebae, ciliates, and flagellates. Besides being major consumers of bacteria and fungi, protozoa have explored all sort of ecological interactions with bacteria from mutualism to intracellular parasitism and phoresy, whether persistent or temporary, specific, or promiscuous (Shi et al. 2021). Thus, the protozoa-bacteria system provides an excellent model to study how intracellular pathogens and endosymbionts evolve. In addition, protists are not only ubiquitous in the environment, but are also part of the gut microbiota in many animals (Wildschutte et al. 2004; Chabé et al. 2017). In fact, experimental evidences suggest that protozoan predation in the intestine of vertebrates might be a selective pressure maintaining O-antigen diversity in *Salmonella enterica* (Wildschutte and Lawrence 2007).

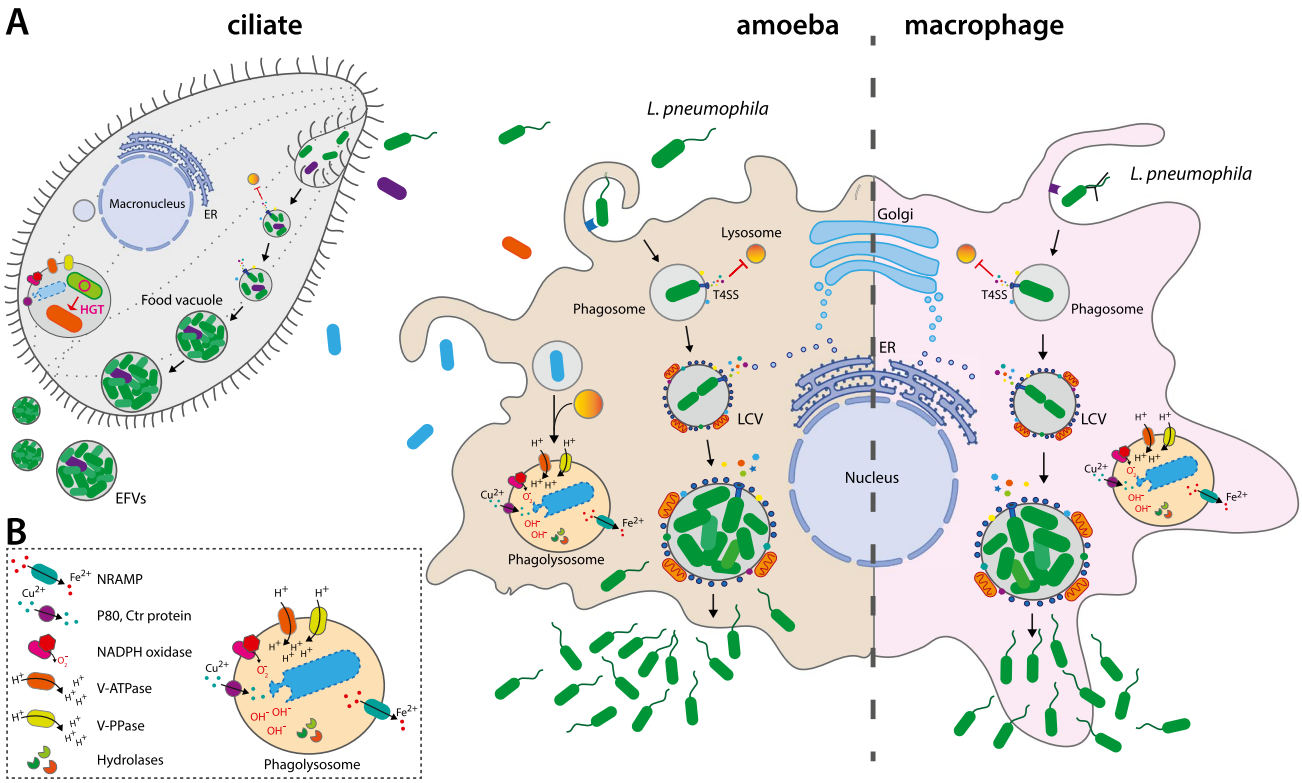


**Fig. 1** Macrophages represent a familiar niche for protozoa-resistant bacteria since the fundamental events of phagocytosis and microbicidal mechanisms are largely conserved with amoebae. **A** As an example, we summarize here the intracellular lifestyle of *L. pneumophila*, which hickjacks similar processes in both amoebae and macrophages by translocating effector proteins into the host cytoplasm via the Icm/Dot type IV secretion system (T4SS). Uptake of Legionella by amoebae and macrophages mainly occurs by coiling phagocytosis after bacterial attachment to the host cell surface. Upon entry, Legionella is enclosed in a phagosome that neither acidifies nor fuses with the lysosome. Instead, the bacterium remodels it into a replicative compartment called Legionella containing vacuole (LCV) that is decorated with recruited mitochondria, RER, and ER-to-Golgi complex-derived vesicles. After several rounds of replication, Legionella breaks out the LCV membrane into the cytosol before lysing the host cell. **B** Besides phagosomal acidification, both macrophages and protozoa challenge ingested bacteria with an oxidative burst, $Fe^{2+}$ and $Mn^{2+}$ depletion from the phagosome with efflux systems, and metal poisoning with $Cu^{+}$ and $Zn^{2+}$ pumps (P80 and Ctr, copper transport system)

## Protozoan predators: from Trojan horses to training grounds for intracellular pathogens

Co-evolution between bacteria and protozoa has occurred for billions of years (Strassmann and Shu 2017). The ancestry of their interactions is supported by data such the presence of genes acquired from amoebae in the genome of *Chlamydiales*, the most ancient group of obligate intracellular bacteria that include notorious intracellular pathogens and endosymbionts of protists (Taylor-Brown et al. 2015). Genomic analysis of *Chlamydiales* and their related amoeba endosymbiont *Protochlamydia amoebophila* UWE25 suggested that the bacterial type III secretion system (T3SS) evolved in the *Chlamydial* linage long before mammals appeared ca. 210 million