Award Number: W81XWH-12-2-0067

TITLE:
The Potential Application and Risks Associated With the Use of Predatory Bacteria as a Bio-control Agent Against Wound Infections

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REPORT DATE: October 2015

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE
October 2015

2. REPORT TYPE
Annual Summary

3. DATES COVERED
1Sep2014 - 31Aug2015

4. TITLE AND SUBTITLE
The Potential Application and Risks Associated With the Use of Predatory Bacteria as a Bio-control Agent Against Wound Infections

5a. CONTRACT NUMBER
W81XWH-12-2-0067

5b. GRANT NUMBER
DM110015

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Rutgers, The State University of New Jersey
Newark, NJ, 7101-0703

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Disease-causing microorganisms that have become resistant to drug therapy are an increasing cause of burn, wound, blast and bone infections, with many traditional antimicrobial agents becoming ineffective. Our main hypothesis is that predatory prokaryotes could serve as a novel therapeutic agent to control wound-related bacterial infections. In a previous study, we confirmed that predatory bacteria *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* are able to prey on a wide range of pathogens including bacteria isolated from Wounded Warriors. The aim of this proposal is to address key questions regarding the safety and efficacy of predatory bacteria and investigating predator prey interactions and resistance. Using enrichment culturing techniques we have verified that no genetically stable predation resistant phenotype developed in host cells following sequential predation. Our data also confirmed that the predators do not breach their host specificity and attack previously resistant bacteria. Cell toxicity assays, using human cell lines, demonstrated that predatory bacteria are significantly less toxic than the control. Finally, using a mouse wound model we determined that administering live predatory bacteria into exposed wounds did not cause any significant adverse effect to the animal or alter wound clinical score compared to a non-infected wound. However, at this point we have seen no statistically significant indication of enhance wound heeling in infected animals treated with the predators based on wound size, and weight change.

15. SUBJECT TERMS
Wounded soldiers, Predatory bacteria, Bio-control, Wound infections, Multi-drug-resistant, *Bdellovibrio bacteriovorus, Micavibrio aeruginosavorus*, Biofilms.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	93	19b. TELEPHONE NUMBER *(include area code)*

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Introduction

Disease-causing microorganisms that have become resistant to drug therapy are an increasing cause of burn, wound, blast and bone infections, while many traditional antimicrobial agents are becoming ineffective. Resistance can be considered as a natural response to the selective pressure of a drug and can develop in both free-floating bacteria, as well as, in surface-attached bacteria or biofilms. One of the major difficulties in controlling surface-attached bacteria is their enhanced resistance to antimicrobial agents i.e. biofilm bacteria can be up to 1000 times more resistant to antimicrobial agents than their planktonic counterparts. Thus, the high doses of antimicrobials required to rid wounds and medical devices of biofilms are impractical. The problem of multidrug-resistant (MDR) bacterial infections in the Wounded Warrior drove researchers to examine other potential anti-bacterial strategies. Among these alternative therapies is the use of biological control agents such as medical maggots, phage, biodebridement, and predatory bacteria.

Our main hypothesis is that predatory prokaryotes are able to serve as a novel topical therapeutic agent in controlling non-treatable, wound-related bacterial infections. In a previous study, we confirmed that predatory bacteria are able to prey on a wide range of pathogens including multidrug resistant bacteria isolated from Wounded Warrior. The aim of this proposal is to address key questions regarding the safety and efficacy of predatory bacteria in *ex vivo* and *in vivo* systems. The proposal is divided into three aims: (I) Investigating predator-prey/host bacteria interactions and resistance, (II) Determining the effect of predatory bacteria on mammalian cells, and (III) Measuring the efficacy of predation and toxicity in animal models.

Keywords
Wounded soldiers, Predatory bacteria, Bio-control, Wound infections, Multi-drug-resistant, Bdellovibrio bacteriovorus, Micavibrio aeruginosavorus, Biofilms.

Overall project summary

Aim-I of the proposed study was to investigate predator-prey/host bacteria interactions and resistance. The goal of this task was to examine key questions regarding adaptation of the host to the predator. The aim is divided into three subtasks:

Subtask 1.1. Development of genetically stable resistance to predation.
Subtask 1.2. Examine the ability of the predator to breach its host specificity and attack previously resistant bacteria.
Subtask 1.3. Enrich for hyper predatory variants.

Aim I. Investigating predator prey interactions and resistance.
The goal of this task was to investigate key questions regarding adaptation of the host to predation.

Subtask 1.1. Development of genetically stable resistance to predation.
Rationale. It is believed that, unlike antibiotics or phage therapy, the selective pressure of predation does not generate genetically stable resistant variants in the host. Since the appearance of host resistance might reduce the efficacy of predation, we conducted experiments aimed at increasing the selective pressure on the host and assessing if any genetically stable predation resistant phenotypes emerge.

Aim-1, Task-1, Subtask 1.1, Experiment 1. Enriching for host resistant phenotypes by culturing.

Host bacteria were cultured with the predator for 24 hrs (predation cycle), thereafter, the remaining host cells were collected by centrifugation, suspended in predator-free media and allowed to grow for an additional 24 hrs (growth cycle). The host cells were collected once more and fresh predators were added (predation cycle). The predation and growth cycle were repeated 20 times. Finally, the reduction in total host was evaluated by CFU enumeration and compared to the initial host reduction measured during the first predation cycle.

Experiment 1.1.1
In these experiments, we have sequentially cultured host bacteria with two predators, *B. bacteriovorus* 109J and *B. bacteriovorus* HD100. The host bacteria used in the experiment was *A. baumannii* NCIMB 12457. Initial predation was determined. Thereafter, host cells were sequentially cultured 20 times. The host population reduction at the final pass was evaluated. All experiments were conducted in triplicates. Data represent the average log change.

Initial predation

Initial reduction (log_{10}) of *A. baumannii* after co-culturing with *B. bacteriovorus* HD100, *B. bacteriovorus* 109J or predator free control.

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Initial average log change	+0.2	-2.3	-4.3

Final predation

Final average population reduction (log_{10}) of *A. baumannii* that was sequentially cultured 20 times on ***B. bacteriovorus* HD100**.

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Average log change	+0.2	-3.6	-3.7

Final average population reduction (log_{10}) of *A. baumannii* that was sequentially cultured 20 times on ***B. bacteriovorus* 109J.**

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Average log change	+0.2	-3.8	-3.6

Results. From the data above, it seems that a stable resistant phenotype didn't develop on *A. baumannii* cells which were sequentially cultured on *B. bacteriovorus* 109J or HD100.

Experiment 1.1.2

In these experiments, we have sequentially cultured host bacteria with two predators, *B. bacteriovorus* 109J and *B. bacteriovorus* HD100. The host bacteria used in this experiment was *K. pneumoniae* ATCC 33495. Initial predation was determined. Thereafter, host cells were sequentially cultured 20 times. The host population reduction at the final pass was evaluated. All experiments were conducted in triplicates. Data represent the average log change. All experiments were conducted in triplicates. Data represent the average log change.

Initial predation

Initial reduction of *K. pneumoniae* (log_{10}) after co-culturing with *B. bacteriovorus* HD100, *B. bacteriovorus* 109J or predator free control.

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Initial average log change	+0.1	-5	-2.7

Final predation

Final average population reduction (log_{10}) of *K. pneumoniae* that was sequentially cultured 20 times on ***B. bacteriovorus* HD100**.

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Average log change	-0.15	-4.4	-3.5

Final average population reduction (log_{10}) of *K. pneumoniae* that was sequentially cultured 20 times on ***B. bacteriovorus* 109J.**

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Average log change	+0.1	-5	-2

In order to confirm that genetically stable predation resistant phenotype did not develop, cultures from the final *B. bacteriovorus* 109J enrichment experiment were stored and re-examined in predation experiments.

Reduction of *K. pneumoniae*, that was sequentially cultured 20 times on *B. bacteriovorus* HD100, and stored.

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Average log change	0	-4.4	-2.7

Reduction of *K. pneumoniae*, that was sequentially cultured 20 times on *B. bacteriovorus* 109J, and stored.

	Control	*B. bacteriovorus* HD100	*B. bacteriovorus* 109J
Average log change	0	-5.0	-3.9

The data confirms that no stable resistance developed in *K. pneumoniae* following predation.

Results. The data obtained suggests that no genetically stable predation resistant phenotype developed in *K. pneumoniae* following sequential predation by *B. bacteriovorus* 109J and *B. bacteriovorus* HD100. Furthermore, no genetically stable predation resistant phenotypes developed in *A. baumannii* following sequential predation by *B. bacteriovorus* HD100 and *B. bacteriovorus* 109J.

Aim-1, Task-1, Subtask 1.1, Experiment 1.1.3

In this experiment, we have sequentially cultured host bacteria with *M. aeruginosavorus*. The host bacteria used in this experiment was *P. aeruginosa* Pa14. Initial predation was determined. Thereafter, host cells were sequentially cultured 20 times. The host population reduction at the final pass was evaluated. All experiments were conducted in triplicates. Data represent the average log change.

Initial predation

Initial reduction of *P. aeruginosa* Pa14 after co-culture with *M. aeruginosavorus* or predator free control.

	Control	*M. aeruginosavorus*
Initial average log change	+0.1	-2

Reduction of *P. aeruginosa* Pa14 after 15 cycle of passage with *M. aeruginosavorus* then co-cultured with *M. aeruginosavorus* or predator free control:

	Control	*M. aeruginosavorus*
Average log change	-0.3	-2.3

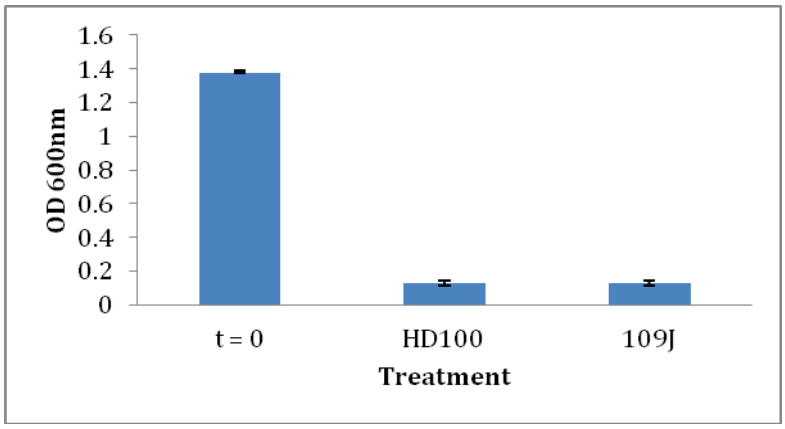
Results: From the above data it seems *P. aeruginosa* Pa14 did not develop resistance to predation by *M. aeruginosavorus*.

Aim-1, Task-1, Subtask 1.1, Experiment 2. *Enriching for biofilm predation resistant phenotypes.*

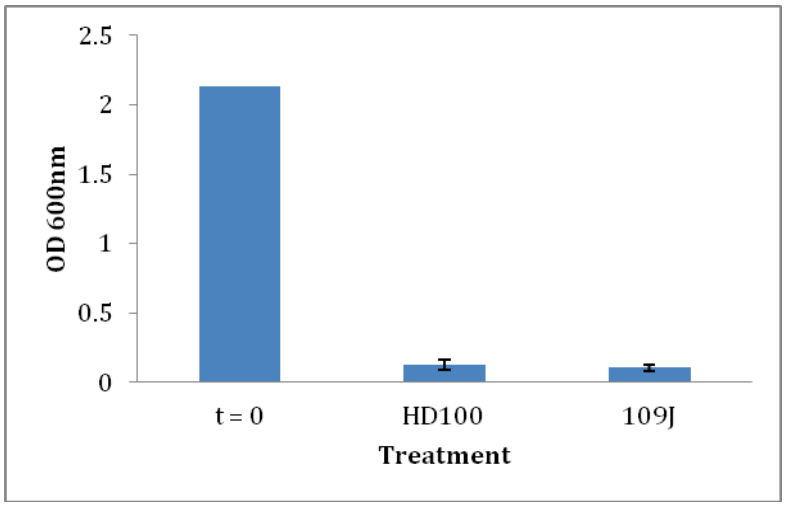
Objective. The aim of this task was to see if biofilm resistant phenotypes develop in biofilm that were cultured multiple times in the presence of the predator.

Bacteria, grown as biofilms, were sequentially grown in the presence of the two predators, *B. bacteriovorus* 109J and *B. bacteriovorus* HD100. The host bacteria used in this experiment was *A. baumannii* NCIMB 12457. The experiment was conducted in 8 wells for each treatment and examined for the emergence of a predation resistant biofilm. CV staining was used to quantify biofilm reduction due to predation.

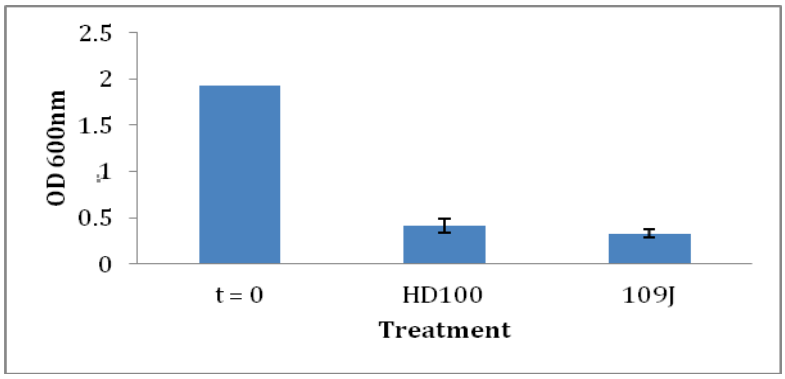
The data below represents the biofilm reduction in host cells in the initial co-culture.



The data below represents the biofilm reduction of host cells that were sequentially cultured 7 times on *B. bacteriovorus* HD100 and then exposed to the predators.



The data below represents the biofilm reduction of host cells that were sequentially cultured 7 times on *B. bacteriovorus* 109J and then exposed to the predators.



Results. The data obtained suggests that host cells grown as a biofilm do not adapt to form predation resistant biofilms.

Aim-1, Task-1, Subtask 1.1, Experiment 3. *Isolating host resistant mutants by induced mutagenesis.*

As culture selection alone did not impose sufficient selective pressure for inducing host resistant phenotypes, we attempted to produce predation resistant mutants by random mutagenesis. UV radiation and transposon mutagenesis were used to introduce mutations. Thereafter, the mutants were evaluated for the emergence of predation resistance. In this set of experiments *K. pneumoniae* ATCC 33495 was mutagenized and used as host for both *B. bacteriovorus* 109J and *M. aeruginosavorus*.

Outcome. At the completion of growth cycle 7 (out of 10) we could conclude that no predation resistant phenotypes were detected in *K. pneumoniae* following sequential predation with *B. bacteriovorus* 109J and *M. aeruginosavorus*.

Subtask 1.2. Investigating the ability of the predator to breach its host specificity and attack previously resistant bacteria.

Rationale. We have previously conducted experiments aimed at investigating the host range of each predator. However, it could be speculated that during the predation process alterations might cause a change in host specificity. Although a breach in host specificity could be less desirable, as it might allow the predators to attack communal non-pathogenic Gram-negative bacteria, it could be favorably used in order to increase the predatory portfolio of the predator

allowing it to attack new desired pathogens. In the following sets of experiments we investigate if a breach or alteration in predator host specificity could arise.

Subtask 1.2. Experiment 1. *Enriching for B. bacteriovorus 109J with altered host specificity.*

The aim of this experiment was to investigate if *B. bacteriovorus* will breach its host specificity and attack a host that initially was resistant to predation. To this end, *B. bacteriovorus* was co-cultured with two hosts *Stenotrophomonas maltophilia* ATCC 13636 and *Streptococcus epidermidis* ATCC 12228. The inability of the predators to prey on the host was confirmed (Table-1 and 3). Thereafter, we have attempted to enrich for *Bdellovibrio* variants that could attack the previously resistant bacteria (Table-2 and 4).

