

RESEARCH LETTER – Food Microbiology

Yersinia pseudotuberculosis IP32953 survives and replicates in trophozoites and persists in cysts of *Acanthamoeba castellanii*

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One sentence summary: *Yersinia pseudotuberculosis*–amoeba interactions.

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ABSTRACT

Yersinia pseudotuberculosis is a foodborne enteric pathogen that causes a mild self-limiting diarrhea in humans. *Yersinia pseudotuberculosis* is able to persist in soil and water and in association with fresh produce, but the mechanism by which it persists is unknown. It has been shown that *Y. pseudotuberculosis* co-occurs with protozoans in these environments; therefore, this study investigates if bacterivorous free-living amoeba (FLA) are able to support persistence of *Y. pseudotuberculosis*. Coculture studies of *Y. pseudotuberculosis* and the prototype FLA, *Acanthamoeba castellanii* revealed that bacteria had an enhanced capacity to survive in association with amoeba and in the absence of any cytotoxic effects. *Yersinia pseudotuberculosis* is able to survive and replicate in trophozoites specifically localized within vacuoles, and persists within cysts over a period of at least a week. These data present the first evidence that *Y. pseudotuberculosis* is able to resist the bacterivorous nature of FLA and instead exhibits an enhanced ability to replicate and persist in coculture with amoeba. This study sheds light on the potential role of FLA in the ecology of *Y. pseudotuberculosis* which may have implications for food safety.

Keywords: *Acanthamoeba*; *Yersinia*; cyst; trophozoite

INTRODUCTION

Yersinia pseudotuberculosis is a facultative anaerobic psychrotrophic bacterium that belongs to the family Enterobacteriaceae. *Yersinia pseudotuberculosis* causes a mild self-limiting enteric disease, called yersiniosis, which typically presents as a fever, abdominal pain and diarrhea. *Yersinia pseudotuberculosis* is frequently isolated from foods, e.g. raw seafood, fresh vegetables, unpasteurized milk and processed pork, and from

animals, soil and water. The ability of *Y. pseudotuberculosis* to survive and proliferate at low temperatures of 0–4 °C within refrigerators, for example on raw vegetables or processed pork poses a challenge for proper food storage and preservation (Kangas *et al.* 2008; Rimhanen-Finne *et al.* 2009; Vaerewijck *et al.* 2010; Laukkanen-Ninios, Fredriksson-Ahomaa and Korkeala 2014). The ecology of *Y. pseudotuberculosis* with regard to its mode of transmission, survival in the environment and on food,

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and its source of contamination on farms, is not well known. The mechanisms of persistence of *Y. pseudotuberculosis* on food, farms and slaughterhouses (Laukkanen et al. 2008) need to be understood if new strategies are to be developed to minimize food contamination by these bacteria.

There is evidence for a role of free-living amoeba (FLA) as a reservoir or vehicle of dissemination of bacterial pathogens which addresses the ecology of these bacteria. FLA are ubiquitous, bacterivorous unicellular eukaryotic organisms that have two characteristic life stages (Khunkitti et al. 1998; Siddiqui and Khan 2012). One life stage is a metabolically active trophozoite form which phagocytoses bacteria (Khunkitti et al. 1998). These trophozoites are akin to macrophages and some other mammalian cell types because phagocytosed bacteria are trafficked through the canonical endocytic pathway to be digested in the phagolysosome. A second life stage is a dormant double-walled cyst form that is resistant to harsh environmental conditions (e.g. extreme temperatures, desiccation and nutrient deprivation) and supports prolonged survival under these conditions (Khunkitti et al. 1998). Exposure to favorable conditions activates excystment and return to the metabolically active trophozoite life stage (Khunkitti et al. 1998; El-Etr et al. 2009). Some pathogenic intracellular bacteria resist being killed by amoeba and instead exploit this association to enable their persistence in the environment. Such interactions have been demonstrated between the foodborne pathogens *Campylobacter jejuni* (Olofsson et al. 2013), *Salmonella* (Douesnard-Malo and Daigle 2011), *Listeria monocytogenes* (Pushkareva and Ermolaeva 2010) and *Yersinia enterocolitica* (Lambrecht et al. 2013) with soil- and water-borne FLA. These pathogens utilize conserved intracellular survival strategies to survive in unicellular amoeba. For instance, *C. jejuni* evades intracellular digestion by avoiding localization to the amoebal lysosomal vacuole (Olofsson et al. 2013), much like how it is trafficked within mammalian epithelial cells (Watson and Galan 2008). Other pathogens like *Francisella tularensis* and *Mycobacterium* spp. are able to survive for weeks within *Acanthamoeba* spp. cysts (El-Etr et al. 2009; Ben Salah and Drancourt 2010; Amisshah et al. 2014).