years ago, most likely to modulate their interactions with protozoa (Horn et al. 2004). Similarly, it was proposed that amoebae may be the natural targets of some effector proteins secreted by *P. aeruginosa* T3SS (Matz et al. 2008). Protozoan predation is now recognized as a key force driving the evolution and ecology of many microbial pathogens, including bacteria and fungi (Wildschutte et al. 2004; O'Connor et al. 2011; German et al. 2013; Erken et al. 2013; Amaro et al. 2015; Laencina et al. 2018; Sun et al. 2018; Casadevall et al. 2019). This is perfectly illustrated by *Legionella* species, the paradigm of bacteria that coevolved with protozoa. It is established that the combined pressure of amoebae and ciliates has shaped the repertoire of effector proteins found in the genomes of *Legionellae*, representing 7–10% of the bacterial genome (Burstein et al. 2016; Gomez-Valero et al. 2019; Park et al. 2020). The so-called effectors are proteins translocated by dedicated secretion systems that subvert host cell processes for the benefit of bacteria.

Bacteria-protist interactions are now gaining attention due to the fact that protozoa serve as a protective niche in which many bacterial and fungal pathogens escape from environmental stresses or even replicate (Table 1) (Sun et al. 2018). Furthermore, many protozoa form dormant cysts that resist harsh conditions, hence protecting the encased bacteria from stress and biocides, and allowing them to go undetected by the standardized culture-dependent protocols (Lambrecht et al. 2015). Under favorable conditions, cysts turn into vegetative cells, releasing internalized bacteria, thus facilitating their persistence in the environment and water distribution systems (Lambrecht et al. 2015). Likewise, bacteria that resist lysosomal digestion in protozoa can be released into the environment freely after host cell lysis or packaged into expelled food vacuoles (EFVs) that serve as vectors for bacterial dissemination. Packaging into EFVs by ciliates or amoebae confers many survival advantages to bacteria such as resistance to different biocides, UV light, acid pH, starvation, or desiccation (Berk et al. 2008; Denoncourt et al. 2014; Espinoza-Vergara et al. 2019). Additionally, the diameter of EFVs (1–5 μm) falls within the range of respirable particles and could penetrate into human lung alveoli, suggesting that EFVs could help propagate pathogens through the air (Denoncourt et al. 2014). The conditions that favor production of packaged bacteria in natural and man-made environments are currently unknown. Although it is thought to be a protozoan-driven process, bacterial virulence factors seem to play an important role in the production of EFVs (Berk et al. 2008; Espinoza-Vergara et al. 2019), and further investigations are required to determine the molecular mechanisms and environmental conditions involved.