B. bacteriovorus 109J was suspended in a culture containing a 1:1 ratio of the non-host bacteria (*S. maltophilia* or *S. epidermidis*) and the host bacteria *E. coli* strain WM3064, a diaminopimelic acid (DAP) auxotroph. The specific *E. coli* was used in order to allow initial *Bdellovibrio* cell growth. After 48 hrs of predation, the *Bdellovibrio* was isolated by filtration (0.45 um filter) and re-cultured in a new co-culture containing a 2:1 ratio of non-host and host (predation resistant bacteria). The predation cycles were repeated 11 times, in each cycle the fraction of the host *E. coli* was reduced. Finally, *Bdellovibrio* cells were isolated and their ability to prey on *S. maltophilia* (Table-1 and 2) or *S. epidermidis* (Table-3 and 4) was examined. All experiments were conducted in triplicates. Data represent the average log change.

Table-1. Initial reduction of *S. maltophilia* after co-culturing with *B. bacteriovorus* 109J.

	Control	*B. bacteriovorus* 109J
Initial average log change	+0.2	-0.2

Table-2. Final population reduction of *S. maltophilia* exposed to predator-free control and the predator *B. bacteriovorus* 109J that was sequentially cultured with *S. maltophilia*.

	Control	*B. bacteriovorus* 109J
Initial average log change	+0.11	+0.11

Table-3. Initial reduction of *S. epidermidis* after co-culturing with *B. bacteriovorus* 109J.

	Control	*B. bacteriovorus* 109J
Final average log change	-0.41	-0.3

Table-4. Final population reduction of *S. epidermidis* exposed to predator-free control and the predator *B. bacteriovorus* 109J that was sequentially cultured with *S. epidermidis*.

	Control	*B. bacteriovorus* 109J
Final average log change	-0.55	-0.55

Result: The data obtained suggests that *B. bacteriovorus* does not have an ability to breach its host specificity and attack previously resistant bacteria.

Subtask 1.2. Experiment 2. *Enriching for M. aeruginosavorus with altered host specificity.*

The aim of this experiment was to investigate if sequential re-culturing of *M. aeruginosavorus* ARL-13 on a non-host bacteria will result in predation. To this end, *M. aeruginosavorus* was co-cultured with two hosts *Stenotrophomonas maltophilia* ATCC 13636 and *Streptococcus epidermidis* ATCC 12228. The inability of the predators to prey on the host was confirmed (Table-1A and 2A). Thereafter, we have attempted to enrich for *Micavibrio* variants that could attack the previously resistant bacteria (Table-3A and 4A).

M. aeruginosavorus was suspended in a culture containing a 1:1 ratio of the non-host bacteria (*S. maltophilia* or *S. epidermidis*) and the host bacteria *E. coli* strain WM3064, a diaminopimelic acid (DAP) auxotroph. The specific *E. coli* was used in order to allow initial *Micavibrio* cell growth. After 48 hrs of predation the *Micavibrio* lysate was re-cultured in a new co-culture containing a 2:1 ratio of non-host and host (predation resistant bacteria). The predation cycles were repeated 11 times, in each cycle the fraction of the host *E. coli* was reduced. Finally, *Micavibrio* cells were isolated and their ability to prey on *S. maltophilia* (Table-1 and 2) or *S. epidermidis* (Table-3 and 4) was examined. All experiments were conducted in triplicates. Data represent the average log change.

Table-1A. Initial reduction of *S. maltophilia* after co-culturing with *M. aeruginosavorus.*

	Control	*M. aeruginosavorus*
Initial average log change	+0.1	+0.1

Table-2A. Final population reduction of *S. maltophilia* exposed to predator-free control and the predator *M. aeruginosavorus* that was sequentially cultured with *S. maltophilia*.

	Control	*M. aeruginosavorus*
Initial average log change	-0.4	-0.4

Table-3A. Initial reduction of *S. epidermidis* after co-culturing with *M. aeruginosavorus.*

	Control	*M. aeruginosavorus*
Final average log change	-0.3	-0.3

Table-4A. Final population reduction of *S. epidermidis* exposed to predator-free control and the predator *M. aeruginosavorus* that was sequentially cultured with *S. epidermidis*.

	Control	*M. aeruginosavorus*
Final average log change	-0.33	-0.2

Result: The data obtained suggests that *M. aeruginosavorus* does not have an ability to breach its host specificity and attack previously resistant bacteria.

Subtask 1.3. Enriching for hyper predatory variants.
Rationale. As the long-term goal of our study is to develop predatory bacteria as a topical bio-control agent, isolating superior predatory isolates is desired. The aim of this task is to enrich for predator variants that exhibit an elevated predation phenotype.

Experiment 1. *Enriching for hyper virulent B. bacteriovorus.* Previous experiment showed that *B.bacteriovorus* 109J was able to reduce *A. baumannii* strain AB3917 and AB5256 by a **single log,** as opposed to a 5-log reduction seen on most isolates. The aim of this task was to sequentially culture the predator on each of the host in order to enrich for hyper virulent predators.

B. bacteriovorus 109J was co-cultured with *A. baumannii* AB3917 and *A. baumannii* AB5256 for 48 hrs and the reduction of host bacteria was measured (Tables 5 and 5A). After 48 hrs the *Bdellovibrio* was isolated by filtration (0.45um Milex) and re-cultured with fresh *A. baumannii*. As a control, *Bdellovibrio* was cultured with *E. coli*. The predation cycles were repeated 10 times. The predation ability of the culture-enriched *Bdellovibrio* was compared to a control *Bdellovibrio*, which was not co-cultured with *A. baumannii*. All experiments were conducted in triplicates. Data represent the average log change.

Table 5. Initial reduction of *A. baumannii* AB3917 after co-culture with *B. bacteriovorus* 109J or predator free control:

	Control	*B. bacteriovorus* 109J
Initial average log change	-0.3	-5.0

** The outcome of this experiment is somewhat unexpected, as we initially had seen only a one log reduction in the ability of *B. bacteriovorus* 109J to reduce *A. baumannii* AB 3917. We could only speculate that the ability of *B. bacteriovorus* 109J to reduce *A. baumannii* AB 3917 altered during storage of the predator in the lab and continuous passing of the predator.

To measure if we can enrich for hyper virulent *B. bacteriovorus*, the 109J strain was cultured on *A. baumannii* AB3917. As seen in Table 5, a slight increase in the ability of *B. bacteriovorus* to reduce AB3917 was seen.

Table 5a. Final reduction of *A. baumannii* AB3917 after co-culturing with predator free control and *B. bacteriovorus* 109J that was sequentially cultured on AB3917.

	Control	*B. bacteriovorus* 109J
Final average log change	+0.1	-5.5

Enriching for hyper virulent *B. bacteriovorus* on *A. baumannii* AB5256

To measure if we can enrich for hyper virulent *B. bacteriovorus*, the 109J strain was cultured on *A. baumannii* AB5256.

Table 6. Initial reduction of *A. baumannii* AB5256 after co-culturing with *B. bacteriovorus* 109J or predator free control:

	Control	*B. bacteriovorus* 109J
Initial average log change	+0.2	-0.9

Table 6a. Final reduction of *A. baumannii* AB5256 after co-culturing with predator free control and *B. bacteriovorus* 109J that was sequentially cultured with AB5256.

Experiment	Control	*B. bacteriovorus* 109J
Final average log change	-0.1	-0.6

Result: The data above shows that after continuous predation cycles there were no increase in the ability *B. bacteriovorus* 109J to reduce this specific strain.

Additional experiment.
Since *B. bacteriovorus* 109J that was cultured on *A. baumannii* AB5256 did not become more aggressive, we were interested in measuring if *B. bacteriovorus* 109J that was passed on AB3917 or *E. coli* will be more aggressive against this strain. Predation experiments were performed using *A. baumannii* AB5256 as host and *B. bacteriovorus* 109J that was passed on AB5256, AB3917 and *E. coli* (Table 7, 7a and 7c respectively) as predator.

Table 7. Reduction of *A. baumannii* AB5256 exposed to predator-free control and the predator *B. bacteriovorus* 109J sequentially cultured with AB5256:

Experiment	Control	*B. bacteriovorus* 109J
Average reduction log change	-0.1	-0.9

Table 7a. Reduction of *A. baumannii* AB5256 exposed to predator-free control and the predator *B. bacteriovorus* 109J sequentially cultured with AB3917.

	Control	*B. bacteriovorus* 109J
Average reduction log change	+0.5	-1.2

Table 7c. Reduction of *A. baumannii* 5256 exposed to predator-free control and the predator *B. bacteriovorus* 109J sequentially cultured with *E. coli*.

	Control	*B. bacteriovorus* 109J
Average reduction log change	+0.09	-1.2

Result: The data obtained suggests that *B. bacteriovorus* 109J could become more virulent on a particular host. However, this increase in predation could develop even in the absence of the prey. Culturing the predator with the prey could slightly enhance predation. On the other hand, increased predation does not develop on all prey with some host bacteria maintaining their tolerance even after continuous culturing. Therefore, we can conclude that enhanced predation is specific and could develop on some host bacteria and not others.

Subtask 1.3. Experiment 2. *Enriching for hyper virulent M. aeruginosavorus.* Previous experiments showed that *M. aeruginosavorus* ARL-13 was able to reduce *A. lwoffii* strain ATCC15309 by less than a log. The aim of this task was to sequentially culture the predator on each of the hosts in order to enrich for hyper virulent predators.

M. aeruginosavorus ARL-13 was co-cultured with *A. lwoffii* strain ATCC15309 for 48 hrs and the reduction of host bacteria was measured (Tables 8 and 8a). After 48 hrs the *M. aeruginosavorus* was isolated by filtration (0.45um Milex) and re-cultured with fresh *A. lwoffii*. The predation cycles were repeated 12 times. The predation ability of the culture-enriched *M. aeruginosavorus* was compared to a control *M. aeruginosavorus*.

Table 8. Initial reduction of *A. lwoffii* after co-culturing with *M. aeruginosavorus ARL-13* or predator free control:

	Control	*M. aeruginosavorus*
Initial average reduction log change	+0.2	-0.3

Table 8a. Final reduction of *A. lwoffii* after co-culturing with *M. aeruginosavorus* ARL-13 or predator free control:

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	Control	*M. aeruginosavorus*
Final average reduction log change	0	-0.24

Result: The data above shows that after continuous predation cycles, there was no increase in the ability of *M. aeruginosavorus* to reduce *A. lwoffii*.

Experiment 3. *Enriching for predatory bacteria variants, more efficient at predation at elevated temperatures.*

The aim of this experiment was to enrich for high temperature predation variants more suitable for medical application. To this end, predation of *Bdellovibrio* and *Micavibrio* at three different temperatures (30°C, 37°C and 39°C) was evaluated.

We observed that predators showed significant host reduction at temperatures 30°C and 37°C but not at 39°C. Hence, enrichment experiment was done using *B. bacteriovorus* 109J lysate and *Micavibrio* lysate from 37°C and then repeated several predation cycles after every 48 hrs until the temperature reached 39°C. In each cycle the incubation temperature was increased by 0.5°C until the temperature reached 39°C. The predators from the last cycle were isolated and their ability to prey at 39°C was measured.

In this experiment predation was measured by the reduction in culture turbidity using Synergy H1 Hybrid Reader.

The following Table-9 shows the average reduction in culture turbidity. Co-cultures were placed at 39°C, the highest temperature reached during the experiment. The following predators were used:
Sample A: Non temperature acclimated *Bdellovibrio.*
Sample B: Acclimated *Bdellovibrio.*
Sample C: Non temperature acclimated *Micavibrio.*
Sample D: Acclimated *Micavibrio*.

Table 9: Culture turbidity change. Data represent the average of three experiments.

Predator used	Non temperature acclimated *Bdellovibrio*	Acclimated *Bdellovibrio*	Non temperature acclimated *Micavibrio*	Acclimated *Micavibrio*
Average change in culture turbidity	3.6± 3%	43±9%	14±9%	37±5%

Summary: The data obtained suggests that *Bdellovibrio* 109J and *Micavibrio* could be acclimated to prey at an elevated temperature of 39°C.

Additionally, we ran the temperature predation experiment with both *Bdellovibrio* 109J and *Micavibrio* using the Synergy H1 Hybrid Reader. *Bdellovibrio* 109J and *Micavibrio lysates were* prepared and incubated in the spectrophotometer at 39°C for 80 hrs. The change in culture turbidity was measured.

The predators used were:
Sample A: Non temperature acclimated *Bdellovibrio.*
Sample B: Acclimated *Bdellovibrio.*

Sample C: Non temperature acclimated *Micavibrio.*
Sample D: Acclimated *Micavibrio*.

Fig 1: *K. pneumoniae* predator-free control incubated at 39°C.

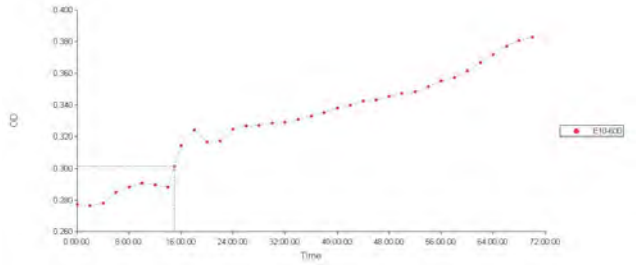


Fig 2: *K. pneumoniae* incubated with a non-temperature acclimated *B. bacteriovorus 109J* set at 39°C.

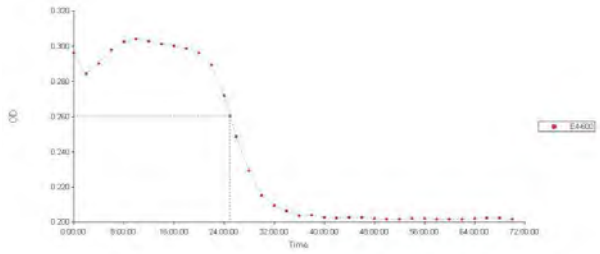


Fig 3: *K. pneumoniae* incubated with a temperature acclimated *B. bacteriovorus 109J* set at 39°C.

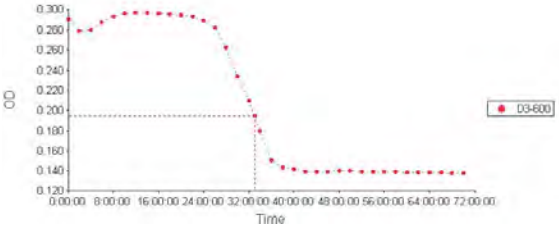


Fig 4: *K. pneumoniae* incubated with a non-temperature acclimated *M. aeruginosavorus* set at 39°C.

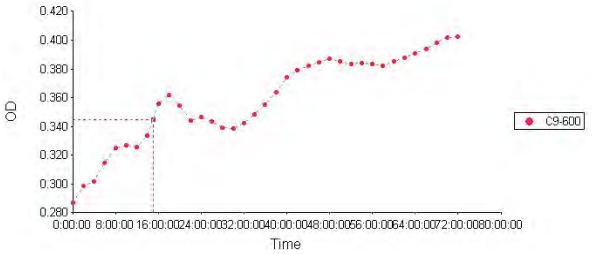
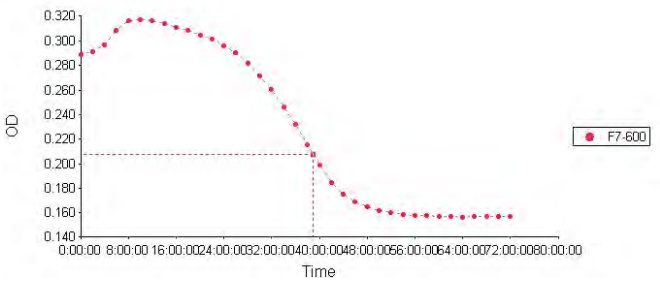


Fig 5: *K. pneumoniae* incubated with a temperature acclimated *M. aeruginosavorus* set at 39°C.



