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Cite this article: Armitage DW. 2016 Bacteria facilitate prey retention by the pitcher plant *Darlingtonia californica*. *Biol. Lett.* **12**: 20160577.
<http://dx.doi.org/10.1098/rsbl.2016.0577>

Received: 6 July 2016

Accepted: 26 October 2016

Subject Areas:

biomechanics, ecology

Keywords:bacteria, pitcher plant, rheology, surface tension, carnivorous plant, *Darlingtonia californica***Author for correspondence:**

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Electronic supplementary material is available online at <https://dx.doi.org/10.6084/m9.fig-share.c.3571608>.

Biomechanics

Bacteria facilitate prey retention by the pitcher plant *Darlingtonia californica*David W. Armitage^{1,2}¹Department of Integrative Biology, University of California Berkeley, 3040 Valley Life Sciences Building, Berkeley, CA 94720-3140, USA²Department of Biological Sciences, University of Notre Dame, 290B Galvin Life Science Center, Notre Dame, IN 46556, USA

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Bacteria are hypothesized to provide a variety of beneficial functions to plants. Many carnivorous pitcher plants, for example, rely on bacteria for digestion of captured prey. This bacterial community may also be responsible for the low surface tensions commonly observed in pitcher plant digestive fluids, which might facilitate prey capture. I tested this hypothesis by comparing the physical properties of natural pitcher fluid from the pitcher plant *Darlingtonia californica* and cultured 'artificial' pitcher fluids and tested these fluids' prey retention capabilities. I found that cultures of pitcher leaves' bacterial communities had similar physical properties to raw pitcher fluids. These properties facilitated the retention of insects by both fluids and hint at a previously undescribed class of plant–microbe interaction.

1. Introduction

Plants have evolved a variety of strategies for living in nutrient-poor environments. One particularly widespread strategy is the close association between a host plant and one or more species of beneficial bacteria and fungi. These interactions commonly involve a mutually beneficial exchange of the host's photosynthates for mineral nutrients scavenged from the soil or fixed from the atmosphere [1]. In addition to assistance with nutrient acquisition, plant-associated microbiota may also perform beneficial secondary functions. For instance, microbes may facilitate disease suppression outside of the host's own immune response [2] or aid in the removal of growth-inhibiting compounds [3]. Outside of these cases, however, novel classes of beneficial plant–microbe interactions remain elusive.

Carnivory is another adaptation to nutrient-poor habitats, and plants have evolved a variety of methods to trap and digest arthropod prey [4]. Although the majority of carnivorous plants produce their own digestive enzymes to break down prey, members of the pitcher plant family Sarraceniaceae are hypothesized to rely heavily on an associated microbial digestive community [5,6]. These plants possess modified, fluid-filled leaves in which prey are retained and digested by a community of mutualistic aquatic invertebrates and bacteria [7,8]. Fluid from these plants' leaves can have lower interfacial (surface) tensions than water [4,5,9]—a property interpreted to facilitate the retention and drowning of prey. Because bacterial biomass can be very high in pitcher plant fluid (10^9 – 10^{11} cells ml^{-1}) [10], and because many bacteria produce biosurfactants hypothesized to aid the digestion of water-insoluble compounds such as lipids [11], it stands that bacteria may be causing a reduction of the pitcher fluid's interfacial tension. Based on the observation that insects added to the pitcher fluid of adult *Darlingtonia californica* Torr. (Sarraceniaceae) leaves experience difficulty escaping, I measured the tensile and rheological properties of pitcher fluid and pitcher bacterial cultures and tested whether prey retention by pitcher plant bacterial cultures was comparable to that of natural pitcher fluids.