Remarkably, many pathogens appear to be more virulent after passing through protozoa (Rasmussen et al. 2005; Koubar et al. 2011; Espinoza-Vergara et al. 2019). For instance, amoeba-grown *L. pneumophila* cells displayed increased resistance to antibiotics, chlorine compounds, and other biocides, and were more infectious compared to in vitro grown bacteria (Cirillo et al. 1994; Chang et al. 2009; Personnic et al. 2021). Similarly, *Vibrio cholerae* cells packaged into EFVs by ciliates exhibited enhanced resistance to antibiotics and stressful conditions such as the acid pH and long-term starvation (Espinoza-Vergara et al. 2019). Importantly, Espinoza-Vergara and coworkers demonstrated that *V. cholerae* cells in EFVs were highly virulent and primed for infection, colonizing the mouse intestine 10 times more efficiently than free-living bacteria, illustrating the potential risks of EFVs. Noteworthy, it has been recently shown that passage through amoebae could revert virulence attenuation of the fungal pathogen *Paracoccidioides* (Albuquerque et al. 2019). Surviving protozoan digestion implies the ability to mount a rapid response to the variety of stresses encountered within the protozoan phagosome, namely acid pH, oxidative stress, starvation, oxygen depletion, and antimicrobial peptides (Koubar et al. 2011; Rehfuss et al. 2011; George et al. 2019). In line with this hypothesis, transcriptome analyses revealed that *S. enterica* exhibited a similar gene expression profile following ingestion by either *Tetrahymena* or human macrophages, conferring acid resistance to egested bacteria (Rehfuss et al. 2011). Therefore, it is thought that harsh conditions faced in the protozoan phagosome might pre-adapt the pathogen to similar stressful conditions that will be encountered in the human body (Espinoza-Vergara et al. 2020). Furthermore, conditions faced within the phagosome of amoebae and macrophages have been recently shown to induce a highly virulent persister state in *L. pneumophila* with an increased tolerance to antibiotics (Personnic et al. 2019) that might be the cause for relapsing forms of Legionnaires' disease.

**Table 1** Reported associations between selected bacterial pathogens and protozoa. Interactions with non-pathogenic bacteria were recently reviewed in Durocher et al. (2020) and Shi et al. (2021)