Result: The data suggests that *B. bacteriovorus* 109J as well as *M. aeruginosavorus* could be acclimated to prey at elevated temperature of 39^{o}C.

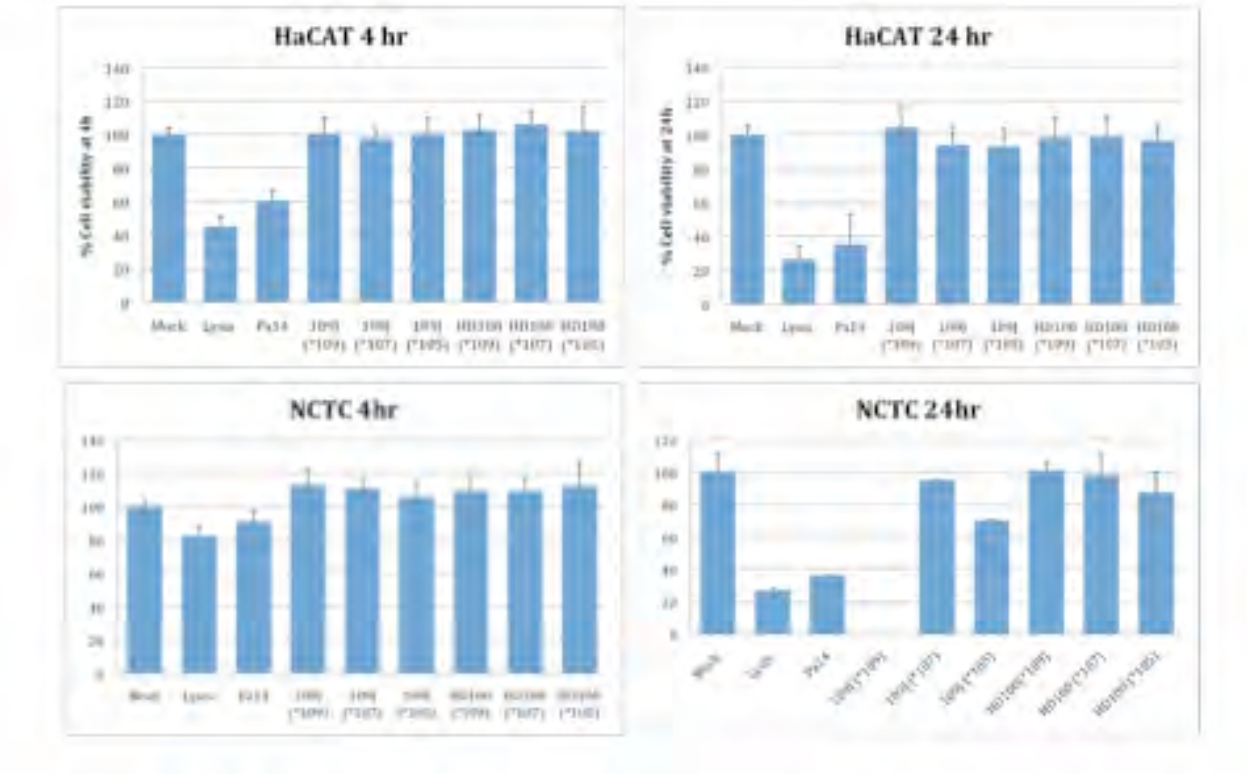
Aim II. Determining the effect of predatory bacteria on eukaryotic cells. Although the effect of predation on prokaryotic Gram-negative cells is documented, limited data is available regarding predation on eukaryotic cells. As the goal of our research is to utilize predatory bacteria as a live antibiotic, examining the potential risk of predatory bacteria on non-microbial host is essential for the development of a safe bio-control agent.
The goal of this aim is to determine if predatory bacteria have an adverse affect on eukaryotic cells. The aim is divided into three subtasks.

Subtask 2.1. *Determining the toxicity of predatory bacteria on mammalian cells.*
Subtask 2.2. *Determining the influence of predator exposure on cell cytokine profile.*
Subtask 2.3. *Cell attachment and invasion assay.*

Subtask 2.1. *Determining the toxicity of predatory bacteria on mammalian cells.*
Rationale. It was previously demonstrated that, when added to human cell lines, some Gram-negative bacteria could induce cell death. In order to investigate whether predatory bacteria have an adverse toxic affect on eukaryotic cells, we introduced predatory bacteria to mammalian cell cultures and examined the change in cell viability.

The effect of *B. bacteriovorus* 109J and HD100 on mammalian cell viability was investigated. Initial experiments were conducted with 3 different concentrations of predatory bacteria following 4 and 24 hrs of exposure. Each experiment was conducted on 2 selected cell lines; HaCAT human epidermal keratinocytes, and NCTC mouse fibroblasts. Three experiments were conducted. The effect of predatory bacteria on mammalian cell viability was examined using PrestoBlue^{TM} cell viability reagent.

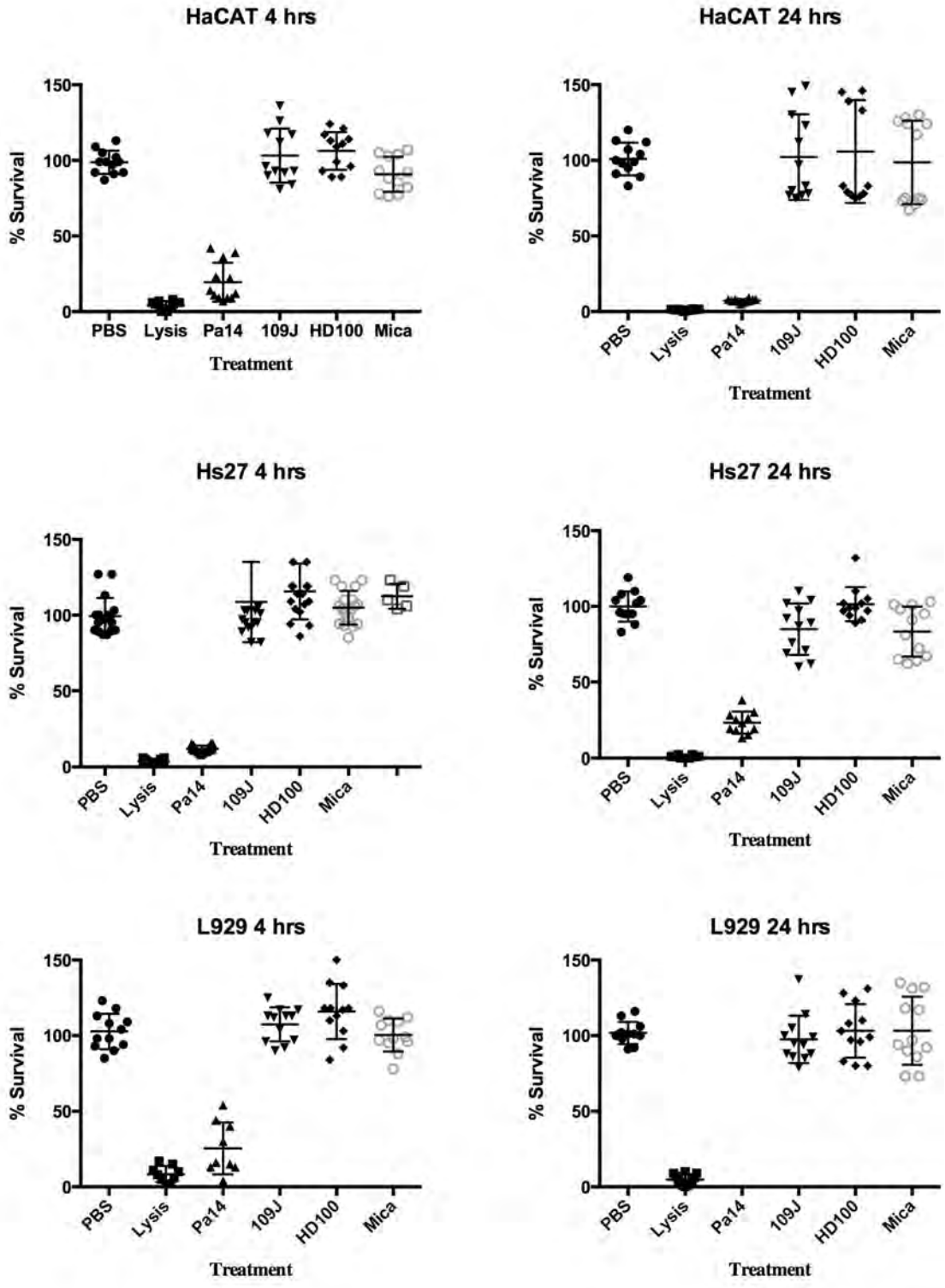
Outcome. The data below represents the result of the cell toxicity assays. The data represents the average and SD of 9 replicates from 3 separate experiments. The data obtained suggests that *B. bacteriovorus* 109J and HD100 are significantly less toxic than the *Pseudomonas aeruginosa* used as control (10^9 CFU/ml).



Since we did not see any significant killing by the predators, we continued the experiment using only the high cell density of the predatory bacteria.

In the following experiment, 3 predatory bacteria ($1x10^9$/ml) were co-cultured with the three selected cell lines: human epidermal keratinocytes (HaCaT), human skin fibroblasts (Hs27) and mouse fibroblasts (NCTC L929).

Outcome. No statistical significant reduction in cell viability was observed when predatory bacteria ($1x10^9$/ml) were co-cultured with any of the examined cell lines. The data below represents the result of the cell toxicity assay. The data represents the average and SD of 12 replicates from 4 separate experiments. The data obtained suggests that *B. bacteriovorus* 109J and HD100 and *Micavibrio* are significantly less toxic than the *P. aeruginosa* (10^9 CFU/ml) used as control.

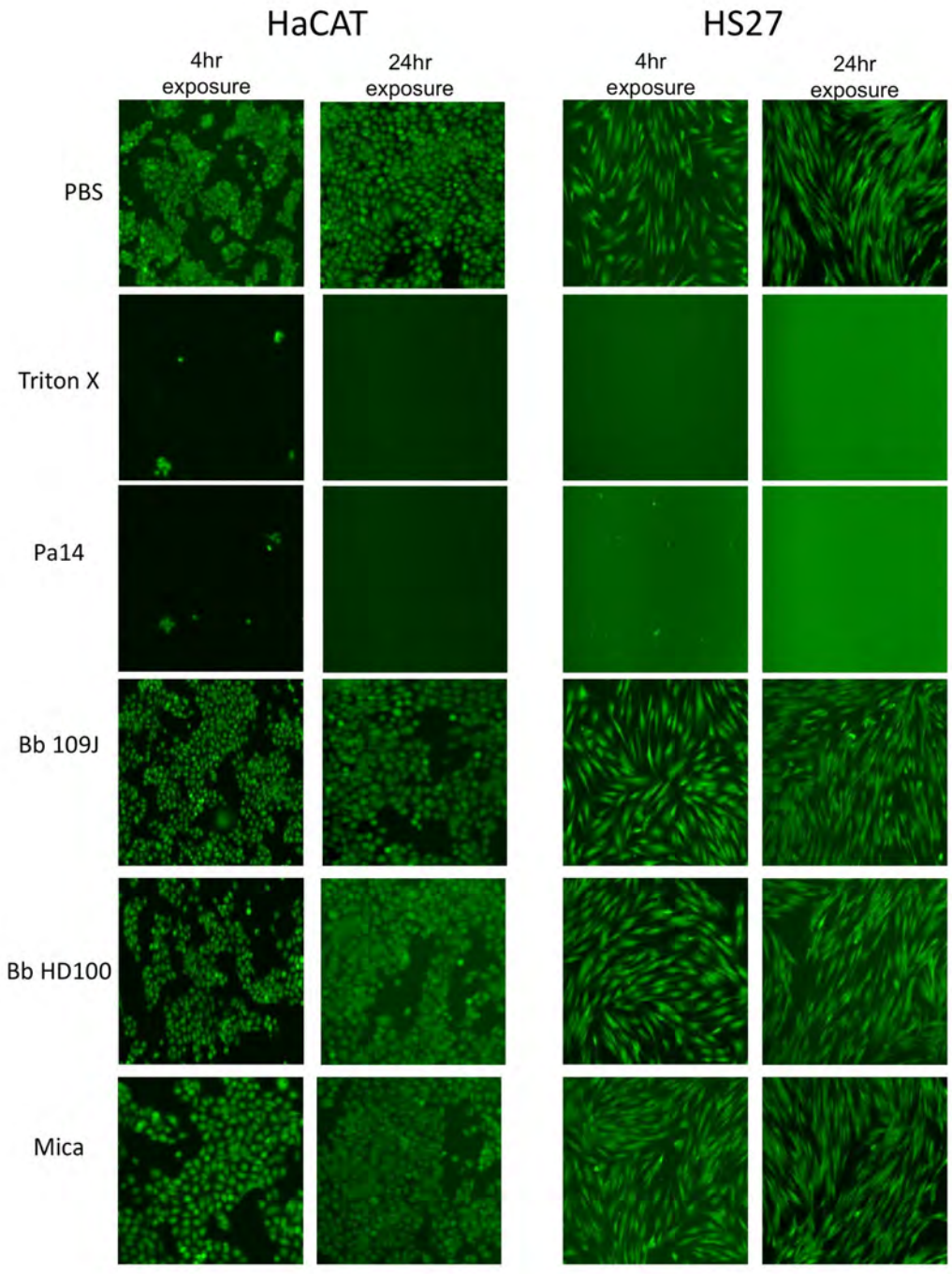


Conclusion. Predatory bacteria do not have a negative effect on eukaryotic cells' viability when examined ex-vivo.