FLA and *Y. pseudotuberculosis* co-occur in natural soil and water environments, on fresh produce, including lettuce and carrots (Kangas et al. 2008; Rimhanen-Finne et al. 2009; Vaerewijck et al. 2011), and they are found in anthropogenic environments, like refrigerators associated with fresh produce (Vaerewijck et al. 2010). It has been shown that several different species of protozoa isolated from lettuce and spinach can harbor the food pathogens *Escherichia coli* O157:H7 and *Salmonella enterica* (Gourabathini et al. 2008) within vesicles that are expelled from protozoa. These bacteria-filled vesicles once expelled not only protect the bacteria from harsh conditions, but also support bacterial replication and subsequent escape back into the environment (Gourabathini et al. 2008). These factors led to the hypothesis that FLA may act as a reservoir or vehicle of dissemination of *Y. pseudotuberculosis* in the environment. To test if this was possible, we investigated the interaction of *Y. pseudotuberculosis* with the laboratory prototype FLA, *A. castellanii* using controlled coculture studies.

MATERIALS AND METHODS

Cultivation of *Acanthamoeba castellanii*

Acanthamoeba castellanii (Douglas) Page amoeba (ATCC® 30010; American Type Culture Collection) was maintained axenically in proteose peptone yeast extract glucose

(PYG) medium (ATCC recipe; <http://www.atcc.org/products/all/30010.aspx#culturemethod>) at ambient room temperature (25°C) in 25-cm² culture flasks. The adherent amoeba were harvested by tapping the flasks vigorously, followed by centrifugation (300 × g for 5 min) to collect dislodged amoeba. Amoeba were then washed with phosphate-buffered saline (PBS) and re-suspended in nutrient-rich PYG medium. A Neubauer counting chamber was used to estimate amoeba numbers. For interaction studies with bacteria, the amoeba culture was diluted to approximately 1 × 10⁵ living trophozoites/ml and seeded into 24-well plates and allowed to adhere to the wells overnight at 25°C ambient temperature; all plates were subsequently maintained at this temperature. The viability of *A. castellanii* trophozoites was confirmed using trypan blue exclusion.

Bacterial cultures

The *Y. pseudotuberculosis* IP32953 strain was grown for 12 h overnight in brain heart infusion (BHI) broth at 25 or 37°C to test if any adaptive influences at temperature conditions mimicking ambient environmental growth and a mammalian host, respectively, occurs. *Escherichia coli* DH5α cultured in Luria-Bertani (LB) medium at 37°C was also used. Bacteria were enumerated using a Petroff Hausser counting chamber. All bacteria are maintained at -80°C glycerol stocks in the laboratory. The *Y. pseudotuberculosis* IP32953 strain was maintained on Yersinia selective agar base (Oxoid) after culture from glycerol stocks.

Extracellular persistence assay

Amoeba were seeded on 24-well plates as described above and infected with *Y. pseudotuberculosis* cultured at 25°C and *E. coli* DH5α cultured at 37°C at a multiplicity of infection (MOI) of 100, i.e. 10⁷ bacteria. Bacteria were also cultured singly. After 24 and 168 h co- and monoculture, serial dilutions were performed and plated without lysing of amoeba.

Bacterial entry and intracellular survival assays in PYG medium

Amoeba that were seeded overnight on 24-well plates were washed and incubated in fresh PYG medium for 1 h prior to infection. To determine if *Y. pseudotuberculosis* could enter or be phagocytosed by amoeba, bacteria were cocultured with amoeba at an MOI of 100. *Yersinia pseudotuberculosis* was cultured at either 25 or 37°C, and *E. coli* at 37°C prior to use in coculture experiments. After coculture of bacteria and amoeba for 1 h, cells were washed once with PBS and incubated with 200 μg ml⁻¹ of gentamicin in PYG medium for a further 2 h to kill extracellular bacteria. The amoebas were then washed once with PBS to remove dead bacterial cells and debris, and then lysed with addition of 0.2% Triton X-100 for 10 min. Lysates were immediately serially diluted and plated on BHI agar. Following incubation for 48 h at 28°C, colony forming units (CFU) were enumerated. To assess if *Y. pseudotuberculosis* could survive intracellularly in amoeba trophozoites over a period of 1 week (168 h), assays in PYG were performed similarly to entry assays except that, following gentamicin treatment, the medium was replaced with fresh PYG medium and incubation of the coculture was allowed to proceed for a further 24 h or 1 week. Amoebas were lysed using 0.2% Triton X-100 immediately after incubation periods, serially diluted and plated. Monocultures of bacteria were included as controls and were treated with gentamicin and serially diluted and plated at the respective time points, to confirm that all