| Pathogen | Phenomenon | Protist | Reference |
|---|---|---|---|
| *Aeromonas hydrophila* | Intracellular survival | *T. thermophila* | (Pang et al. 2016) |
| *Bacillus anthracis* | Intracellular multiplication | *A. castellanii* | (Dey et al. 2012) |
| *Bordetella bronchiseptica* | Intracellular survival | *D. discoideum* | (Taylor-Mulneix et al. 2017) |
| *Burkholderia species* | Symbiosis, farming | *D. discoideum* | (Brock et al. 2018) |
| *B. cepacia complex (Bcc)* | Intracellular survival | *Acanthamoeba spp* | (Marolda et al. 1999) |
| *B. pseudomallei* | Intracellular survival | *Acanthamoeba spp* | (Inglis et al. 2000) |
| *Campylobacter jejuni* | Intracellular multiplication | *A. polyphaga, A. castellanii* | (Bui et al. 2012; Vieira et al. 2017) |
| | Packaging into EFVs | *T. thermophila* | (Trigui et al. 2016) |
| *Chlamydiae* | Endosymbiosis | Several amoebae | (Horn et al. 2004; König et al. 2019) |
| *Chlamydia pneumoniae* | Intracellular growth | *A. castellanii* | (Essig et al. 1997) |
| *Coxiella burnetii* | Intracellular survival | *A. castellanii* | (La Scola and Raoult 2001) |
| *Escherichia coli O157:H7* | Intracellular survival | *A. castellanii, T. thermophila* | (Barker et al. 1999; George et al. 2019) |
| | Packaging into EFVs | *Tetrahymena sp* | (Lainhart et al. 2009; Smith et al. 2012) |
| *Francisella tularensis* | Intracellular growth | *A. castellanii* | (Abd et al. 2003) |
| *Helicobacter pylori* | Packaging into EFVs | *A. astronyxis* | (Denoncourt et al. 2014) |
| *Listeria monocytogenes* | Intracellular survival | *A. castellanii* | (Lambrecht et al. 2015) |
| | Packaging into EFVs | *Colpoda sp* | (Raghu Nadhanan and Thomas 2014) |
| | Survival within cysts | *A. castellanii* | (Lambrecht et al. 2015) |
| *L. pneumophila and other Legionella species* | Intracellular survival and multiplication | Over 20 species: *N. gruberi, A. castellanii, V. vermiformis, D. discoideum, Tetrahymena* | (Rowbotham 1980; Fields 1996; Hägele et al. 2000; Berk et al. 2008; Boamah et al. 2017) |
| | Packaging into EFVs | *T. thermophila, T. tropicalis, A. axtronyxis* | (Berk et al. 2008; Denoncourt et al. 2014; Amaro et al. 2015) |
| | Increased resistance to stress, biocides, antibiotics | Several amoebae and ciliates | (Thomas et al. 2004; Chang et al. 2009; Koubar et al. 2011; Personnic et al. 2021) |
| | Increased virulence | *A. castellanii* | (Cirillo et al. 1994; Personnic et al. 2019) |
| | Resuscitation | *A. castellanii, A. polyphaga* | (Steinert et al. 1997; Personnic et al. 2019) |
| | Intracellular digestion | *S. palustris* | (Amaro et al. 2015) |
| *Mycobacterium avium* | Intracellular multiplication | *A. castellanii* | (Cirillo et al. 1997) |
| | Increased stress resistance and virulence | *A. castellanii* | (Cirillo et al. 1997; Salah et al. 2009) |
| *M. leprae* | Survival within cysts | *A. castellanii* | (Wheat et al. 2014) |
| *M. marinum* | Intracellular multiplication | *A. castellanii* | (Salah et al. 2009) |
| | Survival in cysts and enhanced stress resistance | *A. castellani, Tetrahymena* | (Salah et al. 2009) |
| *M. tuberculosis* | Intracellular survival | *A. castellanii, A. polyphaga* | (Mba Medie et al. 2011) |
| *M. ulcerans* | Intracellular survival and enhanced virulence | *A. polyphaga* | (Azumah et al. 2017) |
| *Pseudomonas aeruginosa* | Intracellular survival | *A. castellanii, A. polyphaga, D. discoideum* | (Pukatzki et al. 2002; Matz et al. 2008) |
| | Enhanced resistance to stress and biocides | *A. castellanii* | (Leong et al. 2020; Sarink et al. 2020) |
| | Mobbing | *A. castellanii* | (Shteindel and Gerchman 2020) |
| *Salmonella enterica* | Intracellular survival | *Tetrahymena, D.discoideum, A. castellanii* | (Wildschutte et al. 2004; Riquelme et al. 2016) |
| | Packaging into EFVs | *Tetrahymena sp* | (Brandl et al. 2005; Rehfuss et al. 2011) |
| | Enhanced resistance to stress and biocides | | |
| | Survival within cysts | *A. castellanii* | (Lambrecht et al. 2015) |
| | Enhanced virulence | *A. castellanii* | (Carlson et al. 2007) |
| *Staphylococcus aureus* MRSA | Intracellular survival | *A. polyphaga* | (Huws et al. 2006) |
| *Stenotrophomonas maltophila* | Intracellular multiplication | *V. vermiformis* | (Cateau et al. 2014) |
| *Vibrio cholerae* | Intracellular multiplication | *A. castellanii* | (Abd et al. 2005; Van Der Henst et al. 2016) |
| | Intracellular survival | *T. thermophila* | (Espinoza-Vergara et al. 2019) |
| | Enhanced stress resistance | | |
| | Increased virulence | | |
| *V. parahemolyticus* | Intracellular survival | *C. roenbergensis* | (Matz et al. 2011) |
| *Yersinia pestis* | Intracellular survival | *A. castellanii* | (Benavides-Montaño and Vadyvaloo 2017) |
| | | | |
| *Aspergillus fumigatus* | Intracellular survival | *A. castellanii* | (Van Waeyenberghe et al. 2013) |
| *Cryptococcus neoformans* | | *A. castellanii, D. discoideum* | (Steenbergen et al. 2001, 2003) |
| *Blastomyces dermatitidis* | Intracellular survival | *A. castellanii* | (Steenbergen et al. 2004) |
| *Histoplasma capsulatum* | Intracellular survival | *A. castellanii* | (Steenbergen et al. 2004) |
| *Paracoccidioides spp* | Intracellular survival and enhanced virulence | *A. castellanii* | (Albuquerque et al. 2019) |
| *Sporothrix schenkii* | Intracellular survival | *A. castellanii* | (Steenbergen et al. 2004) |
| | | | |
| *Adenovirus* | Intracellular survival | *A. castellanii* | (Scheid and Schwarzenberger 2012) |
| *Coxackie B3 virus* | Intracellular survival | *A. castellanii* | (Mattana et al. 2006) |