Imaging cell lines following incubation with predatory bacteria:

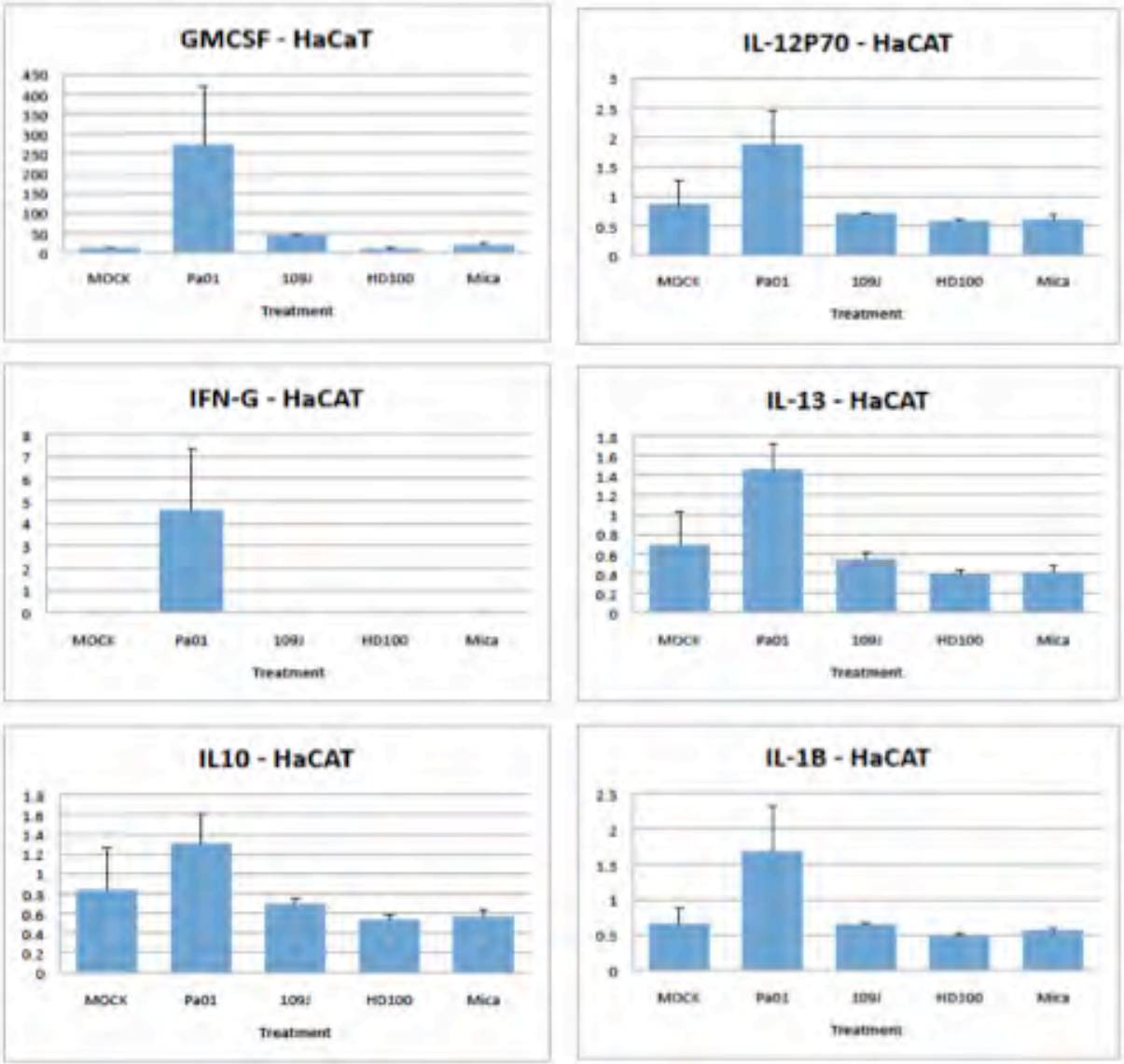
To further demonstrate that predatory bacteria have no negative effect on mammalian cell viability and structure, HaCAT human epidermal keratinocytes and Hs27 human skin fibroblasts were exposed for 4 and 24 hrs to predatory bacteria ($1x10^9$/ml). Thereafter, light microscopy and Fluorescent microscopy were used to evaluate cell morphology. In these experiments PBS was used as a negative control while Triton X and *P. aeruginosa* Pa14 were used as a positive control.

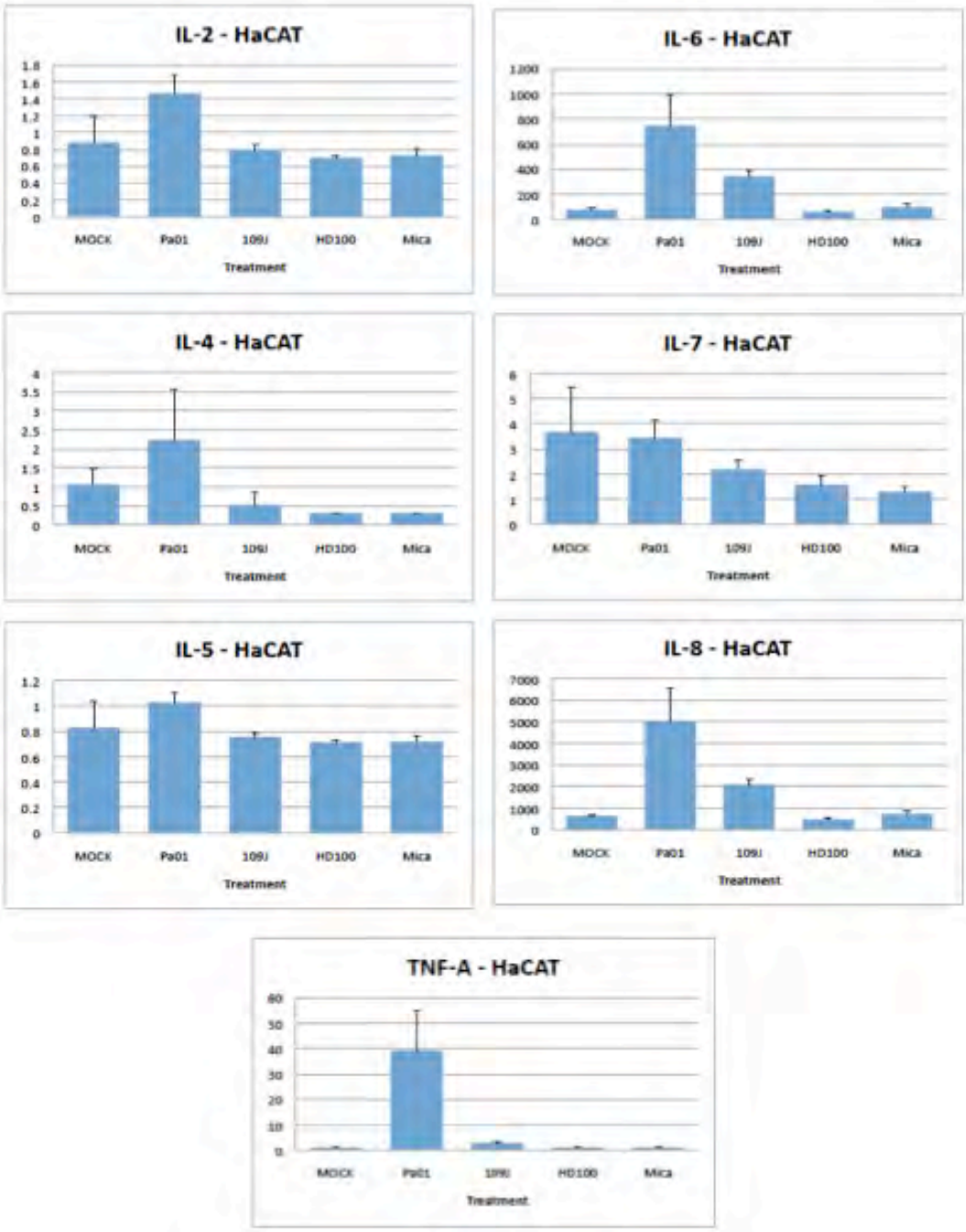
Result and conclusion. As seen in the figure below, total cell detachment was seen when the examined cell lines were exposed for 4 hrs or more to the Triton X or *P. aeruginosa* Pa14. However, no cell detachment or morphological changes were seen in cells which were incubated with the predators, conforming that predatory bacteria are non toxic to mammalian cell lines.


HaCAT
HS27
4hr exposure
24hr exposure
4hr exposure
24hr exposure
PBS
Triton X
Pa14
Bb 109J
Bb HD100
Mica

Subtask 2.2. *Determine the influence of predator exposure on cell cytokine profile.*
Rationale. Exposing cells to microbial challenge could alter cell physiology. Among the changes less desired in wounds, which are already challenged by bacteria, is the overproduction of proinflammatory immune mediators. To examine the effect of predatory bacteria on cell cytokine profile, HaCAT and Hs27 cell lines were grown in 24 well plates and exposed for 4 hrs to 10^9 PFU/ml of each of the 3 predators. Thereafter, the culture media was collected and the pro-inflammatory cytokine profile was measured using the Human High Sensitivity Cytokine Panel (Millipore). Non-bacteria PBS (Mock) exposed cells were used as negative control. For positive control, cells were exposed to *P. aeruginosa* PAO1 which is known to be less cytotoxic than the PA14 strain, thus allowing positive cytokine induction while limiting cell death. Each experiment was conducted with 4 wells for each treatment.

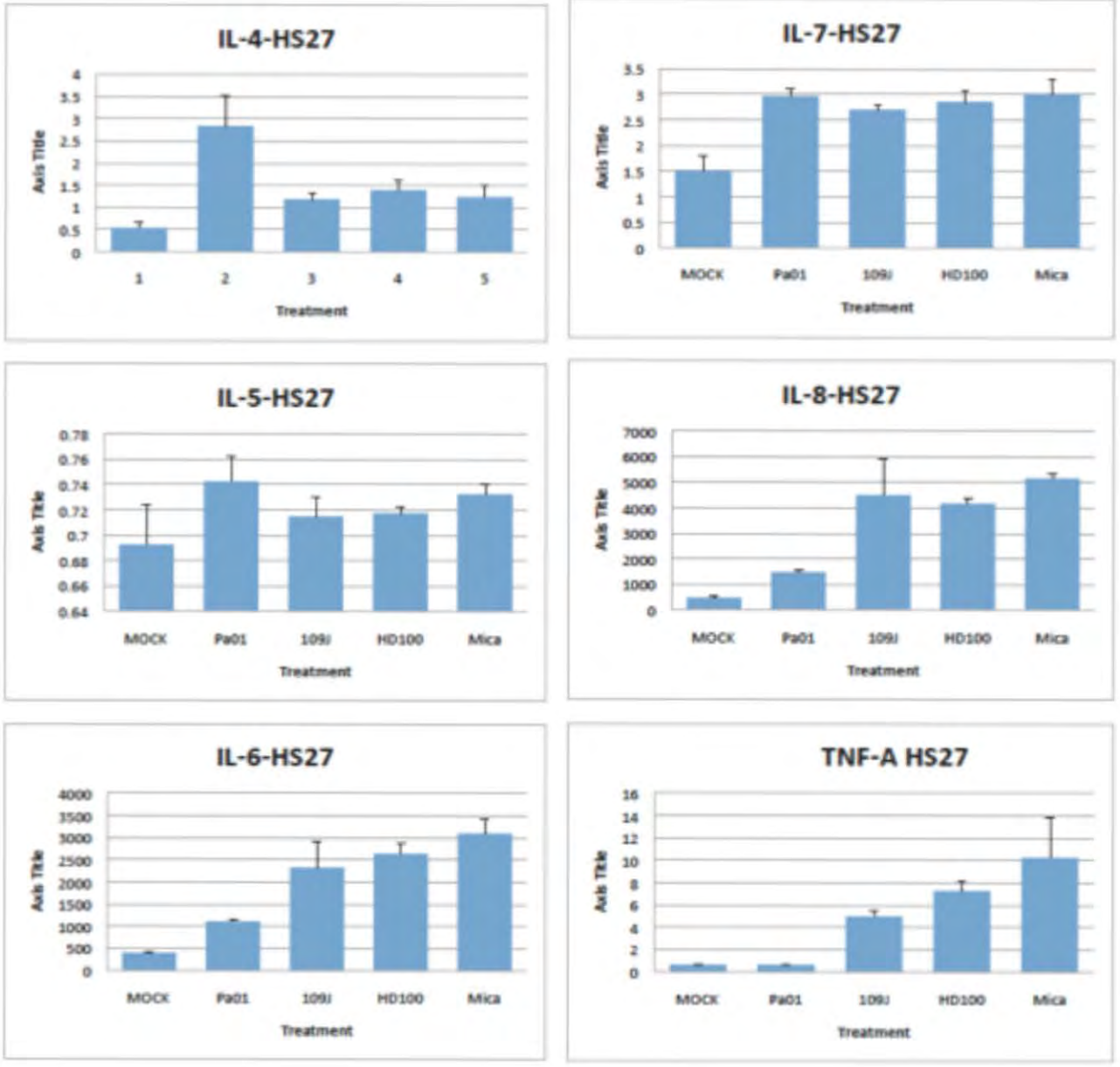
Cytokine profile of HaCAT cells following incubation with predatory bacteria.

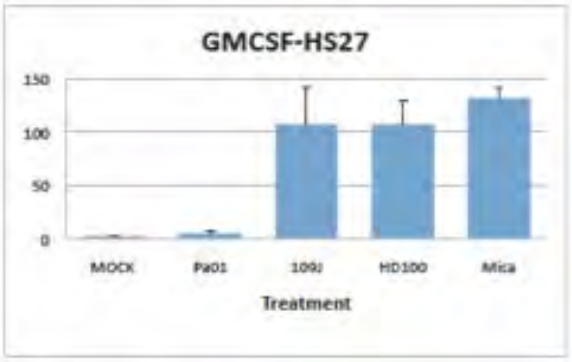


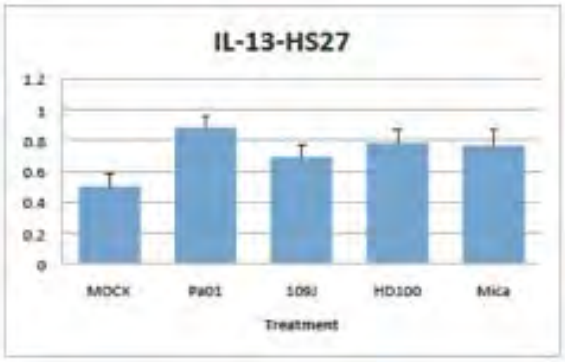


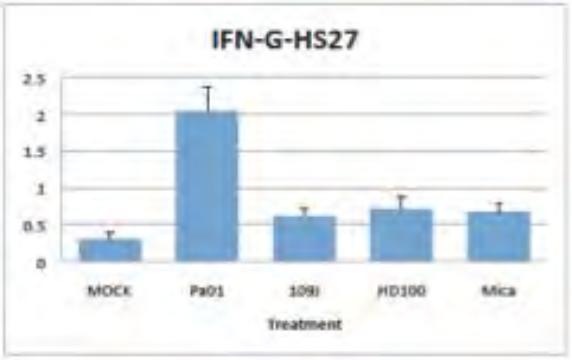
Results. Our data shows that exposing HaCAT cells to high levels of predatory bacteria causes no elevation in cytokine production in 11 out of the 13 examined cytokines. However, both IL-8 and IL-6 were slightly elevated, compared to the control, in cells exposed to *Bdellovibrio* 109J. An increase in cytokine production was seen in 11 out of the 13 examined cytokines after exposure to *P. aeruginosa* PAO1. For IL-5 and IL-7, comparable cytokine levels were measured for the control and *P. aeruginosa* treated sample.

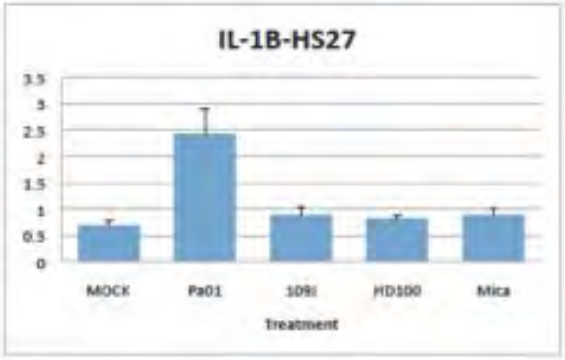
Cytokine profile of Hs27 cells following incubation with predatory bacteria.