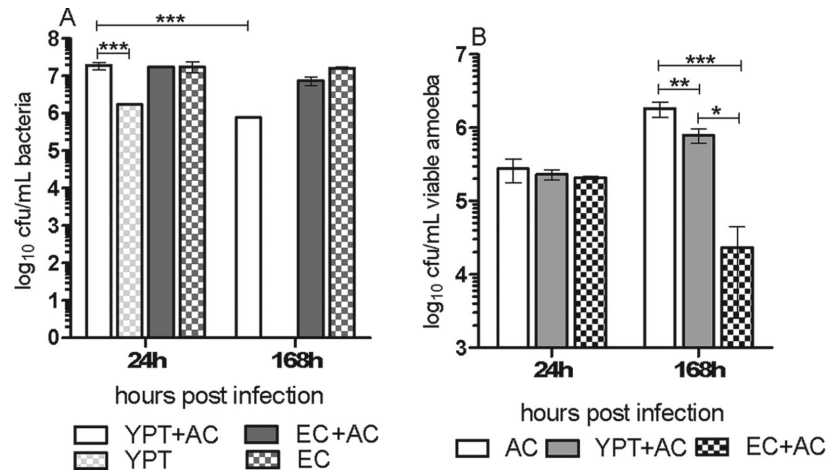


Figure 1. *Yersinia pseudotuberculosis* persists longer in association with *A. castellanii* than during monoculture. (A) Viable bacteria grown with *A. castellanii*, or grown singly. (B) Viable *A. castellanii* grown singly or in coculture with bacteria. Error bars indicate mean \pm SD of at least three independent biological replicates. * = $P < 0.01$, ** = $P < 0.001$, *** = $P < 0.0001$. AC = *A. castellanii*, EC = *E. coli* DH5 α , YPT = *Y. pseudotuberculosis* IP32953.

bacteria succumbed to gentamicin treatment. All experiments were performed using three independent biological replicates, each consisting of two technical replicates.

Bacterial entry and intracellular survival assays in high salt (HS) buffer

Incubation in nutritive-deficient HS buffer (95 mM NaCl, 5 mM KCl, 8 mM MgSO₄, 0.4 mM CaCl₂, 1 mM NaHCO₃, 20 mM Tris-HCl, pH 9.0) results in encystment of amoeba (El-Etr et al. 2009) and allows testing of survival of *Y. pseudotuberculosis* in cysts. Entry and survival assays in HS buffer were performed similarly as above but with the following modifications: following 24 and 168 h of coculture, amoeba were washed once with PBS, and HS medium was replaced with fresh PYG medium and supplemented with IsoVitaleX (BD Biosciences) to allow excystment and bacterial recovery over 48 h. Amoeba were then lysed with 0.2% Triton X-100 immediately after incubation periods, serially diluted and plated. Monocultures of bacteria were included as controls and were treated with gentamicin and serially diluted and plated at the respective time points to confirm that all bacteria succumbed to gentamicin treatment. All experiments were performed using three independent biological replicates each consisting of two technical replicates.

Electron microscopy

For preparation of samples for electron microscopy (EM), bacteria that were cocultured with amoeba in PYG (for trophozoites) or HS medium (for cysts) for 168 h were pelleted and fixed with 2% paraformaldehyde/2.5% glutaraldehyde in 0.2 M cacodylate buffer (El-Etr et al. 2009). After fixing, samples were washed, dehydrated and embedded in an Epon resin. Ultrathin sections (~90–100 nm) were prepared and placed on a grid for microscopic analysis.

Cytotoxicity assays

A Cytotox-96 non-radioactive kit (Promega) was used as per the manufacturer's instructions. Cocultures and monocultures were prepared as above but without gentamicin treatment and sampled after 24 h.