Collectively, the aforementioned facts have led to the concept of protozoa as evolutionary "training grounds" for bacterial virulence and "Trojan horses" of the microbial world (Molmeret et al. 2005), meaning that pathogens can escape from stress, develop, and "train" their virulence traits in environmental protozoa before facing human phagocytic cells. Yet, while experimental data demonstrated that coevolution with protists have shaped bacterial genomes and virulence traits (Wildschutte et al. 2004; Danelishvili et al. 2007; O'Connor et al. 2011; Park et al. 2020), it is thought that pathogens likely evolved their ability to subvert the "more sophisticated" immune defenses of metazoans that are not found in protists through adaptation to multicellular organisms (Best and Abu Kwaik 2019). In line with this hypothesis, recent findings indicate a role for metazoans in Legionella's ability to manipulate innate immunity processes absent in protists such NF-kB and Toll-like receptor (TLRs) signaling pathways or the inflammasome (Losick et al. 2010; Asrat et al. 2015; Mallama et al. 2017).

## The natural choice: protozoa as host models for intracellular pathogens

Research over the last decades has proved that amoebae and ciliates not only represent an important ecological niche for pathogenic bacteria and fungi, but are also excellent and cost-effective models to study host–pathogen interactions at the molecular and cellular level (Leoni Swart et al. 2018; Espinoza-Vergara et al. 2019). Amoebae share with macrophages similar mechanisms to phagocytize and kill bacteria in acidic phagolysosomes by hydrolases combined with reactive oxygen species (ROS) and copper ions (German et al. 2013; Hao et al. 2015; Espinoza-Vergara et al. 2020) (Fig. 1). The acidic pH (5–5.5) is maintained by vacuolar-type ATPases (V-ATPases) that translocate protons into the phagosome lumen. In addition to V-ATPases, recent research has shown that heterotrophic protists also possess vacuolar-type $H^+$-translocating pyrophosphatases (V-PPases) whose expression is upregulated during bacterivorous growth and likely contribute to phagolysosome acidification (Pérez-Castieira et al. 2002; Massana et al. 2021). ROS in the phagosome are generated by the NADPH oxidase complex and copper transporters (Ctr) which are present in macrophages and protists as well (Jacobs et al. 2006; German et al. 2013). Indeed, ROS production in the phagosome of *T. thermophila* during predation on *Escherichia coli* O157:H7 has been recently visualized and quantified (George et al. 2019). Consequently, deletion of copper and zinc resistance genes impaired survival of intracellular bacteria within protozoa (Hao et al. 2016). Additionally, phagosomes are depleted of essential metal nutrients for bacteria such as $Fe^{2+}$ and $Mn^{2+}$ by the action of the natural resistance-associated macrophage protein (NRAMP). This antimicrobial mechanism is thought to occur in the protozoan phagosome as well, since homologs of mammalian NRAMP1 were found in amoebae and ciliates (German et al. 2013). In fact, studies have shown that deletion of *Nramp1* genes rendered the amoeba *D. discoideum* more susceptible to infection by intracellular bacteria (Peracino et al. 2006, 2013; Brenz et al. 2017).