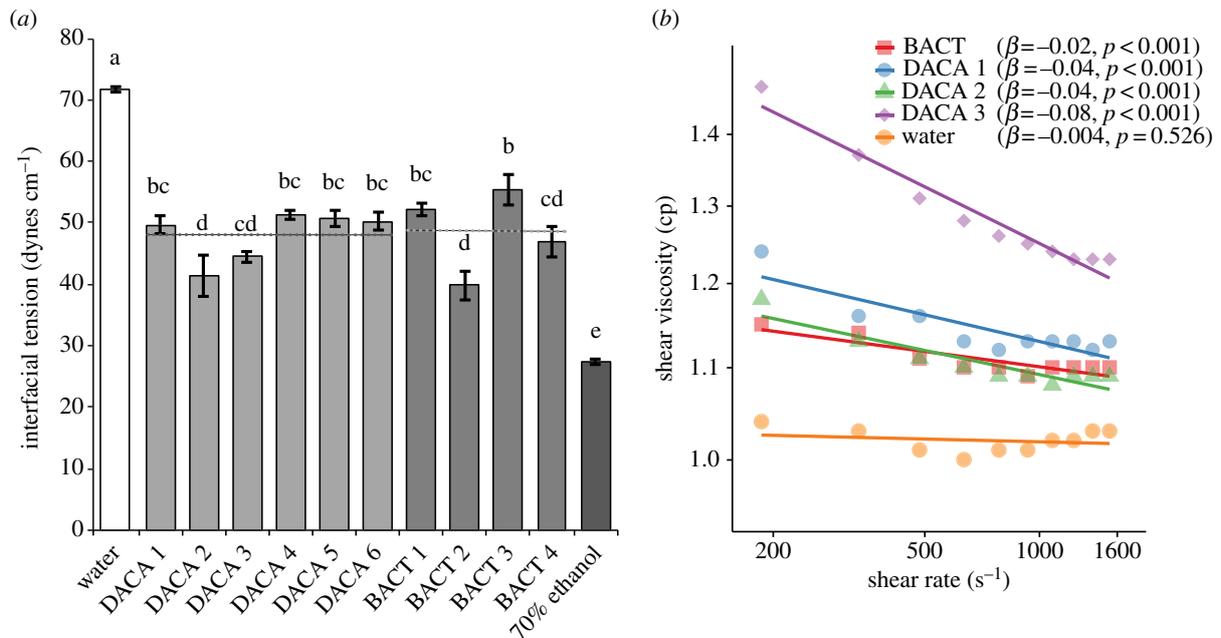


Figure 1. (a) Interfacial tension measurements for fluid samples. Samples sharing letters do not significantly differ from one another ($p > 0.01$). The dotted line denotes mean value for aggregated *Darlingtonia* (DACA) fluid and bacterial culture samples. BACT sample numbers correspond to the DACA samples from which they were inoculated. DACA samples 5 and 6 are from young leaves. 1 dyne = 1×10^{-5} N. (b) Shear viscosity measurements on selected fluid samples. Significantly negative slopes (β) of *Darlingtonia* fluid and bacterial cultures indicate very slight shear-thinning properties. 1 cp (centipoise) = 10^{-3} Pa s. (Online version in colour.)

2. Material and methods

In July 2014, I collected fluid from six individual *D. californica* leaves growing in Plumas National Forest, California. Four leaves belonged to the year's first cohort, which began trapping prey in mid-June. Two additional samples were collected from two-week-old leaves.

To create 'artificial' pitcher fluid, I inoculated four glass tubes with 10 ml of autoclaved, $0.2 \mu\text{m}$ -filtered water collected from my field site. Into this water, I added 3 g l^{-1} autoclaved powder from freeze-dried, ground crickets and a $50 \mu\text{l}$ aliquot from one of four month-old $150 \mu\text{m}$ -filtered pitcher fluid samples. To simulate the oxygen environments of pitcher leaves [12], I briefly bubbled cultures every 2 h from 08.00 to 20.00. After 30 days, the cultures were stored at 5°C . Dilutions of each culture were also plated on R2A agar and colony-forming units (CFUs) were counted.

To measure the interfacial tension of pitcher fluid samples and bacterial cultures, I used a pendant-drop tensiometer [13] to photograph the profiles of individual $1.0 \mu\text{m}$ -filtered droplets suspended from clean glass capillary tubes [14] (electronic supplementary material, figure S1). I repeated this process for 10 drops per sample. I estimated the interfacial tension, γ , of each drop photograph using the method of Stauffer [15]. This method requires the equatorial diameter, D_e , of the drop, and D_s , the diameter of the drop at a vertical distance D_e from the drop's lower apex. The ratio $D_s/D_e = S$ relates to a correction factor H required by the equation