Statistical analyses

Graphpad Prism 5 was used for statistical evaluation of the data. One-way ANOVA with a post-hoc Tukey's Test was used to determine differences in viable bacteria or amoeba at each time point.

RESULTS

Persistence of *Y. pseudotuberculosis* in coculture with *A. castellanii*

To establish if *Y. pseudotuberculosis* could survive in association with amoeba, coculture of bacteria and amoeba was allowed to proceed free of gentamicin treatment and amoeba were not lysed before plating. After 24 h of coculture, bacterial numbers remained the same in coculture and monoculture for both *Y. pseudotuberculosis* and *E. coli* (Fig. 1A). At 1 week of coculture, *Y. pseudotuberculosis* numbers were reduced by ~1 log but viable bacteria were absent from the monoculture. In contrast, *E. coli* survived at equivalent numbers in both coculture and monoculture at 1 and 7 days.

The amoeba in the *Y. pseudotuberculosis* amoeba coculture and amoeba monoculture were able to replicate ~0.5–1 log greater over the 1 week period, but viable amoeba numbers decreased by 1 log in coculture containing *E. coli* (Fig. 1B). Many cysts were visible in cocultures of *E. coli* and amoeba at 1 week. Bacterial cytotoxicity assays were performed to determine if this accounted for the general decreases in viable amoeba present after 1 week of coculture. Cytotoxicity assays which measure release of lactate dehydrogenase (LDH) revealed that bacteria were not cytotoxic to amoeba because comparable levels of LDH were produced by single cultures of amoeba, or bacteria and the cocultures at both pre-culture temperatures (Fig. 2).

Yersinia pseudotuberculosis replicates and survives up to 1 week within trophozoites

To establish if *Y. pseudotuberculosis* is able to survive in intracellular association with *A. castellanii*, we determined if *Y. pseudotuberculosis* was phagocytosed by trophozoites using a gentamicin protection assay to kill extracellular bacteria that had not entered trophozoites. Monocultures of bacteria were treated

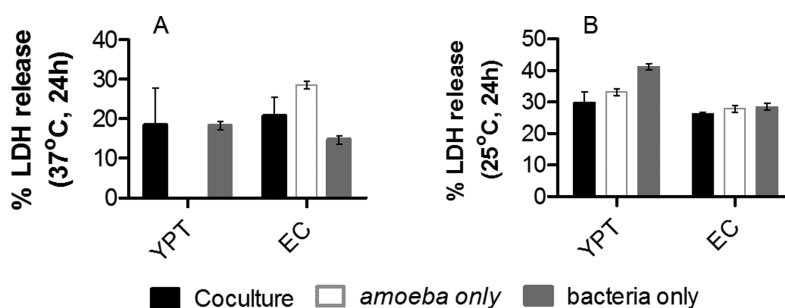


Figure 2. *Yersinia pseudotuberculosis* is not cytotoxic to *A. castellanii*. Percentage of LDH released by bacteria, *A. castellanii*, or coculture of bacteria and *A. castellanii* at 37°C (A) or 25°C (B). Error bars indicate mean \pm SD of at least three independent biological replicates. YPT = *Y. pseudotuberculosis* IP32953, EC = *E. coli* DH5 α .

with the same concentration of gentamicin but viable bacteria were not recovered. Irrespective of pre-culture temperature, *Y. pseudotuberculosis* entered amoeba at equal numbers (Fig. 3A), but *E. coli* was not similarly recovered from trophozoites.

Next the survival of bacteria was assessed over a period of 1 week. Although pre-culture at 37 and 25°C resulted in equal replication of *Y. pseudotuberculosis* by 1 week, pre-culture at 37°C enabled faster replication as demonstrated by the significant difference ($P < 0.01$) in bacteria between the two pre-culture temperatures after 24 h of coculture.

To determine the localization of *Y. pseudotuberculosis* and *E. coli* in trophozoites of *A. castellanii*, EM was performed after coculture at 1 week. Intact *Y. pseudotuberculosis* cells were observed to reside intracellularly within vacuoles (Fig. 3B) of *A. castellanii* but this was not observed for *E. coli* (data not shown).