Remarkably, homologs of pattern recognition receptors (PRRs) and interferon-γ-inducible lysosomal thiol reductase (GILT), an enzyme used by *L. monocytogenes* during macrophage infection (Singh et al. 2008), have been recently identified in the genomes of amoebae *A. castellanii*, *Dictyostelium discoideum*, and *Willaertia magna* (Clarke et al. 2013; Peracino et al. 2013; Pan et al. 2018; Hasni et al. 2020), supporting the use of amoebae as model systems to study host–pathogen interactions. Importantly, *D. discoideum*, *A. castellanii*, and *T. thermophila* are molecularly amenable and genetically tractable protist hosts (Hägele et al. 2000; Karrer 2000; Solomon and Isberg 2000; Eisen et al. 2006; Leoni Swart et al. 2018) that have been used to screen bacterial genome libraries and antibacterial compounds (Kicka et al. 2014; Harrison et al. 2016; Kebbi-Beghdadi et al. 2019; Thewes et al. 2019; Park et al. 2020; Espinoza-Vergara et al. 2020). Bacterial virulence factors can be unraveled using amoebae as hosts in a simple amoeba plaque assay (Pukatzki et al. 2002; Leoni Swart et al. 2018). In addition, these protists have been successfully employed to discover novel intracellular bacteria and potentially pathogenic microorganisms (Pagnier et al. 2008; Tosetti et al. 2014). In fact, the amoebal co-culture is an established method for isolating intracellular bacteria from clinical and environmental samples (Corsaro and Venditti 2009; Lienard et al. 2017; Thewes et al. 2019). Lastly, although research has focused on host–pathogen interactions, the social amoebae *D. discoideum* is currently being used as model to understand microbiome formation and the interplay between host and its microbiota (Farinholt et al. 2019; Sallinger et al. 2021).

## Protists as hotspots for genetic exchanges

Amoebae have been proposed to serve as genetic *melting pots* where intra-amoebal microorganisms can exchange genes between themselves but also with the amoeba host (Moliner et al. 2010; Bertelli and Greub 2012; Gomez-Valero and Buchrieser 2013). Multiple lines of evidence support this hypothesis. For instance, the closest homolog of *L. pneumophila* ankyrin-containing protein Lpg2416 is

found in the *A. polyphaga* Mimivirus, a giant virus that infects *Acanthamoeba*, suggesting intra-amoebal horizontal gene transfer (HGT) between Legionella and Mimivirus (Lurie-Weinberger et al. 2010). Additionally, genes likely acquired from amoebae-related bacteria have also been identified in the Mimivirus genome (Moliner et al. 2010). Moreover, HGT between amoeba-associated bacteria have been evidenced by several studies. Genome analysis of *Rickettsia belli*, the earliest diverging species of *Rickettsiae*, revealed gene transfer events between the ancestors of *Rickettsia* and phylogenetically distant amoeba-resistant bacteria (ARB) such as *L. pneumophila* and *P. amoebophila* (Ogata et al. 2006; Gimenez et al. 2011; Wang and Wu 2017). Moreover, *L. pneumophila* phospholipases PlcA (*lpg0502*) and PlcB (*lpg1455*) have not been found in prokaryotic genomes except *P. aeruginosa* (which resist protozoan digestion) and other amoebae-associated bacteria, supporting intra-amoebal gene transfer (Gomez-Valero et al. 2019). Interestingly, although *Rickettsiales* and *Legionellales* represent excellent models supporting the amoeba melting pot hypothesis (Moliner et al. 2010; Gimenez et al. 2011; Wang and Wu 2017), HGT has also been reported for other bacterial species such as *Bartonella rattaustraliani* and *Rhizobium radiobacteri* within *A. polyphaga* vacuoles (Saisongkorh et al. 2010). Amoebae and ciliates are thought to favor genetic exchange by bringing resistant microorganisms in close proximity within the same protozoan cell or compartment. Research has evidenced that a single protist can harbor several phylogenetically different endosymbionts (Heinz et al. 2007; Matsuo et al. 2010a). Moreover, the indiscriminate feeding of ciliates makes likely that a mixture of different bacterial species are encased in a single food vacuole, which might favor gene transfer between packaged bacteria.

