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Interaction between Food-borne Pathogens (*Campylobacter jejuni*, *Salmonella* Typhimurium and *Listeria monocytogenes*) and a Common Soil Flagellate (*Cercomonas* sp.)

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Abstract

Free-living protozoa may harbor, protect, and disperse bacteria, including those ingested and passed in viable form in feces. The flagellates are very important predators on bacteria in soil, but their role in the survival of food-borne pathogens associated with fruits and vegetables is not well understood. In this study, we investigated the interactions between a common soil flagellate, *Cercomonas* sp., and three different bacterial pathogens (*Campylobacter jejuni*, *Salmonella* Typhimurium, and *Listeria monocytogenes*). Rapid growth of flagellates was observed in co-culture with *C. jejuni* and *S.* Typhimurium over the time course of 15 days. In contrast, the number of *Cercomonas* sp. cells decreased when grown with or without *L. monocytogenes* for 9 days of co-culture. Interestingly, we observed that *C. jejuni* and *S.* Typhimurium survived better when co-cultured with flagellates than when cultured alone. The results of this study suggest that *Cercomonas* sp. and perhaps other soil flagellates may play a role for the survival of food-borne pathogens on plant surfaces and in soil.

Keywords: *Cercomonas* sp., *C. jejuni*, *L. monocytogenes*, *S.* Typhimurium, Flagellate

1. Introduction

Outbreaks of food-borne disease caused by *Campylobacter*, *Salmonella* or *Listeria* associated with the consumption of contaminated vegetables have recently been reported and received worldwide attention (Beuchat, 1996; Crook et al., 2003; Pakalniskiene et al., 2009; Gajraj, Pooransingh, Hawker & Olowokure, 2011; Gardner et al., 2011). Fresh produce consumed raw or minimally processed, such as fruits and vegetables, provide an ideal route for the transmission of certain enteric pathogenic bacteria including *Salmonella* spp., *Escherichia coli*, *Campylobacter jejuni*, and *Listeria monocytogenes* (Beuchat, 2002; Islam et al., 2004; Berger et al., 2010; Newell et al., 2010; Brassard, Guévremont, Gagné & Lamoureux, 2011). Primary sources of pre-harvest contamination include soil-improvement with untreated or improperly composted manure and contaminated irrigation water (Buck, Walcott & Beuchat, 2003; Islam et al., 2004; Berger et al., 2010; McLaughlin, Casey, Cotter, Gahan & Hill, 2011). It has been reported that the microbiota of soil-grown fruits and vegetables may be reflecting the microbiota of soils in which they grow (Jay, Loessner & Golden, 2005).

Protozoa, traditionally divided on the basis of their means of locomotion into four broad categories ciliates, flagellates, sporozoans, and amoebae, are the primary bacterial predators in soil. Of these groups, flagellates and amoebae are thought to be the most abundant and are able to enter soil pore necks as small as 3 μm (Ekelund & Rønn, 1994; Gaze, Burroughs, Gallagher & Wellington, 2003). Flagellates as well as amoebae are important bacterial grazers, and flagellates have been shown to change the composition of the bacterial community in a different manner than the soil amoebae *Acanthamoebae* spp. They play an important role in microbial degradation processes and nutrient flow in soil (Pedersen, Nybroe, Winding, Ekelund & Bjørnlund, 2009). Recent studies have suggested that free-living amoebae are important players in the evolution of obligate and facultative bacterial pathogens (Zhou, Elmose & Call, 2007). Although it has been shown that amoebae can prolong the survival of food-borne pathogens (Gaze et al., 2003; Zhou et al., 2007; Baré et al., 2010), relatively little is known about the role of flagellates in the epidemiology of food-borne diseases. Furthermore, it has been reported that flagellates are present in high numbers on vegetables such as lettuce and spinach (Gourabathini, Brandl, Redding, Gunderson & Berk, 2008; Vaerewijck, Sabbe, Baré & Houf, 2011). These protists ingest only a few bacteria at a time and their role in the survival of food-borne pathogens on plant surface and in soil remains to be investigated (Gourabathini et al., 2008). Accordingly, we investigated the ability of three different food-borne pathogens (*C. jejuni*, *S.* Typhimurium, and *L. monocytogenes*) to survive in co-culture with *Cercomonas* sp. - a common soil flagellate. These bacterial pathogens were selected because they have caused recent outbreaks (Beuchat, 1996; Crook et al., 2003; Gajraj, Pooransingh, Hawker & Olowokure, 2011; Gardner et al., 2011). Although flagellates are the most abundant and widespread soil mesofauna, relatively little is known regarding the impact of this free-living protozoan on fresh produce.