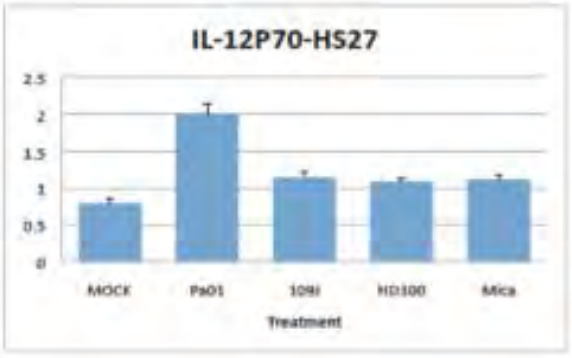


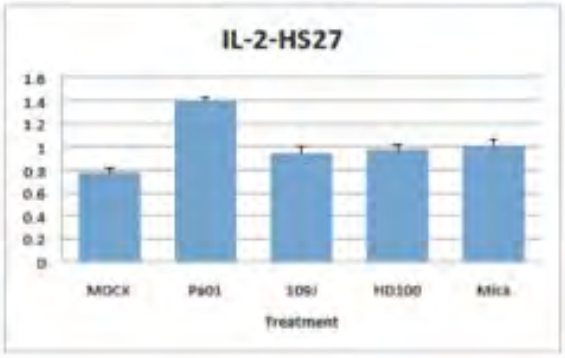

GMCSF-HS27
150
100
50
0
MOCK
Pa01
109J
HD100
Mica
Treatment

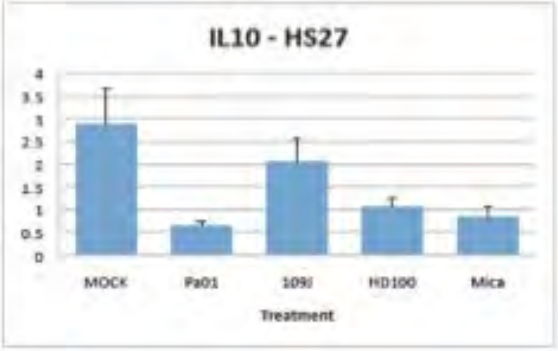

IL-13-HS27
1.2
1
0.8
0.6
0.4
0.2
0
MOCK
Pa01
109J
HD100
Mica
Treatment


IFN-G-HS27
2.5
2
1.5
1
0.5
0
MOCK
Pa01
109J
HD100
Mica
Treatment


IL-1B-HS27
3.5
3
2.5
2
1.5
1
0.5
0
MOCK
Pa01
109J
HD100
Mica
Treatment


IL-12P70-HS27
2.5
2
1.5
1
0.5
0
MOCK
Pa01
109J
HD100
Mica
Treatment


IL-2-HS27
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
MOCK
Pa01
109J
HD100
Mica
Treatment


IL10 - HS27
4
3.5
3
2.5
2
1.5
1
0.5
0
MOCK
Pa01
109J
HD100
Mica
Treatment

Results. The cytokine profile of Hs27 cells exposed to predatory bacteria was found to be different than the HaCAT cells. Three of the 13 cytokines (IL-2, IL-12 and IL-1β) showed no change compared to the PBS control. IL-10 (an anti-inflammatory cytokine) was found to be reduced in cells exposed to the bacteria compared to the PBS control (Mock). Four out of the 13 cytokines were slightly elevated, compared to the PBS control, but less than the *P. aeruginosa*. IL-7 expression was found to be stimulated to similar levels by all bacteria treatments. Finally, exposing the cells to predatory bacteria had caused elevation in IL-6, IL-8, TNF-A and Granulocyte-macrophage colony-stimulating factor (GM-CSF).

Subtask 2.3. Cell attachment and invasion assay.
Rationale. As the biology of predatory bacteria involves the attachment and invasion of the microbial prey, the predatory bacteria might also be able to attach and invade mammalian cells. To determine if cell attachment and invasion does occur, cell cultures were grown in 24 well plates. Cells were removed and placed in a tube (100,000 per tube). Thereafter purified predatory bacteria were added. After 45 min, the cells were washed, to remove unattached bacteria, sonicated briefly and plated to determine microbial load. Experiments were conducted twice in triplicates. *P. aeruginosa* PaO1 was used as a positive control.

Results.

Attachment of predatory bacteria to HaCAT human epidermal keratinocytes cells:

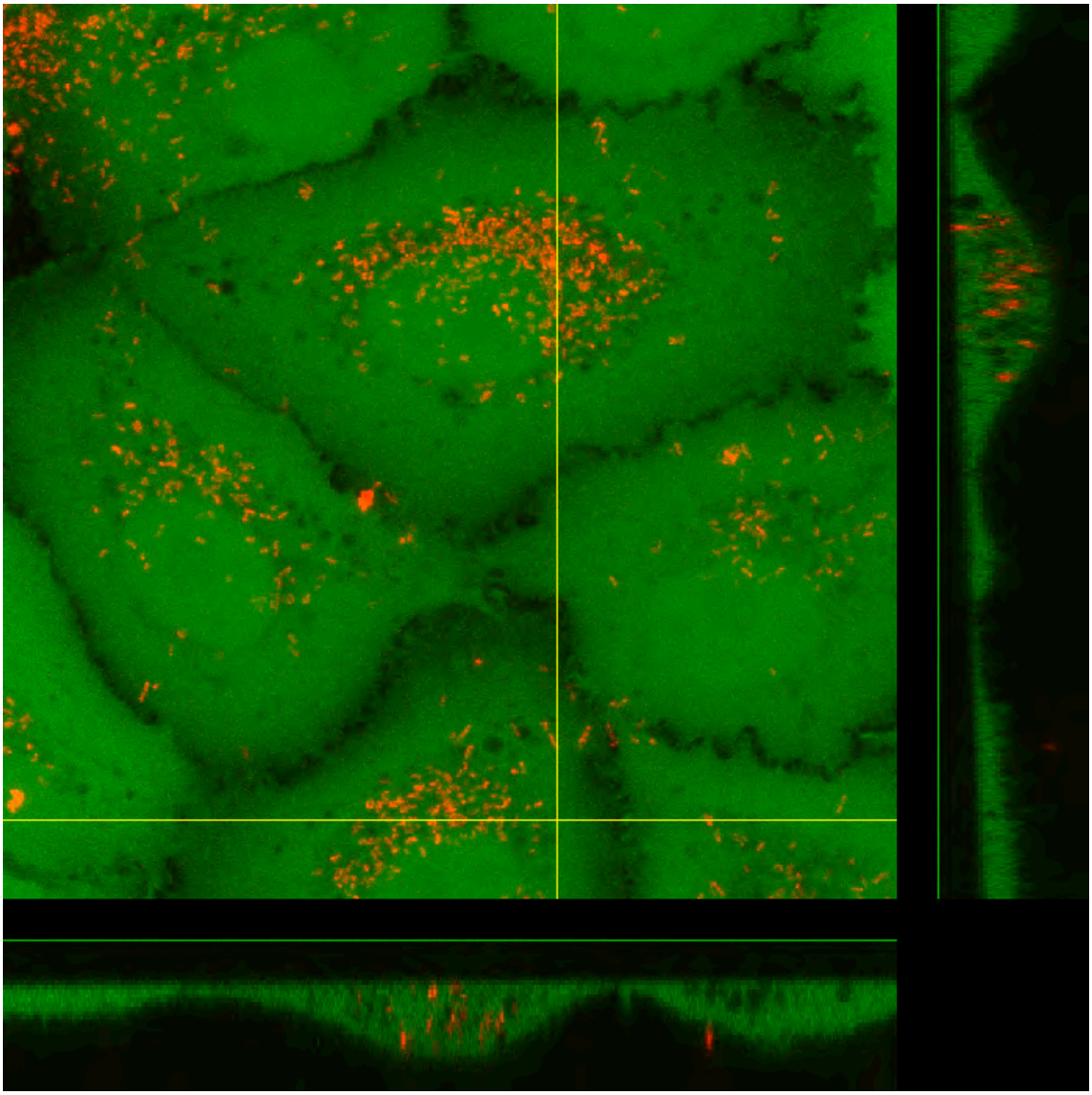
Predator	Initial bacteria concentration	Average predators attached following 45 min of incubation
B. bacteriovorus 109J	3.5×10^{11}	$2.7 \pm 0.7 \times 10^{5}$
B. bacteriovorus HD100	2.4×10^{10}	$4.8 \pm 0.8 \times 10^{5}$
M. aeruginosavorus ARL-13	1.8×10^{9}	$2.5 \pm 0.5 \times 10^{4}$
P. aeruginosa control	6.2×10^{10}	$3.1 \pm 0.2 \times 10^{7}$

Attachment of predatory bacteria to Hs-27 human skin fibroblasts cells:

Predator	Initial bacteria concentration	Average predators attached following 45 min of incubation
B. bacteriovorus 109J	3.75×10^{11}	$8 \pm 0.9 \times 10^{5}$
B. bacteriovorus HD100	1.2×10^{10}	$3.9 \pm 1.4 \times 10^{5}$
M. aeruginosavorus ARL-13	7×10^{8}	$1.7 \pm 0.5 \times 10^{4}$
P. aeruginosa control	7.1×10^{10}	$3.9 \pm 0.6 \times 10^{7}$

Result and conclusion. Our finding demonstrates that predatory bacteria are capable of attaching to human cell lines. However, based on the fact that cell death was not observed in any of our experiments, we could conclude that the attachment does not lead to cell loss.

To confirm *Bdellovibrio* attachment to mammalian cells, we have introduced the pMQ414 plasmid, expressing the tdTomato fluorescent reporter protein, into *B. bacteriovorus* 109J. The fluorescent predator cells were incubated with mammalian cells and examined using a confocal microscopy. Red fluorescent predators attached to mammalian cells could be clearly seen. The image confirms our finding that predatory bacteria could attach to the eukaryotic cells without causing cell damage.



Aim III. Efficacy and toxicity in animal models.

Subtask 3.1. *Determining the toxicity/efficacy of predatory bacteria in a mouse puncture wound model.*

The goal of this aim was to evaluate whether predatory bacteria have a toxic effect on small animals using a range of doses as inoculum into open wounds. In this task we had used a murine wound model of infection developed by collaborators at WRAIR (Zurawski Lab). This part of the study was done and reported by Dr. Zurawski at WRAIR.

Objective: The primary objective of these experiments is to assess the safety of live *Bdellovibrio bacteriovorus* or *Micavibrio aeruginosavorus* administration application on exposed wounds in the mouse wound model. The primary measures of safety in these experiments are weight loss / gain over time of the mice, mice clinical score (recorded daily), Histopathology report on wound samples taken 24h post-surgery, Cytokine profile, and mice wound size. These experiments use either cyclophosphamide treated mice (immunocompromised model) or normal mice. Treatment was done with a single application of predatory bacteria in PBS.

Questions to be answered

Safety of *Bdellovibrio bacteriovorus (*Bb) or *Micavibrio aeruginosavorus (MA)* application and dose effect on immunocompromised mouse health.

Time-to-close – Is the kinetic of wound closure in this immunocompromised model changes upon treatment with *Bdellovibrio bacteriovorus* or *Micavibrio aeruginosavorus*?

Clinical signs of wounding – Is there a change in clinical scores upon treatment with *Bdellovibrio bacteriovorus* or *Micavibrio aeruginosavorus*?

Weights – Is the weight of mice treated with *Bdellovibrio bacteriovorus* or *Micavibrio aeruginosavorus* altered compared to mock treated mice?

Gross pathology – Does gross wound appearance of mice change with *Bdellovibrio bacteriovorus* or *Micavibrio aeruginosavorus* application?

Histopathology of wound healing post wound closure – Does the wound appear in any way different from histopathology perspective upon treatment with *Bdellovibrio bacteriovorus* or *Micavibrio aeruginosavorus*?

Does exposure of the wound to *Bdellovibrio bacteriovorus* or *Micavibrio aeruginosavorus* affects the cytokine profile in the mouse once the wound closes?

Experiment 1.

Objective. The primary objective of the experiment was to assess the safety of live *Bdellovibrio bacteriovorus* administration on exposed wounds in the mouse wound model. The primary measures of safety in these experiments were weight loss of mice and mice clinical score taken daily.

The experimental groups were:

- G1 - 5 mice wounded and treated once with $5x10^8$ PFU/wound *Bdellovibrio bacteriovorus 109J*.
- G2 - 5 mice wounded and treated once with $5x10^4$ PFU/wound *Bdellovibrio bacteriovorus 109J*.
- G3 - 5 mice wounded and treated once with $5x10^8$ PFU/wound *Bdellovibrio bacteriovorus HD100*.
- G4 - 5 mice wounded and treated once with $5x10^4$ PFU/wound *Bdellovibrio bacteriovorus HD100*.
- G5 – 4 Mice wounded and treated with PBS to serve as controls for treatments

Time points and samples collected:
Mice wound size and weights were recorded at the designated time intervals (Day 0,1,3,6,8,10, and 13).
Serum samples were taken at the end of the experiment (when wounds were closed).

Outcome.
No Significant difference with respect to weight and or wound size was detected.

Experiment 2.

Objective: The objective of this experiment is to assess the safety of live *Bdellovibrio bacteriovorus* administration application on exposed wounds in the mouse wound model. The primary measures of safety in these experiments were weight loss of mice and mice clinical score taken daily. This experiment was a biological repeat and includes 9 additional mice used for the 24h post infection time point cytokine and histological analysis.

The experimental groups were:

- G1 – 5+3 mice wounded and treated once with $2.5x10^8$ PFU/wound *Bdellovibrio bacteriovorus 109J*.

- G2 - 5 mice wounded and treated once with $2.5x10^4$ PFU/wound *Bdellovibrio bacteriovorus 109J*.

- G3 – 5+3 mice wounded and treated once with $1.62x10^8$ PFU/wound *Bdellovibrio bacteriovorus HD100*.

- G4 - 5 mice wounded and treated once with $1.62x10^4$ PFU/wound *Bdellovibrio bacteriovorus HD100*.

- G5 – 5+3 Mice wounded and treated with PBS to serve as controls for treatments

Time points and samples collected: Mice wound size and weights were recorded at the designated time intervals (Day 0,1,3,6,8,10, and 13).
Serum samples were taken at the end of the experiment (when wounds were closed).
Three mice from groups G1, G3, and G5 were sacrificed at 24h post-surgery. Wounds were extracted and submitted for Histological analysis.

Outcome.
No Significant difference with respect to weight and or wound size was detected. No difference in the histology analysis at 24h post-surgery was detected.

Experiment 3.

Objective: The primary objective of this experiment is to assess the safety of live *Bdellovibrio bacteriovorus* administration application on exposed wounds in the mouse wound model. The primary measures of safety in these experiments will be weight loss of mice and mice clinical score (recorded daily). This experiment uses cyclophosphamide (CP) treated mice (immune-compromised model) to assess safety.

The experimental groups were:

- G1 – 5+3 CP pre-treated mice wounded and treated once with $5x10^8$ PFU/wound *Bdellovibrio bacteriovorus 109J*.

- G2 - 5 CP pre-treated mice wounded and treated once with $5x10^4$ PFU/wound *Bdellovibrio bacteriovorus 109J*.

- G3 – 5+3 CP pre-treated mice wounded and treated once with $8x10^8$ PFU/wound *Bdellovibrio bacteriovorus HD100*.

- G4 - 5 CP pre-treated mice wounded and treated once with $8x10^4$ PFU/wound *Bdellovibrio bacteriovorus HD100*.

- G5 – 5+3 CP pre-treated mice wounded and treated with PBS to serve as controls for treatments.

Time points and samples collected:
Mice wound size and weights were recorded at the designated time intervals (Day 0,1,3,6,8,10, and 13).
Serum samples were taken at the end of the experiment (when wounds were closed).
Three mice from groups G1, G3, and G5 were sacrificed at 24h post-surgery. Wounds were extracted and submitted for Histological analysis.

Outcome.
No Significant difference with respect to weight and or wound size was detected.
No difference in the histology analysis at 24h post-surgery was detected.

Experiment 4.

Objective: The primary objective of this experiment is to assess the safety of live *Micavibrio aeruginosavorus* administration application on exposed wounds in the mouse wound model. The primary measures of safety in these experiments will be weight loss of mice and mice clinical score (recorded daily). This experiment uses cyclophosphamide (CP) treated mice (immune-compromised model) to assess safety.