$$\gamma = \frac{gD_e^2\Delta\rho}{H}, \quad (2.1)$$

where g is the acceleration due to gravity (9.81 m s^{-2}) and $\Delta\rho$ is the difference in densities between the droplet (997 kg m^{-3}) and air at room temperature. The correction factor H was estimated with the equation

$$\frac{1}{H} = k_e S^{-k_m} + k_3 S^3 + k_2 S^2 + k_1 S + k_0, \quad (2.2)$$

where k_i are empirical constants [16]. I averaged six estimates from a single drop's photographs for a per-drop estimate of γ , and then averaged these estimates across 10 replicate drops per sample to

estimate each sample's interfacial tension. The measurements of pure water and 70% ethanol were nearly identical to their standard theoretical values. I used ANOVA and Tukey's range test to investigate differences between sample means.

I used a cone-plate viscometer (Brookfield Engineering) to measure the shear viscosities of 0.5 ml pitcher fluid samples at shear rates between 100 and 1600 s^{-1} [17]. I plotted samples' viscosities against their log shear rates and fitted linear regression lines to each series using R [18]. I expected shear-thinning or thickening fluids to have non-zero slopes [17].

I conducted an experiment to test the effects of pitcher plant fluid and bacterial cultures on prey retention of the red harvester ant *Pogonomyrmex barbatus*—a close relative of species found trapped in plants at the sample collection site. Individual ants were dropped into 15 ml centrifuge tubes containing 5 ml of one of the following substances: pure deionized water, natural pitcher fluid and fluid pooled from the pitcher bacterial cultures. Escape behaviour of the ants was filmed for 10 min, at which point they were removed and allowed to recover. The experiment was repeated 30 times (using fresh ants for each trial) and I calculated the frequency of escapes for each sample.

Next, I tested the minimal concentration of bacterial culture (BACT) required for retaining ants. I created serial dilutions of pooled cultures in pure water at regular increments from 10^{-1} to 10^{-3} , added ants into 5 ml of each dilution, and filmed their behaviour for 10 min. This was repeated 12 times, using new ants for each treatment and replicate, and the number of successful escapes was scored. I used logistic regression analysis to test whether an ant's probability of escape from the fluid was associated with its dilution.

3. Results and discussion

Pitcher fluid from *Darlingtonia* had interfacial tensions significantly lower than water and higher than 70% ethanol (figure 1a; all statistical results are presented in the electronic supplementary material, data). The same pattern was observed for bacterial cultures seeded with *Darlingtonia* bacteria. These bacterial cultures had very similar mean interfacial