2. Materials and Methods

2.1 Bacteria and conditions

The reference strains of *C. jejuni* NCTC 11168, *L. monocytogenes* VDL 148, and *S.* Typhymurium NCTC 12023 were used in this study to investigate the interactions of these pathogens with a common soil flagellate, *Cercomonas* sp. Before each experiment, *C. jejuni* was grown under microaerobic conditions for 24 h on blood agar (BA) plates (Tryptic soy agar containing 5% [vol/vol] whole sheep blood, 10 μg/ml vancomycin and 5 μg/ml trimethoprim) at 37ºC. *L. monocytogenes* and *S.* Typhimurium were grown on BA plates for 16 h in aerobic conditions.

2.2 Protozoan

The flagellate *Cercomonas* sp. reference strain ATCC 50334 was used as an axenic culture and is maintained at 15ºC on a mixture of heat-killed cells of a soil isolate *Pseudomonas putida* reference strain ATCC 17426. as *Pseudomonas* spp. can be a food source of *Cercomonas* sp. as previously described (Pedersen et al., 2009). and a nutrient medium (ATCC medium 802). The bacteria were harvested and washed twice with modified Neff's Amoeba Saline (AS) buffer (Lekfeldt & Rønn, 2008) and then killed at 80ºC for 15 min. The heterotrophic flagellate *Cercomonas* sp. cells from an actively growing axenic culture was washed three times with AS buffer and subsequently added to 25 cm^2 cell culture flask (Nunc, Roskilde, Denmark) containing 5 ml of ATCC medium 802 to reach the final concentration of 2×10^3 flagellate cells/ml.

2.3 Co-culture experiments

An inoculum of each food-borne pathogen was added to separate flagellate flask with an estimated starting concentration of 10^8 CFU/ml. For control experiments, 100 μl of 5×10^9 CFU/ml heat-killed *P. putida* was added to a flagellate flask as a positive control, while 100 μl of AS buffer was added to another flagellate flask as a

negative control. All flasks were incubated at 15ºC in aerobic conditions. The number of bacterial cells and flagellates were determined at day 3, 6, 9, 12, and 15 of the co-cultures.

2.4 Survival of bacteria and flagellate

The growth of the flagellate was measured by counting the concentration of flagellates (cells/ml) at different time points in the cell culture flasks using an inverted light microscope with LED illumination at ×200 magnification (Leica DM IL LED, Leica Microsystems GmbH, Wetzlar, Germany). For *C. jejuni*, aliquots of 100 µl of 10-fold serial dilutions of co-culture medium were spotted on BA plates and incubated at 37ºC in microaerobic conditions for 36 h until bacterial colonies formed. For *S.* Typhimurium and *L. monocytogenes*, aliquots of 100 µl of 10-fold serial dilutions of co-culture were spread on BA plates and incubated at 37ºC in aerobic conditions for 16 and 24 h, respectively.

2.5 Statistical analysis

A Student's *t*-test was used to compare the numbers of bacteria in co-culture. *P*-values of < 0.05 were considered statistically significant.