Importantly, amoebae and ciliates not only serve as a place for HGT between intracellular microorganisms, but can also participate in genetic exchanges themselves. Hundreds of genes exhibiting best homology match with viral genes have been found in the genomes of amoebae *Vermamoeba vermiformis* (188 genes), *W. magna* (50 genes), and different *Acanthamoeba* species (261 genes), supporting the hypothesis of amoeba-virus genetic transfer (Moreira and Brochier-Armanet 2008; Chelkha et al. 2018, 2020; Hasni et al. 2019). Likewise, genes believed to be acquired from bacteria have been also identified in the genome of *D. discoideum* (Eichinger et al. 2005), *Naegleria gruberi*, and *A. castellanii* (Clarke et al. 2013). The strongest evidences of eukaryote to prokaryote gene transfer are found in the genome of *Legionellales* (Burstein et al. 2016; Gomez-Valero et al. 2019). Remarkably, *Legionella* species contain a significant number of proteins with highest sequence homology to protozoan proteins, the so-called eukaryotic-like proteins (ELPs), which were likely acquired from protozoan hosts. Most genes encoding ELPs have G + C biases compared to other *Legionella* genes and cluster with eukaryotic proteins in phylogenies, supporting the protozoa-to-Legionella transfer hypothesis (Cazalet et al. 2004; De Felipe et al. 2005; Burstein et al. 2016; Gomez-Valero et al. 2019). ELPs contain domains found only in eukaryotes such as ankyrin repeats (ANK), leucine-rich repeats, F-box, or U-box domains, and are believed to allow bacterial survival by subverting host cellular processes in a phenomenon called molecular mimicry (Mondino et al. 2020). Importantly, comparative analysis of 514 prokaryotic proteomes revealed that ELPs are significantly enriched in the genomes of bacteria that replicate in protozoa (Schmitz-Esser et al. 2010), suggesting that phylogenetically distant bacteria that infect amoebae may exploit similar strategies to interact with their hosts: molecular mimicry and HGT (Gomez-Valero and Buchrieser 2019). The ability of some amoeba-resistant bacteria (e.g., *L. pneumophila*, *B. cenocepacia*) to develop natural competence for transformation (Stone and Abu Kwaik 1999) might have facilitated genetic exchanges within amoebae. However, how eukaryotic genes are taken up by intracellular bacteria remains to be investigated.

## Protozoa as key players in the dynamics of the community resistome

Until recently, the interactions between bacteria and protozoa have received little attention regarding the spread of antibiotic resistance. However, studies carried out over the last years point out that protozoan predation might play a key role on the dissemination of antibiotic resistance genes (ARGs) and the dynamics of the community resistome (Cairns et al. 2018a; Nguyen et al. 2020). Remarkably, work done by Cairns and collaborators clearly evidenced the relevance of protozoan predation for plasmid persistence in bacterial communities (Cairns et al. 2016, 2018b). Their experiments in laboratory microcosms revealed that ciliates foster bacterial conjugation frequency and prevent loss of resistance plasmids even in the absence of antibiotic pressure (Parry 2004; Cairns et al. 2016). This can be explained because protozoan grazing prevents bacteria from reaching the stationary phase when conjugation rate is lower (Wright 1988; Šimek et al. 1997; Cairns et al. 2018a). Therefore, under continuous protozoan pressure, surviving bacteria exhibit the higher metabolic activity required for maintaining the conjugation apparatus (Lopatkin et al. 2016), and thus an enhanced conjugation rate. Experimental data from two independent research groups supported this hypothesis (Bellanger et al. 2014; Cairns et al. 2016). Additionally, different laboratories have reported a dramatic increase (100–1000 × fold) in

the transfer of resistance plasmids between *E. coli* and other bacterial species when a protozoan predator is present (Schlimme et al. 1997; Matsuo et al. 2010a; Bien et al. 2017; Matsushita et al. 2018).

Protists can serve as vectors for multidrug-resistant microorganisms from soil consumers to higher trophic levels via food chains (Chen et al. 2019; Zhang et al. 2019), but also as places where antibiotic resistance likely evolves. Although it remains to be investigated, protozoan predation might indirectly promote antibiotic tolerance by selecting genes conferring resistance to both intracellular digestion and antibiotics such as copper efflux pumps (Vieira et al. 2017). Heavy metals have been shown to co-select for ARGs in bacteria (Poole 2017; Dickinson et al. 2019). As stated before, macrophages and bacterivorous protists use copper and zinc to kill their prey in the phagosome. Hence, the ancient relationships between bacteria and protists suggest that grazing might represent a strong selective force for maintaining metal resistance genes. Indeed, given the acknowledged role of protozoan predation in shaping the structure and diversity of bacterial communities (Gao et al. 2019), it has been proposed that protists might play a significant role in the dynamics of the metal resistome in the environment as well (Hao et al. 2021).