Yersinia pseudotuberculosis can survive for up to 1 week within cysts

During amoeba encystment, which lasts several hours, digestive vacuoles and other components (e.g. golgi, nucleus) are broken down and extruded leaving only lipid droplets to remain in the double-walled mature cyst (Bowers and Korn 1969). Hence, any intracellular bacteria that were not digested or expelled during encystment would represent bacteria that remained in cysts upon initial entry into trophozoites in nutritive-deficient HS buffer. Upon coculture with bacteria, encystment occurred within 24 h, which is typical for *Acanthamoeba* growing in HS medium (El-Etr et al. 2009). *Yersinia pseudotuberculosis* entered into trophozoites and was recovered from cysts at 24 and 168 h at both 25 and 37°C (Fig. 4A). Interestingly, the pre-culture of *Y. pseudotuberculosis* at 37°C, which is representative of mammalian physiological conditions, favors entry into trophozoites cultured in HS medium and faster growth rate in PYG medium. *Escherichia coli* was never recovered from cysts, which correlates with its inability to survive in trophozoites. Consistent with these data, EM confirmed that *Y. pseudotuberculosis* was localized within mature double-walled cysts at 1 week of coculture (Fig. 4B), but *E. coli* was not observed within cysts.

DISCUSSION

Our findings reveal that the foodborne pathogen, *Y. pseudotuberculosis* is capable of resisting grazing by bacterivorous protozoa in a non-cytotoxic manner. Instead, these bacteria show enhanced persistence in extracellular association with amoeba. The enhanced ability to persist in association with *A. castellanii* but not in monoculture has also been demonstrated for *Salmonella enterica* serovar Typhi and *Y. enterocolitica* (Douesnard-

Malo and Daigle 2011; Lambrecht et al. 2013). Simultaneously, we demonstrated that *Y. pseudotuberculosis* is able to survive and replicate within trophozoites, especially at 37°C, similar to its phenotype in macrophages (Moreau et al. 2010; Ligeon et al. 2014). Bacterial factors expressed at 37°C might trigger improved uptake and replication of *Y. pseudotuberculosis* within amoeba. As such, because HS buffer is nutritionally deficient, turnover of the expression factors is slower than during PYG growth, thus sustaining higher entry. The intravacuolar residence of *Y. pseudotuberculosis* was reminiscent of its infection in macrophages within which the bacteria can survive by inhibiting acidification of the vacuole (Wang and Ahearn 1997). Other bacterial pathogens, such as *C. jejuni* (Axelsson-Olsson et al. 2005), *F. tularensis* (El-Etr et al. 2009) and *Legionella pneumophila* (Greub and Raoult 2004) survive within *Acanthamoeba* vacuoles and prevent acidification of the intravacuolar niche as an intracellular survival strategy. Interestingly, this is in contrast to *Y. enterocolitica* which resides in the cytosol of *A. castellanii* (Lambrecht et al. 2013) although both species of *Yersinia* are intracellular foodborne pathogens that cause yersiniosis.

Our data demonstrating the maintenance of viable *Y. pseudotuberculosis* in cysts is a relatively rare observation because not many other human pathogens have been observed in cysts. Several *Mycobacterium* spp. (Ben Salah and Drancourt 2010; Amisshah et al. 2014) are able to survive in the exocyst while *F. tularensis* was shown to survive within cysts for a period of at least 3 weeks (El-Etr et al. 2009). *Legionella pneumophila* survival in cysts has been proposed (Skinner et al. 1983; Kilvington and Price 1990) but is contentious because viable *L. pneumophila* was demonstrated in vesicles expelled from encysting amoeba (Bouyer et al. 2007; Hoffmann, Harrison and Hilbi 2014). Cysts are supposedly dormant bodies; however, it is not known whether bacteria are metabolically active while inside. Our data suggest that *Y. pseudotuberculosis* is not replicating within cysts. This observation is based on the experimental design in which amoeba excystment and bacterial recovery is allowed to ensue for 48 h prior to plating, but the number of *Y. pseudotuberculosis* in trophozoites remains similar to those recovered from cysts. During long-term persistence in a soil microcosm, *Y. pseudotuberculosis* has been observed to adopt physiological characteristics that are indicative of lower metabolic activity, which is reversed following several passages in a nutritive medium (Buzoleva and Somov 2003).