The experimental groups were:

- G1 – 5+3 mice wounded and treated once with $1x10^8$ PFU/wound *M. aeruginosavorus*.
- G2 – 5+3 mice wounded and treated once with $1x10^4$ PFU/wound *M. aeruginosavorus*.
- G3 – 5+3 Mice wounded and treated with PBS to serve as controls for treatments
- G4 – 5+3 CP pre-treated mice wounded and treated once with $5x10^8$ PFU/wound *M. aeruginosavorus*.
- G5 – 5+3 CP pre-treated mice wounded and treated once with $5x10^4$ PFU/wound *M. aeruginosavorus*.
- G6 – 5+3=8 CP pre-treated mice wounded and treated with PBS to serve as controls for treatments.

G1, G3-G6 were sacrificed at 24h post-surgery. Wounds were extracted and submitted for Histological analysis.

Time points and samples collected:
Mice wound size and weights were recorded at the designated time intervals (Day 0,1,3,6,8,10, and 13).
Serum samples were taken at the end of the experiment (when wounds were closed).
Three mice from groups G1, G3-G6 were sacrificed at 24h post-surgery. Wounds were extracted and submitted for Histological analysis.

Outcome.

No Significant difference with respect to weight and or wound size was detected.
No difference in the histology analysis at 24h post-surgery was detected.

Conclusion. Predatory bacteria do not seem to have a negative effect on mice well-being or wound healing when administered to open wounds of healthy animals or animals which are immunocompromised.

Average data collected throughout the study was analyses summarized and reported below.

Results

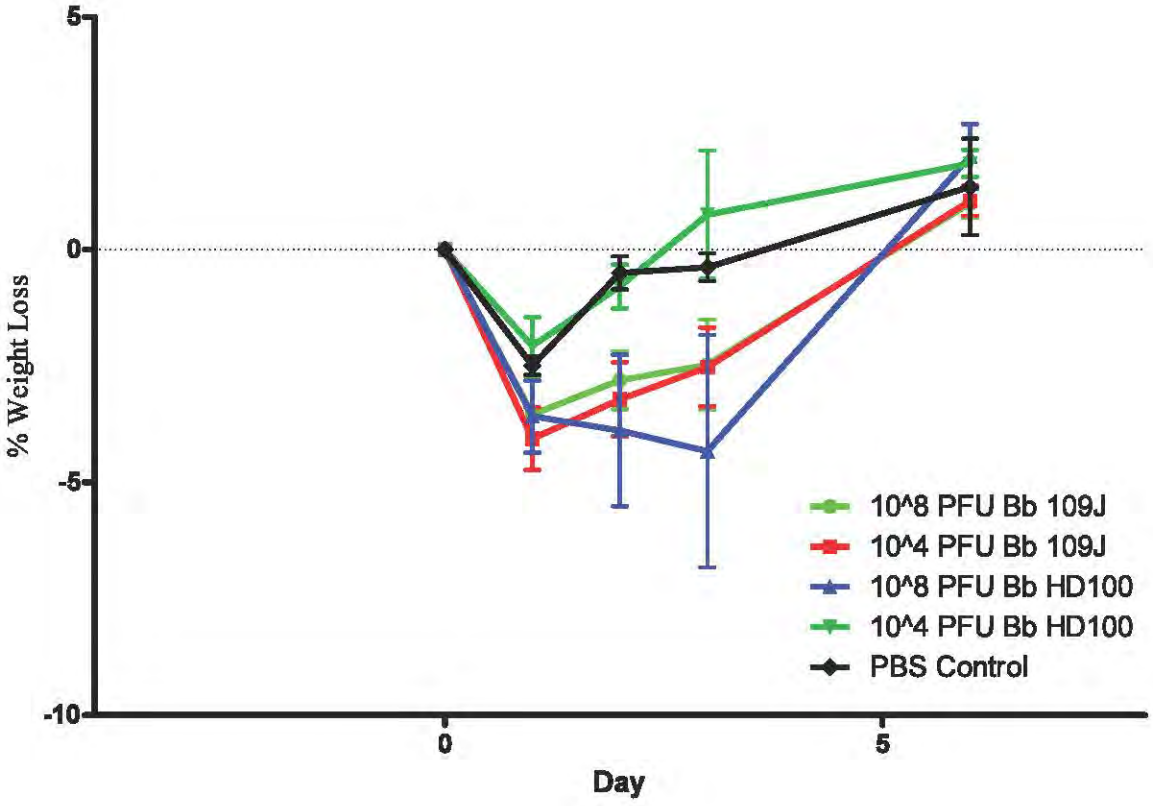
All mice survived the treatment and were on course to full recovery or fully recovered by the end of the experiment.

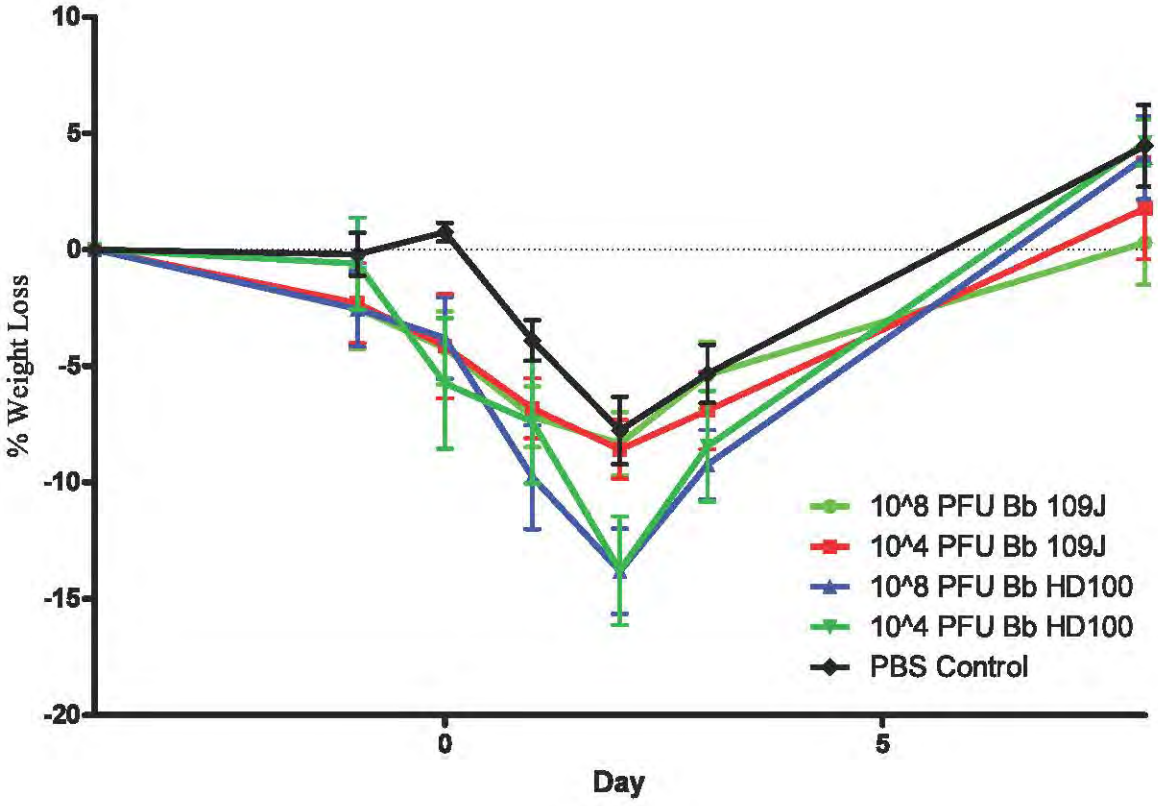
Clinical scores were similar in all groups during the first days until no clinical signs could be observed.

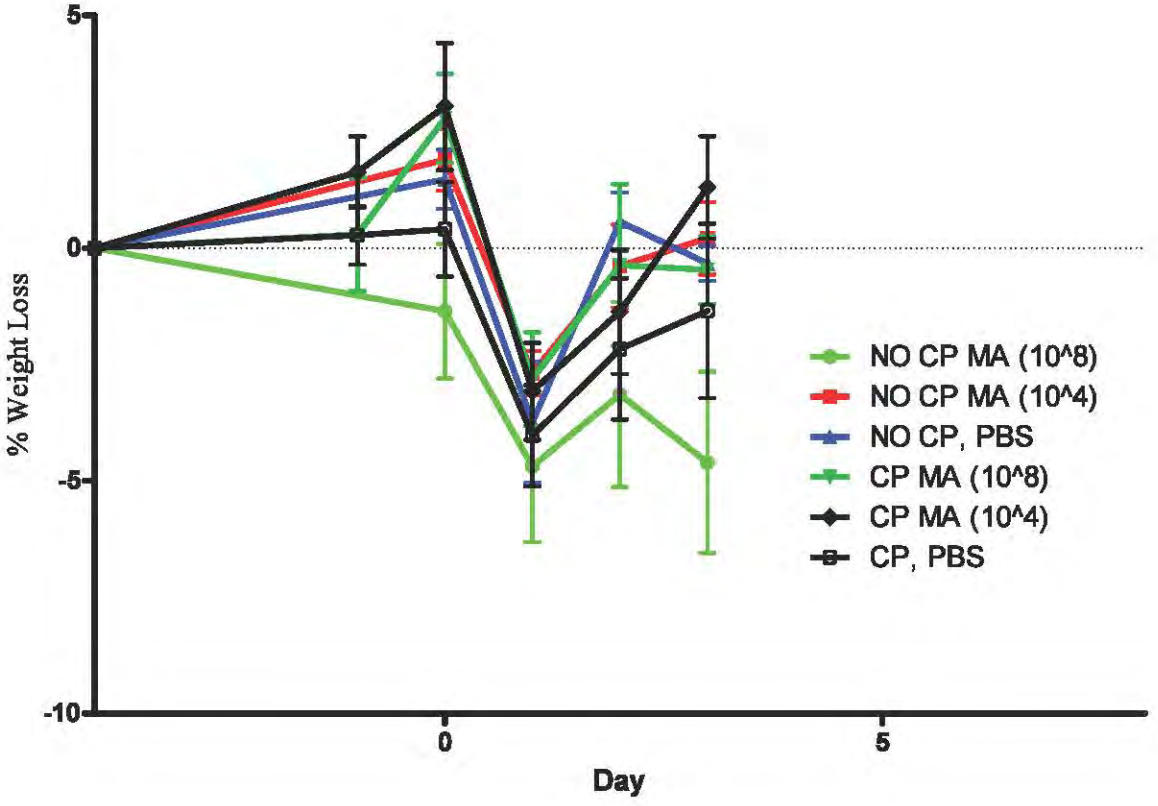
Weight loss trends from day (-4 or 0) to the last day that measurements were taken post operation are similar among the groups. Treatment with high dose of HD100 seems to result in a transient increased weight loss that is most obvious at day 2.

There seems to be no difference between treatment and control with respect to the effect on weight later in the time course.

There seems to be little to no effect of CP treatment on the weight and no real difference among the groups except for the CP treated 10^4 applied *Micavibrio aeruginosavorus*. We assume this is as a result in the variance between the animal and the specific execution of that experiment.


5
0
-5
-10
% Weight Loss
0
5
Day
10^8 PFU Bb 109J
10^4 PFU Bb 109J
10^8 PFU Bb HD100
10^4 PFU Bb HD100
PBS Control


10
5
0
-5
-10
-15
-20
% Weight Loss
0
5
Day
10^8 PFU Bb 109J
10^4 PFU Bb 109J
10^8 PFU Bb HD100
10^4 PFU Bb HD100
PBS Control


5
0
-5
-10
% Weight Loss
0
5
Day
NO CP MA (10^8)
NO CP MA (10^4)
NO CP, PBS
CP MA (10^8)
CP MA (10^4)
CP, PBS