3. Results and Discussion

To investigate the interaction of food-borne pathogens with flagellates, we first determined whether these bacteria have an effect on the growth of *Cercomonas* sp. As shown in Figure 1, the flagellate *Cercomonas* sp. did not grow in the co-culture with *L. monocytogenes* and lost the viability after day 3 and decreased more after 6 days until no cells were detectable by day 12. There was no significant difference in the number of *Cercomonas* sp. cells when cultivated with or without *L. monocytogenes* for flagellate cells decreased rapidly in both cases (Figure 1). Interestingly, the rapid growth of flagellates was observed in the co-culture with *C. jejuni* and *S.* Typhimurium as well as in the positive control. The numbers of flagellates counted in flasks cultivated with *C. jejuni* and *S.* Typhimurium were almost equal to numbers of flagellate cells obtained in positive control flasks (where heat-killed *P. putida* was added) over the time course of 15 days. These results are in agreement with a previous study that described Gram-negative bacteria including *Pseudomonas* spp. as a good food source for the growth of *Cercomonas* sp. (Lekfeldt & Rønn, 2008; Pedersen et al., 2009).

The effect of flagellates on survival of food-borne pathogens in co-culture was determined by conventional bacterial plate counting (CFU) at different time points. As shown in Figure 2, no significant difference was obtained with the number of *L. monocytogenes* cultivated with or without *Cercomonas* sp. after 12 days (Figure 2). This corresponded well to the decreased number of *Cercomonas* sp. cells, suggesting that this bacterium is not a food source and may be toxic for the flagellates. Cytotoxicity of haemolytic *Listeria* spp. in protozoa was originally demonstrated by Ly & Muller, (1990). They have shown that haemolytic *L. monocytogenes* and *L. seeligeri* induce lysis of *Tetrahymena pyriformis* and *Acanthamoeba castellanii* during 8-15 days, while only few protozoa underwent lysis in the presence of non-haemolytic *L. innocua*. Interestingly, the number of *C. jejuni* cells in co-culture with *Cercomonas* sp. decreased slowly and remained approximately 2×10^2 CFU/ml at day 15. This corresponded well with the higher final number of flagellate cells when grown with this bacterium of apparent high food source (Figure 1). In contrast, in the absence of flagellates, CFU number of *C. jejuni* decreased rapidly and 2.6×10^4 and 3.4×10^2 CFU/ml were obtained at day 3 and day 6, respectively. The number of *S.* Typhimurium cells obtained in the co-culture with *Cercomonas* sp. was significantly higher ($P<0.05$) than those obtained in the culture without flagellates on day 9, 12 and 15 (Figure 2). This bacterium seems to be a good food source for the flagellate as a higher number of *Cercomonas* sp. was observed over the time course of 15 days. Although flagellates ingest *C. jejuni* and *S.* Typhimurium in the co-cultures, these bacteria still seem to survive longer in the presence of this protozoan than when cultivated without protozoan. Our data suggest that the flagellates use *C. jejuni* and *S.* Typhimurium as food sources, but there seems to be a mutual benefit in the relationship. By enhancing bacterial survival, the protozoa do not run out of food, while the bacteria "enjoy" the more favorable conditions generated by the flagellates and use the flagellates as temporary protective structures and vehicles for dissemination. It has been reported that flagellates ingest only a few bacteria at a time (Gourabathini et al., 2008), and thus they do not hinder the survival of *C. jejuni* and *S.* Typhimurium, which are in agreement with our data. Our data suggest that flagellates may play a role in the transmission of food-borne pathogens as they may enter the human food chain following the application of animal manures to agricultural land with raw consumed crops such as salads, fruit and vegetables. Furthermore, it has been reported that food-borne pathogens originating from animal manures could survive for a long time in soil after application (Nicholson, Groves & Chambers, 2005). Alongside amoebae which have been demonstrated to promote the survival of these pathogens (Gaze et al., 2003; Baré et al., 2010); our study suggests that flagellates may also play a similar role as amoebae.