In contrast to the interaction of *Y. pseudotuberculosis* with *A. castellanii*, *E. coli* DH5 α was able to survive in extracellular association with *A. castellanii* but resisted being phagocytosed as indicated by our inability to quantify viable bacteria from intracellular assays, or observe *E. coli* within trophozoites or cysts. This may indicate an alternate mechanism of grazing resistance for

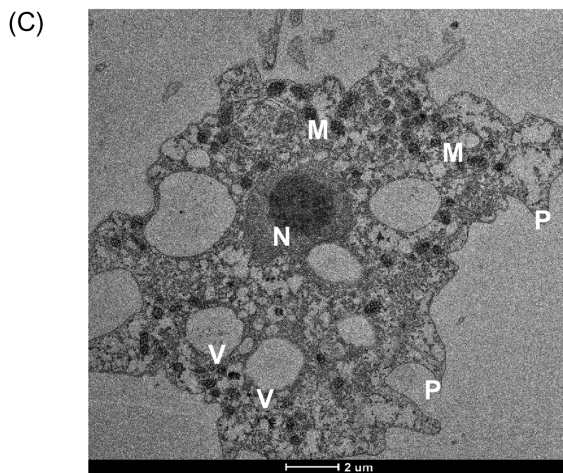
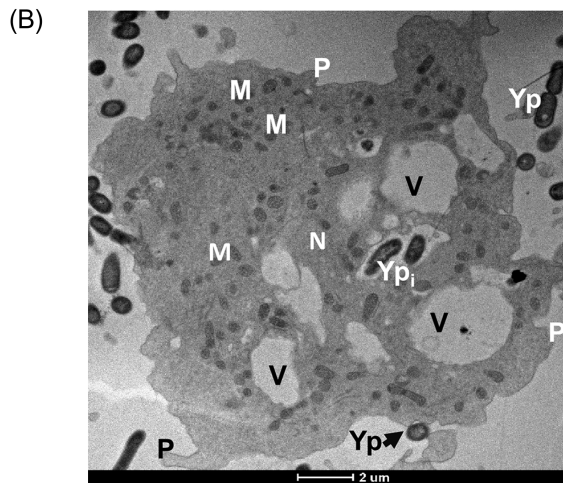
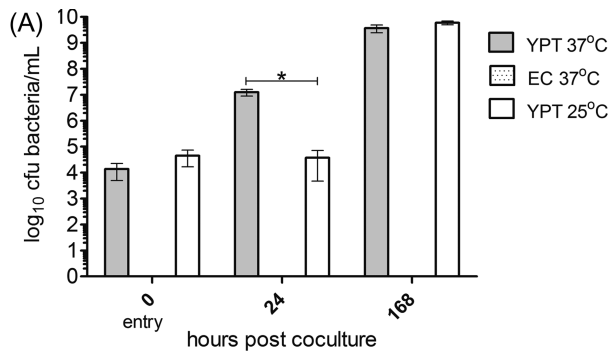


Figure 3. *Yersinia pseudotuberculosis* can enter, replicate and survive up to 1 week within *A. castellanii* trophozoites. (A) Entry and survival of bacterial strains in trophozoites after pre-culture at 37 or 25°C. Error bars indicate mean \pm SD of at least three independent biological replicates. * = $P < 0.0001$. EC = *E. coli* DH5 α , YPT = *Y. pseudotuberculosis* IP32953. (B) Transmission electron micrographs of *Y. pseudotuberculosis* IP32953 localized within *A. castellanii* trophozoite. (C) Uninfected trophozoite. M = mitochondria, Yp = extracellular *Y. pseudotuberculosis*, N = nucleus, V = vacuole, P = pseudopodia, Yp_i = intracellular *Y. pseudotuberculosis*. Scale bar representing 2 μ m is indicated.

E. coli DH5 α , since the pathogenic *E. coli* O157:H7 is able to survive within amoeba vacuoles (Gourabathini et al. 2008). However, *E. coli* survives at high numbers extracellularly with a concomitant decrease in trophozoites over 1 week. The faster growth of *E. coli* may inhibit the growth and promote encystment of the amoeba due to competition for media nutrients and/or by