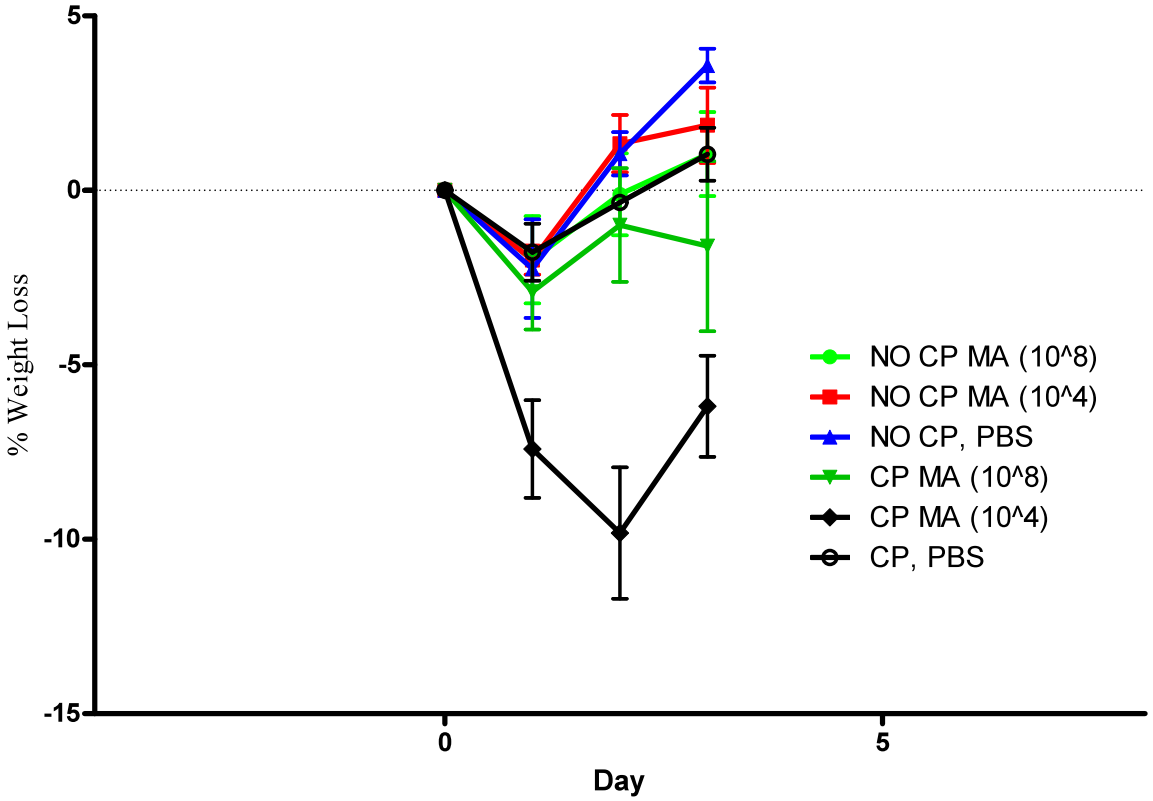
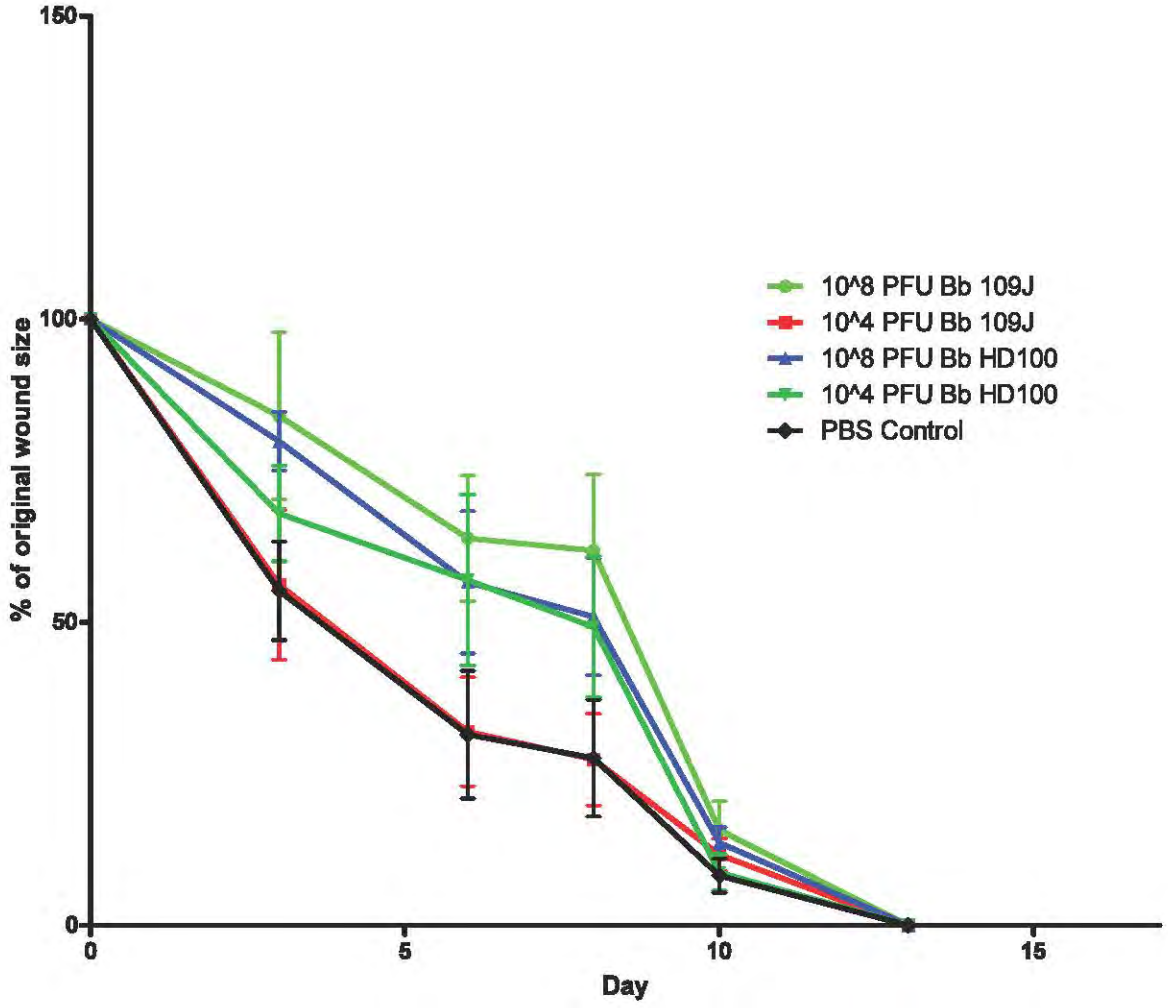
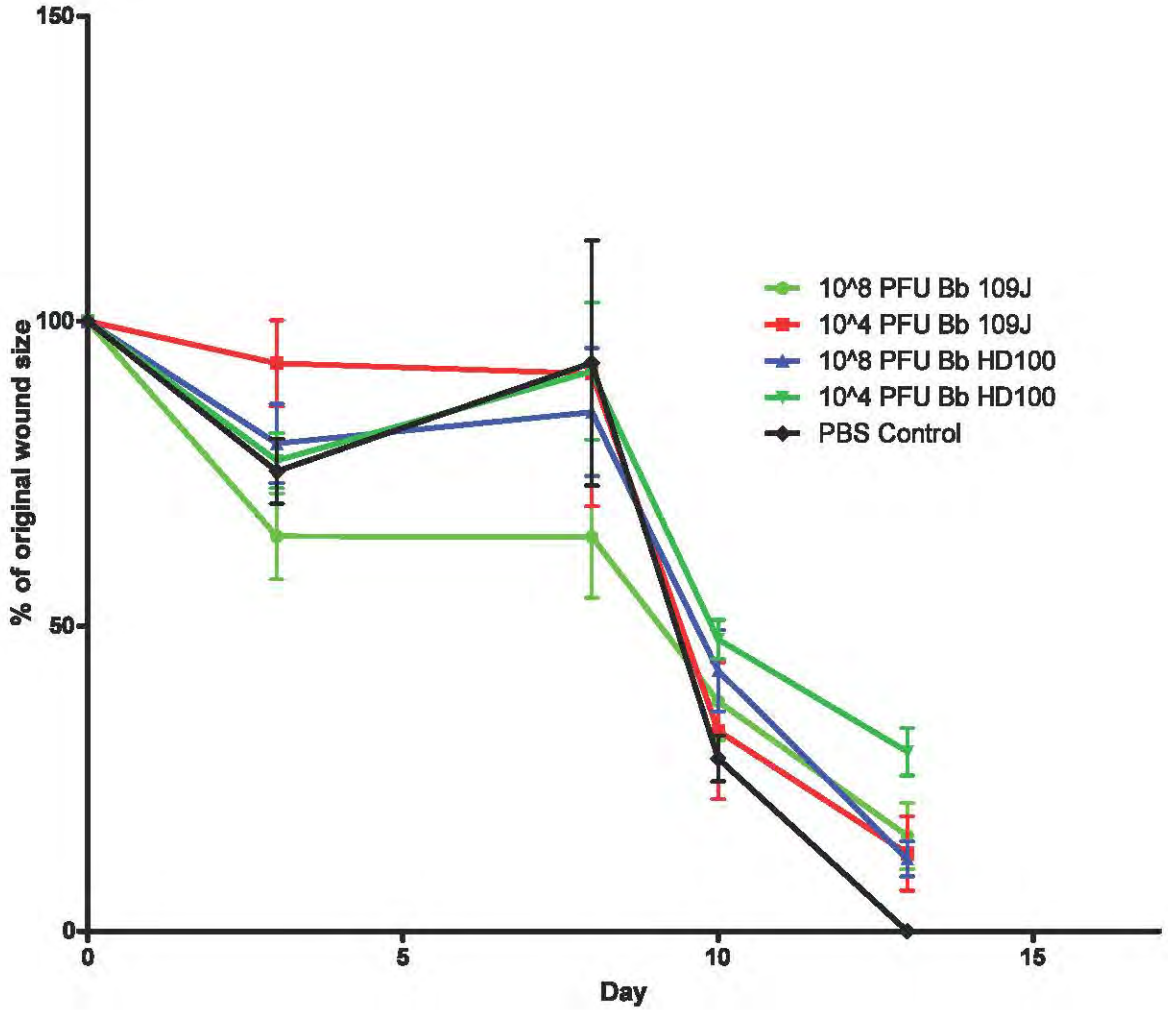
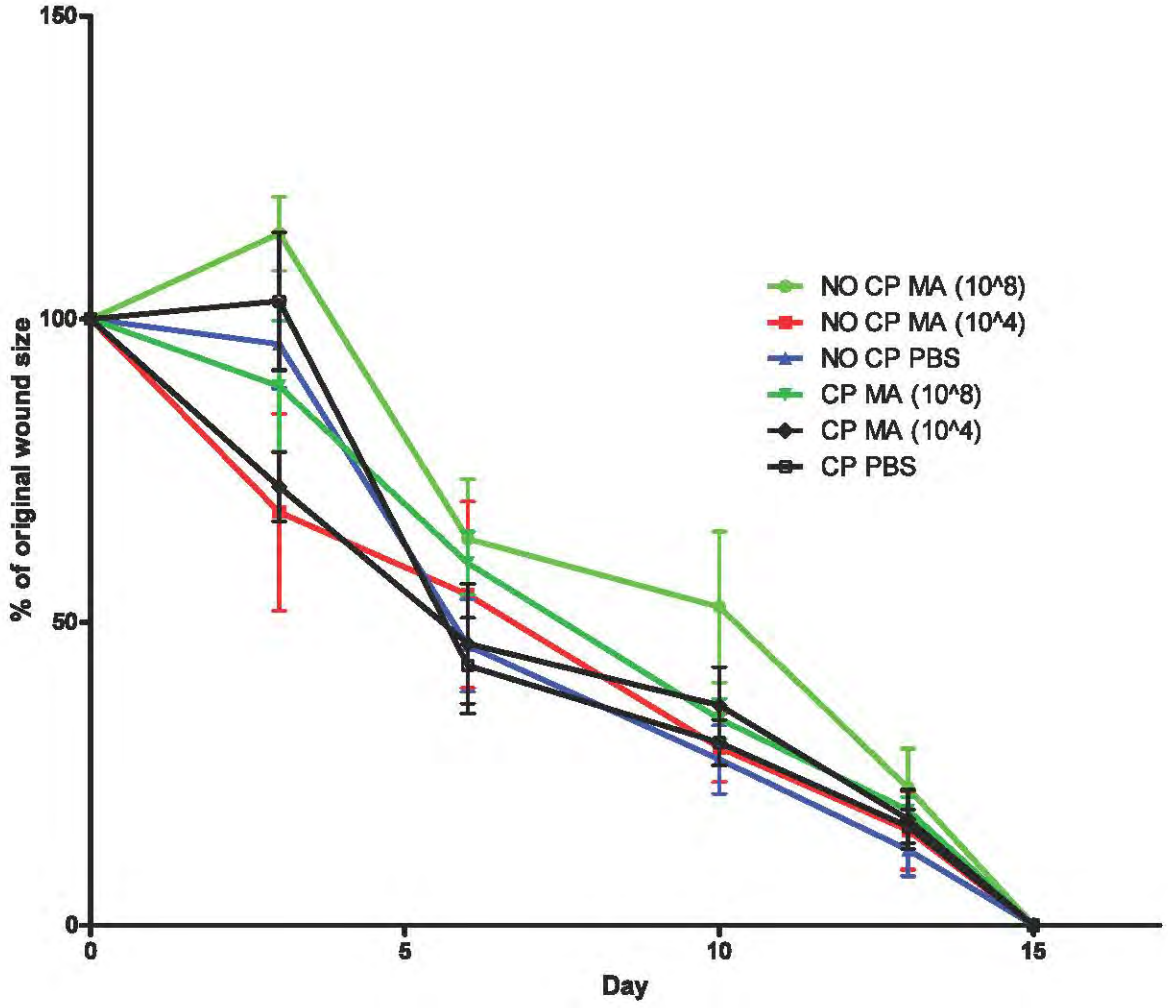


Figure 1 – Percentage weight loss in groups 1-5 over 12 days. Day (-4) & Day (-1) CP treatment, Day 0 – surgery, Day 0 treatment. Data for euthanized mice (if any) is not included.

The different treatments were almost identical in the time required for the wound to completely close. However, differences in the healing process between the Non-CP groups treated with *Micavibrio aeruginosavorus* and the rest of the groups in that experiment can be seen – this is a favorable trend and thus we see it as a positive indicator. Other minor differences in the time to close between treated with Bd and the control PBS treated group can be observed in Figure 2.


150
100
50
0
% of original wound size
0
5
10
15
Day
10^8 PFU Bb 109J
10^4 PFU Bb 109J
10^8 PFU Bb HD100
10^4 PFU Bb HD100
PBS Control


150
100
50
0
% of original wound size
0
5
10
15
Day
10^8 PFU Bb 109J
10^4 PFU Bb 109J
10^8 PFU Bb HD100
10^4 PFU Bb HD100
PBS Control


150
100
50
0
% of original wound size
0
5
10
15
Day
NO CP MA (10^8)
NO CP MA (10^4)
NO CP PBS
CP MA (10^8)
CP MA (10^4)
CP PBS

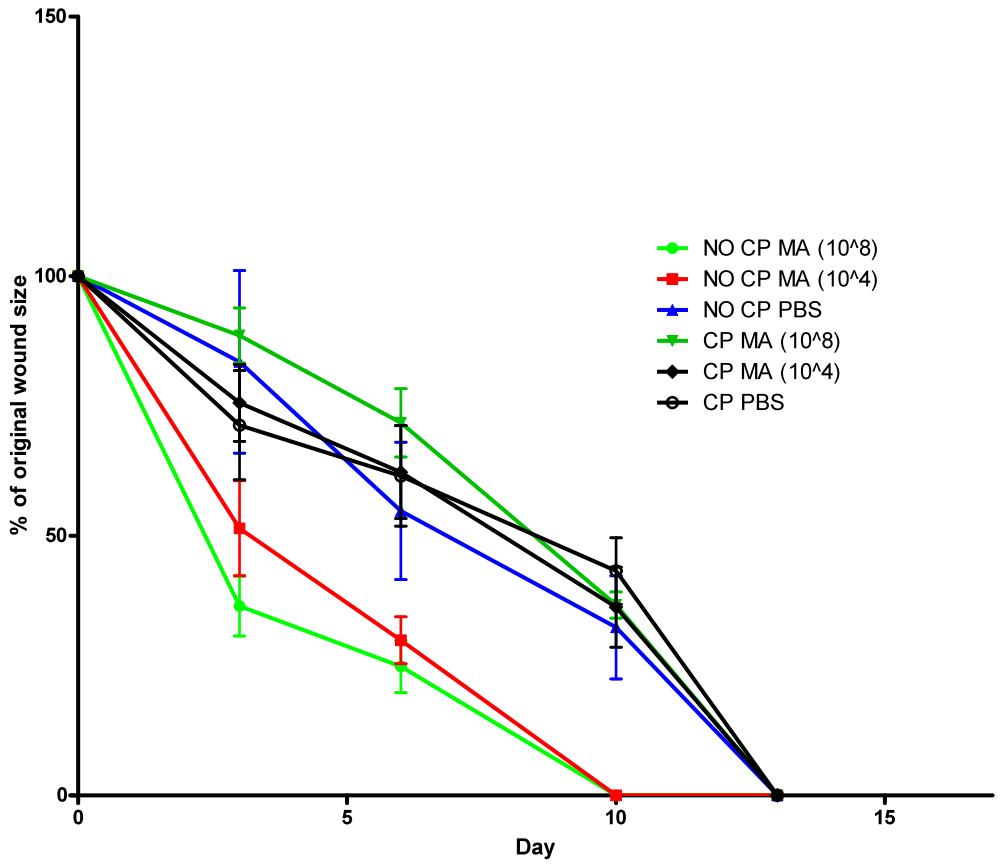


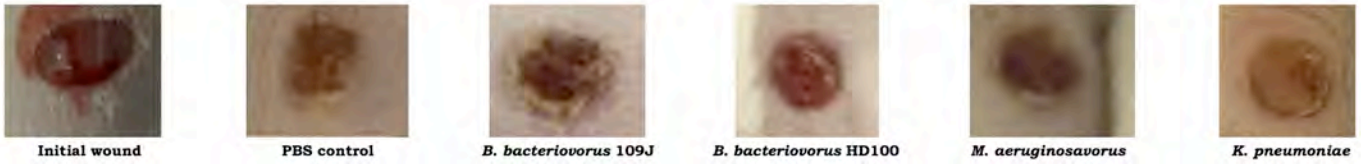
Figure 2 – Percentage of wound size in groups 1-5 over 17 days. Data for euthanized mice (if any) is not included.

Conclusions.

Predatory bacteria are safe when applied topically to open wounds of CP or non CP treated mice (only MA was examined in non-CP treated mice) with doses ranging up to 10^8 PFU causing, if anything, minor effects on weight and a possible positive effect on time it takes the wound to heal. We recommend using the J109 strain as it did not deviate from the PBS control as much as the deviations of the HD100 strain.

Predatory bacteria are non pathogenic in a mice wound model.

Administering high doses ($1x10^8$) of *B. bacteriovorus* 109J, HD100 and *M. aeruginosavorus* to open wounds of healthy animals and animals who are immunocompromised did not have a negative effect on mice well-being or wound healing.



Wound closure 8 days following inoculation with predatory bacteria or control.

PATHOLOGY REPORT

Walter Reed Army Institute of Research, Silver Spring, MD
Naval Medical Research Center, Silver Spring, MD

Accession Number	Animal ID	Protocol Number		Investigator	Pathologist
14-1484	FY1412880	14-BRD-01S		ZURAWSKI	CLH
Species	**Strain**	**Sex**	**Weight**	**Room**	**Method of Euthanasia**
MOUSE	BALB/C	FEMALE	<50 G	GW171	KET/XYL OVERDOSE
Date Received	**Date Necropsied**	**Prosector**		**Type of Necropsy**	**Report Date**
5/27/2014	5/27/2014	HONNOLD		TRIM IN	6/24/2014

History

A 6mm excisional wound was created, mice wounded and treated once with 5.0X10^8 PFU Micavibrio aeruginosavorus.