Observations reported here demonstrate that *Cercomonas* sp., a common soil flagellate, is strongly attracted to and consumes both *C. jejuni* and *S.* Typhimurium which can be introduced into agricultural soil through the deposition of animal faeces, untreated irrigation water, or runoff water from livestock feeding lots (Islam et al., 2004; Berger et al., 2010). Our data indicate that *Cercomonas* sp. consumed *C. jejuni* and *S.* Typhimurium as food sources but not *L. monocytogenes*. Furthermore, *Cercomonas* sp. not only consumes but also significantly prolonged the survival of both *C. jejuni* and *S.* Typhimurium in co-culture up to 15 days while *L. monocytogenes* died after 3-6 days. We did not determine the internal location of bacterial pathogens inside *Cercomonas* sp., but our data support and suggest that by prolonging the survival of bacterial pathogens when cultivated with *Cercomonas* sp. can open a window for the possibility of a cross contamination of these pathogens from soil to the human food chains. The cross contamination could be due to *Cercomonas* sp. itself acting as a vector for carrying the bacteria, but it needs to be proved and examined by different methods. In addition, prolonging the survival of food-borne pathogens in soil by *Cercomonas* sp. could increase the risk of other protozoa, insects, worms or wild birds to be a vector for the pathogens. Also, it is very interesting to study what factors contribute to prolonging the survival of the bacterial pathogens in co-culture with *Cercomonas* sp. The experiments in this direction are in progress. Furthermore, the results of this study could open a new direction for studying the interaction between protozoa and bacterial pathogens from the environments such as fertilized soil, water and animal manures to human foods, specially the consumption of raw crops.

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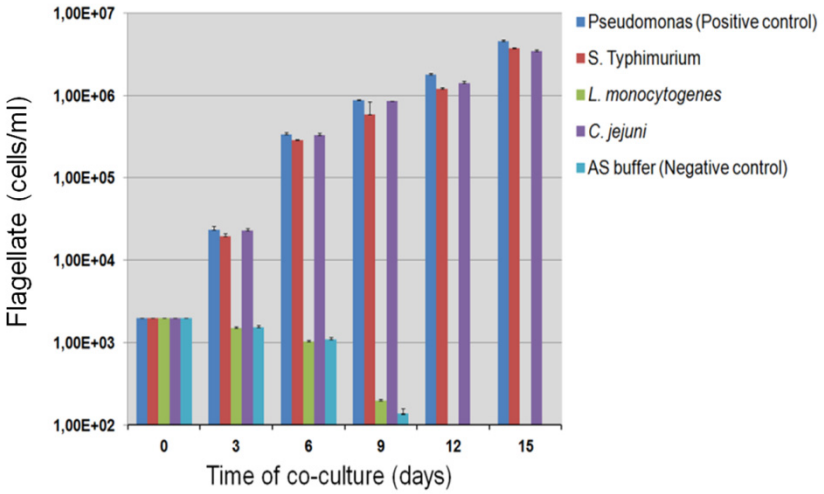

Figure 1. Growth of flagellates in co-culture with or without bacteria at different time points at 15ºC in aerobic conditions. Data are means and standard errors of at least three independent experiments

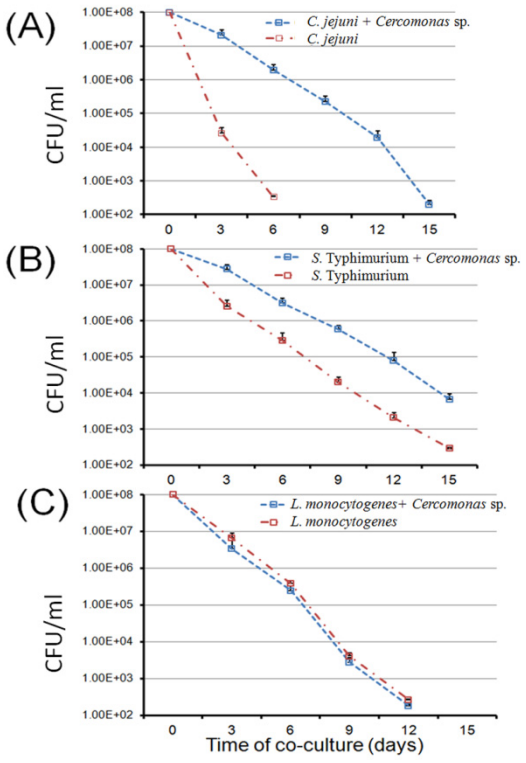

Figure 2. The survival of food-borne pathogens in co-culture with or without *Cercomonas* sp. at different time points at 15ºC in aerobic conditions. CFU counts are present as (A) *C. jejuni*, (B) *S.* Typhimurium, and (C) *L. monocytogenes*. Data are means and standard errors of at least three independent experiments