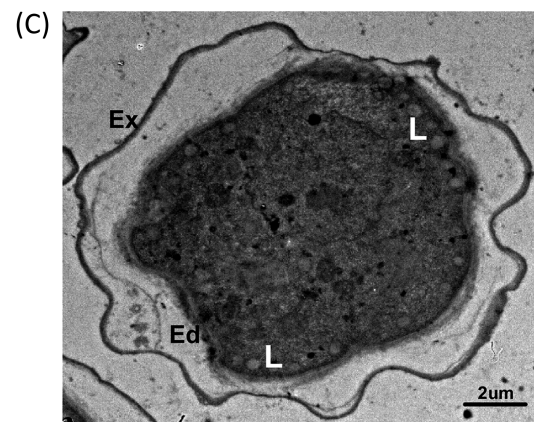
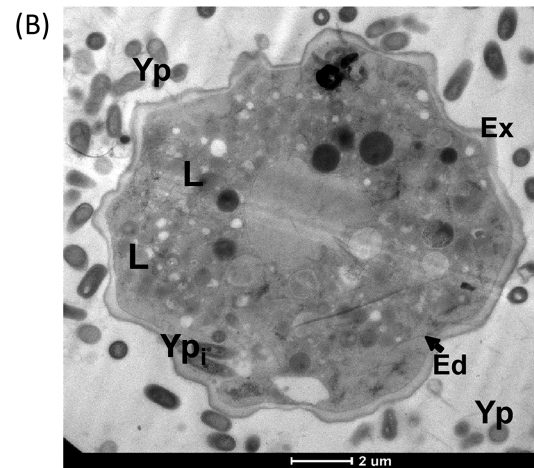
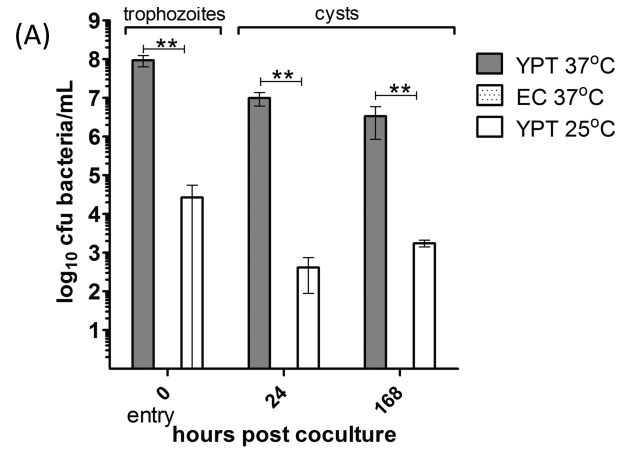


Figure 4. *Yersinia pseudotuberculosis* can survive up to 1 week within *A. castellanii* cysts. (A) Entry into trophozoites and survival of bacteria in cysts after pre-culture at 37 or 25°C. Error bars indicate mean \pm SD of at least three independent biological replicates. ** = $P < 0.001$. EC = *E. coli* DH5 α , YPT = *Y. pseudotuberculosis* IP32953. (B) Transmission electron micrographs of *Y. pseudotuberculosis* IP32953 localized within an *A. castellanii* cyst. (C) An uninfected *A. castellanii* cyst. L = lipid droplet, Yp = extracellular *Y. pseudotuberculosis*, Yp_i = intracellular *Y. pseudotuberculosis*, Ed = endocyst wall, Ex = exocyst wall. Scale bar representing 2 μ m is indicated.

pH decreases caused by metabolic by-products. This has been shown previously in amoeba coculture studies using MOIs of 100 or above (Avery, Harwood and Lloyd 1995; Wang and Ahearn 1997).

Collectively, it appears that *Y. pseudotuberculosis* is able to survive both intracellularly and in extracellular association with *A. castellanii*, and our data imply that this interaction is favourable and enhance persistence and survival of *Y. pseudotuberculosis* in the environment. Interestingly, some intracellular bacteria are able to cycle in and out of the trophozoite (Greub and Raoult 2004) within vesicles that are passively expelled from amoeba. These bacteria containing vesicles have been demonstrated to allow bacterial persistence in the environment by providing protection from harsh conditions, and support both replication and escape of bacteria (Gourabathini et al. 2008). Bacteria that have escaped are expected to be phagocytosed by the same or adjacent amoeba. Cycling of *L. pneumophila* (Bouyer et al. 2007), *S. enterica* (Brandl et al. 2005) and *F. tularensis* within vesicles has been implicated in the dissemination of these pathogens in the environment. Similarly the ability of *Y. pseudotuberculosis* to be sustained at high numbers both intracellularly and extracellularly may imply that these bacteria are cycling in and out of trophozoites.

In conclusion, our study demonstrates that the *Y. pseudotuberculosis*–FLA association favours persistence and replication of this foodborne pathogen. Taken together with data that *Y. pseudotuberculosis* and FLA co-occur, our findings indicate that the protozoa may play a significantly role in the ecology of *Y. pseudotuberculosis*.

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Conflict of interest. None declared.

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