Furthermore, apart from the selection pressure imposed by grazing, it has been evidenced that protozoa may promote horizontal transfer of ARGs among bacteria encased within a food vacuole (Oguri et al. 2011). Successful transference of plasmids bearing extended-spectrum-β-lactamase $bla_{CTX\text{-}M\text{-}27}$ and metallo-β-lactamase $bla_{IMP\text{-}1}$ genes have been observed between *E. coli* and *Aeromonas caviae*, and between clinical *E. coli* isolates within *Tetrahymena* food vacuoles (Matsuo et al. 2010b; Oguri et al. 2011; Matsushita et al. 2018). Notably, the addition of phagocytosis inhibitors (latrunculin B and cytochalasin D) abolished conjugation, suggesting that accumulation of donor and recipient bacteria within protozoan vacuoles was required for conjugation to occur (Oguri et al. 2011). Likewise, McCudding and coworkers described conjugative transfer of the β-lactamase gene $bla_{CMY\text{-}2}$ between *Klebsiella* and *Salmonella* within cattle rumen ciliates (McCuddin et al. 2006). Similar observations were made for different conjugative ARG plasmids under grazing by diverse protozoa (Bien et al. 2017).

Bacteria can also acquire ARGs coded on extracellular DNA (eDNA) via natural transformation (Chen et al. 2005). Studies have shown that eDNA released from bacteria can persist in natural and anthropogenic systems for months (Zhu 2006; Mao et al. 2014; Bien et al. 2017). Interestingly, research indicates that grazing activity by ciliates and nanoflagellates represents a considerable source of bacterial eDNA either in soil or aquatic environments (Ishii et al. 1998; Kawabata et al. 1998; Bien et al. 2017). By using real-time PCR, Bien and collaborators demonstrated an increase in the release of the tetracycline resistance gene *tet(M)* as eDNA by marine bacteria upon addition of protozoan grazers (Bien et al. 2017). The released eDNA remained stable in seawater over the course of the experiment, up to 30 days, suggesting that protozoa may contribute to the formation of an ARG pool in natural systems.

Nevertheless, although the contribution of protozoa to antibiotic resistance in bacteria is beginning to be appreciated, further studies at community level are required to understand the impact of bacteria-protozoa interactions on the dynamics of the resistome in natural and man-made systems. Special attention should be focused on those environments where protozoa and antibiotics co-exist, for instance wastewater treatment plants (Rodriguez-Mozaz et al. 2020). The seminal work done by Cairns and collaborators have demonstrated that protozoan predation and other ecological factors might impose stronger effects on ARG dynamics than sub-inhibitory levels of antibiotics do (Cairns et al. 2018b). Therefore, this finding emphasizes the need to include trophic interactions in studies addressing the selective forces driving dynamics and evolution of ARGs in natural and anthropogenic environments.

## Concluding remarks and future perspectives

Over the last decades, a plethora of data has evidenced the role of protozoa as drivers for the evolution of bacterial pathogens. The dramatic impact of protozoa on pathogen virulence and persistence highlights the importance of bacteria-protist interactions as evolutionary forces shaping bacterial genomes and virulence traits. Besides serving as reservoirs and vectors for bacterial dissemination in the environment, the voracious feeding activity and promiscuous relationships of protists make them protective microniches where genetic exchanges between hosted microorganisms take place. Moreover, although their contribution to antibiotic resistance in bacteria is beginning to be appreciated, recent studies point out that protozoan predation may foster transference of antibiotic resistance genes in microbial communities as well. Amoebae are demonstrated useful models for the study of host–pathogen interactions and we argue that more attention should be paid to bacteria-protist relationships to identify *clues* that favor bacterial virulence, persistence, and transmission in both natural environments and anthropogenic systems.

A remaining challenge in the study of bacteria-protist interactions is the largely underestimated diversity of the associated bacteria since most of them are non-culturable. Likewise, most research focused on a limited number of ciliates and amoebae species, and more studies characterizing environmental isolates are needed. Studies have traditionally relied on classical approaches such as

microscopy, fluorescence in situ hybridization (FISH), and rRNA gene sequencing. However, the incursion of single-cell genomics and other high-throughput sequencing (HTS)-based approaches is expected to provide new opportunities to unravel unseen interactions by capturing genomic data of physically associated microorganisms and exploring a large number of microorganisms directly from environmental samples.

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