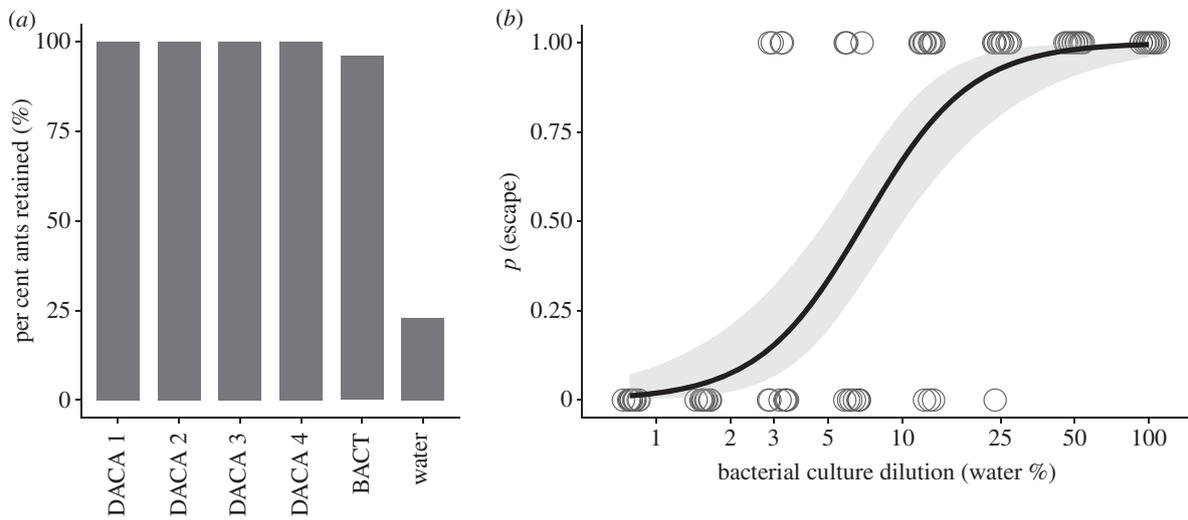


Figure 2. (a) Ants were unable to escape *Darlingtonia* pitcher fluid and cultures seeded with *Darlingtonia* bacteria after 10 min, whereas the majority were able to escape from pure water. (b) The probability of an ant escaping increases as *Darlingtonia* BACT is diluted with water. Black line denotes the fitted logistic regression line and the shaded region covers the 95% confidence interval.

tensions to those of raw pitcher fluid (48.5 ± 3.4 dyne cm^{-1} versus 47.9 ± 1.7 dyne cm^{-1} (1 dyne = 1×10^{-5} N)), and pairwise *post-hoc* analyses revealed individual *Darlingtonia* fluid samples were nearly indistinguishable from their bacterial cultures (figure 1a). Furthermore, these cultures contained approximately similar numbers of bacterial cells (approximately 10^{11} CFUs ml^{-1}) to samples collected from natural pitcher plants (10^9 – 10^{11} cells ml^{-1}) [10]. I encountered significantly negative slope estimates for shear viscosity measurements in *Darlingtonia* fluids and their bacterial cultures, suggesting shear-thinning (non-Newtonian) properties (figure 1b). These slopes, however, were weak in magnitude and may not be biologically relevant.

In prey capture experiments, all ants were retained in raw *Darlingtonia* pitcher fluid, regardless of its leaf of origin (figure 2a). Similarly, 97% of ants introduced into pitchers' bacterial cultures were also unable to escape. Upon contact with either fluid, ants immediately broke the liquid's surface tension and were completely submerged. This property was also observed—in informal field trials—for small ants and volant insects introduced into pitcher fluid. Ants were immobile by the end of the 10 min trial but recovered normal motor function after a period of 10–30 min following their removal from the fluid. While oxygen deficit is the most likely cause for this behaviour, the presence of some other stunning compound cannot be ruled out. None of the ants introduced into pure water broke the surface tension of the water. Those that did not exit the water remained on its surface and were active upon removal. Using logistic regression, I determined that the ratio of water to BACT was significantly positively associated with an ant's probability of escape ($\beta = 2.04 \pm 0.40$, $z = 5.04$, $p < 0.0001$). The response surface of this regression indicates that water : culture ratios below 10% were sufficient to retain the majority of ants (figure 2b).

In concert, these results suggest that the natural digestive bacterial associates of *D. californica* may additionally benefit their hosts by facilitating prey retention by the pitcher fluid. A molecular survey of microbial diversity in *Darlingtonia* fluid has previously demonstrated a high abundance of putative biosurfactant-producing genera [10] (e.g. *Pseudomonas* [19], *Pedobacter* [20], *Serratia* [21]), though further study is required to demonstrate their biosurfactant production *in situ*. A similar retentive property was described in *Nepenthes rafflesiana* [22], though its fluid was found to be highly viscoelastic—a property hypothesized to be caused by plant-secreted mucilage, rather than by bacteria. While all members of the family Sarraceniaceae possess foliar structures (e.g. downward-facing hairs) purportedly functioning to direct insects into the fluid [23], the altered physico-chemical properties of the fluid help to drown insects that would otherwise fail to break the surface tension and escape. Although the prey capture role provided by the bacterial community may not be as critical to the host plant's fitness as its digestive role, these results nonetheless highlight a potentially novel class of beneficial plant–microbe interactions worthy of continued study.

Ethics. This research did not make use of any organisms covered by UC Berkeley animal care and use regulations.

Data accessibility. All data are provided in the accompanying supplemental material.

Competing interests. The author declares no competing interests.

Funding. Funding was provided by an NSF Graduate Research Fellowship.

Acknowledgements. I thank W. Sousa, M. Badger and T. Dolinajec for instrumentation and J. Belsher-Howe (United States Forest Service, USFS) for field collection permits.

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