Macroscopic Findings

Only skin sample with wound submitted for evaluation.

This report will serve as a final report for the following consecutive accession numbers: 14-1484 through 14 -1501.

Microscopic Findings

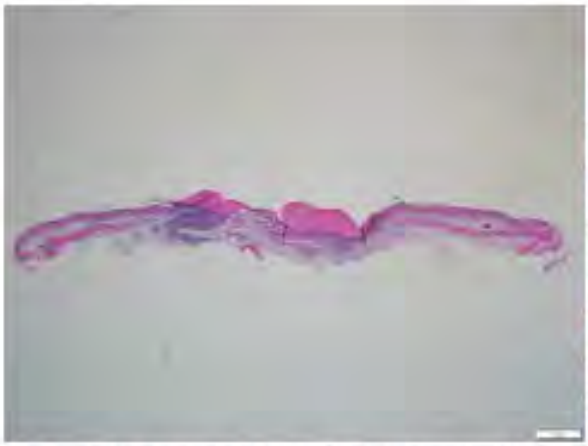
The following morphologic fits for all mice identified as 'Day 2': Haired skin, site of experimental wound: Dermatitis, acute (neutrophilic), focally extensive, marked to severe, with dermal edema, serocellular exudate and crusting.

Comments

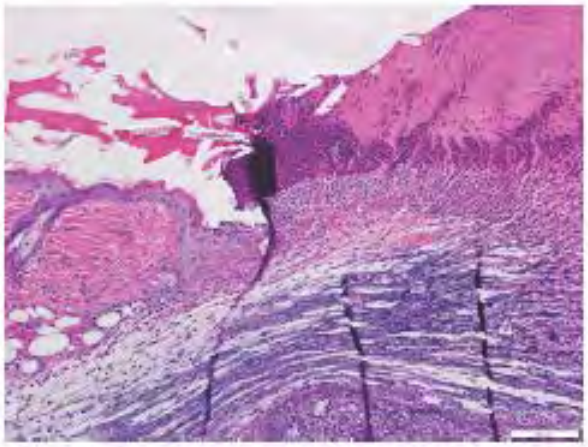
Histologic evaluation of the day 2 wounds did not reveal any differences between groups. The experimental lesion, from a histologic standpoint, is consistent with typical wounding. The predominant inflammatory response is neutrophilic. Unfortunately, I was unable by light microscopy to appreciate any discernible difference between the groups.

Cary Honnold, DVM, Diplomate ACVP, ACVPM

Pathologist Signature: CARY HONNOLD

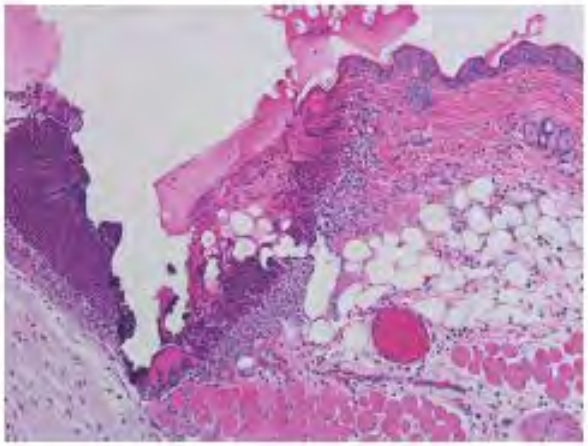


Haired skin from 14-1485, 2 day wound, 1B2, Non CP treated.



Haired skin from 14-1485 demonstrating acute inflammation at wound edge. Typical for Day 2 wounds.

Wound from PBS treated.



Wound edge from PBS treated.

PATHOLOGY REPORT

Walter Reed Army Institute of Research, Silver Spring, MD
Naval Medical Research Center, Silver Spring, MD

ACCESSION NUMBER: 14-0541	ANIMAL ID: FY132472

ACCESSION	CONTRIBUTOR	DIVISION/DEPARTMENT			DATE OF REPORT	
14-0541	ZURAWSKI	BRD-WI			13-Dec-13	
PROTOCOL	**TYPE**	**DATE SUBMITTED**			**DATE COMPLETED**	
14-BRD-01S	TRIM IN	27-Nov-13			13-Dec-13	
SPECIES	**STRAIN**	**AGE**	**SEX**	**WEIGHT**	**ANIMAL ID**	**ROOM NO**
MOUSE	BALB/C	6-12WKS	F	<40 LBS	FY132472	GW173

HISTORY

TREATED 109J, A 6MM EXCISIONAL WOUND WAS CREATED, MICE WOUNDED AND TREATED ONCE WITH 10^8 PFU. BDELLOVIRIO BACTERIOVIRUS 109J

This report is for the following pathology accession numbers, animal numbers, and tube number reference:
1. 14-0541/FY132472/109J
2. 14-0542/FY132474/109J
3. 14-0543/FY132474/109J
4. 14-0544/FY132475/HD100
5. 14-0545/FY132476/HD100
6. 14-0546/FY132477/HD100
7. 14-0547/FY132478/HD100
8. 14-0548/FY132479/Control PBS
9. 14-0549/FY132480/Control PBS

MACROSCOPIC FINDINGS

The submitted skin samples were from the back of the mouse and contained a focal wound with overlying crusting.

MICROSCOPIC FINDINGS

The following morphological diagnosis is essentially representative for all 9 skin wounds examined.
1. Haired skin: Dermatitis, ulcerative, neutrophilic (acute), focal, severe, with edema, serocellular exudate, and underlying neutrophilic myositis.

COMMENTS

The 9 submitted skin samples were examined histologically and there was no discernable difference between control and treated groups. Histologically, the wounds consist of a robust, acute, neutrophilic inflammation, marked edema in surrounding tissue, and serocellular exudate. The neutrophils are also present within the underlying skeletal muscle.

Cary Honnold, DVM, Diplomate ACVP, ACVPM

Subtask 3.1. *Determining the **efficacy** of predatory bacteria in a mouse puncture wound model.*

The effect of predatory bacteria on infected wounds was tested in the mouse puncture model at the Dept. of Wound Infections at WRAIR. Puncture wounds (full thickness) were generated on the back of mouse and inoculated with set doses of *K. pneumoniae* gram-negative bacteria that generate a wound infection. Predatory bacteria were applied once to each wound per group. Mice were evaluated for clinical signs of infection and time to wound closure. The experiments were conducted twice however only the results of the first experiments are summarized. Results and data for the second experiment will be reported in the next report period.

Detailed information regarding each experiment is presented below:

Experiment I.

Objective: The primary objective of these experiments is to assess the efficacy of live *Bdellovibrio bacteriovorus* or *Micavibrio aeruginosavorus* administration application on exposed wounds infected with *Klebsiella pneumoniae* strain 4640 in the mouse wound model. The primary measures of efficacy in these experiments are weight loss / gain over time of the mice, & mice wound size. These experiments use cyclophosphamide treated mice (immunocompromised model). Treatment was applied either as a single dose of predatory bacteria in PBS at 4 h post infection. For Kb4640 the wounds were not covered.

Results.

Not all mice survived the procedure and some succumbed to the infection. Deaths occurred in the first few days post-surgery. All other animals were on course to full recovery or fully recovered by the end of the experiment. Clinical scores were similar in all groups during the first days until no clinical signs could be observed.

Weight loss trends from day (-4 or 0) to the last day that measurements were taken post operation are discussed hereon. For mice infected with KP4640 – the change in weight trends of the mock treated group overlap those of each of the groups treated with predatory bacteria (109J, HD100 and Mica). Single treatment with Colistin (antibiotic) reduces weight loss and is approaching the trends of the Un-infected mock treated group (Figure 1). Similar trend is seen when examining the change in wound size over time (Figure 2). There seems to be no difference between treatment and control with respect to the effect on weight or wound size during the time course.

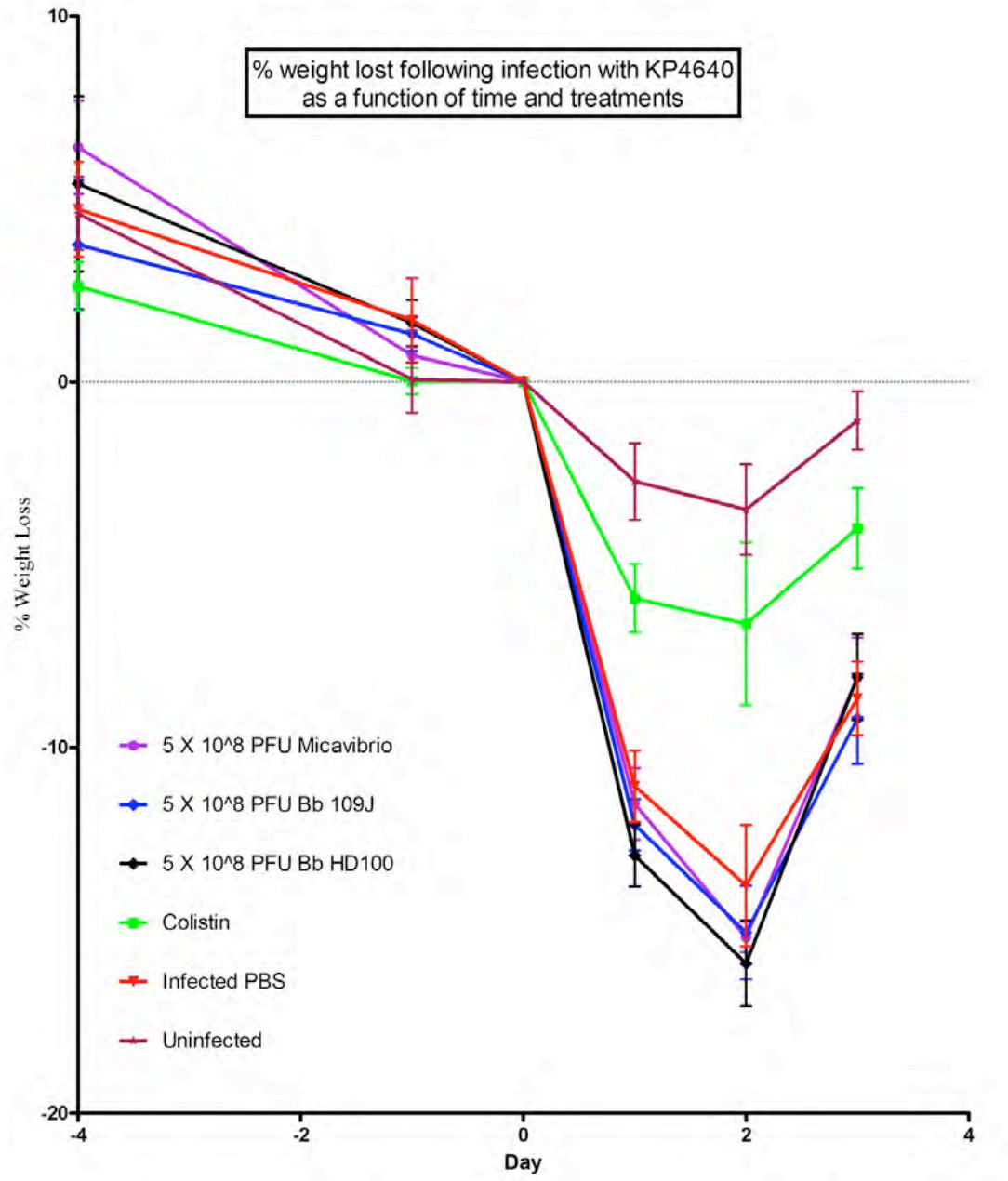


Figure 1 – Percentage weight loss in groups 1-5 over 10 days. Day (-4) & Day (-1) CP treatment, Day 0 – surgery, Day 0 treatment. Data for euthanized mice (if any) is not included.

The different treatments were almost identical in the time required for the wound to completely close. However, differences in the healing process between groups can be seen. Other minor differences in the time to close between group can be observed in Figure 2.

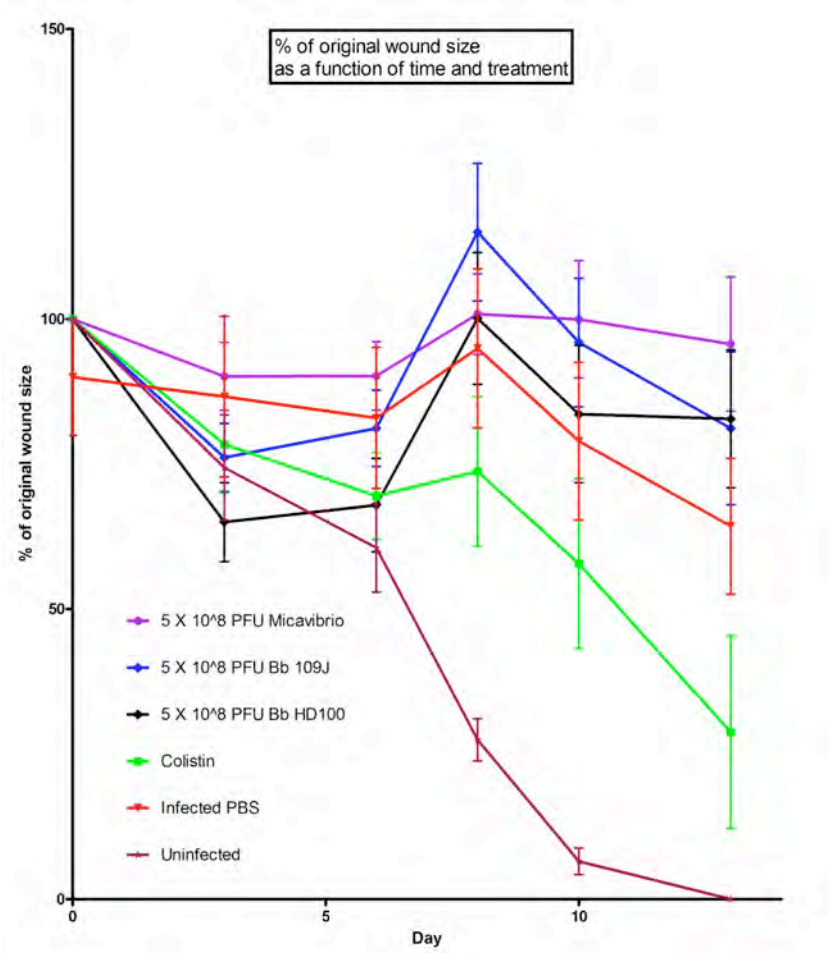


Figure 2 – Percentage of wound size in groups 1-5 over 13 days. Data for euthanized mice (if any) is not included.

Conclusions.

Predatory bacteria are safe when applied topically to open wounds of CP treated mice. However, in this experiment there was no indication that a single dose of predatory bacteria can enhance weight gain or wound closure.

Experiment II results.

Not all mice survived the procedure and some succumbed to the infection. Deaths occurred in the first few days post-surgery. All other animals were on course to full recovery or fully recovered by the end of the experiment. Clinical scores were similar in all groups during the first days until no clinical signs could be observed. Weight loss trends from day (-4 or 0) to the last day that measurements were taken post operation are discussed hereon. For mice infected with KP4640 – the change in weight trends of the mock treated group overlap those of each of the groups treated with predatory bacteria (109J, HD100 and Mica). Single treatment with Colistin (antibiotic) reduces weight loss and is approaching the trends of the Un-infected mock treated group (Figure

1). Similar trend is seen when examining the change in wound size over time (Figure 2). There seems to be no difference between treatment and control with respect to the effect on weight or wound size during the time course.

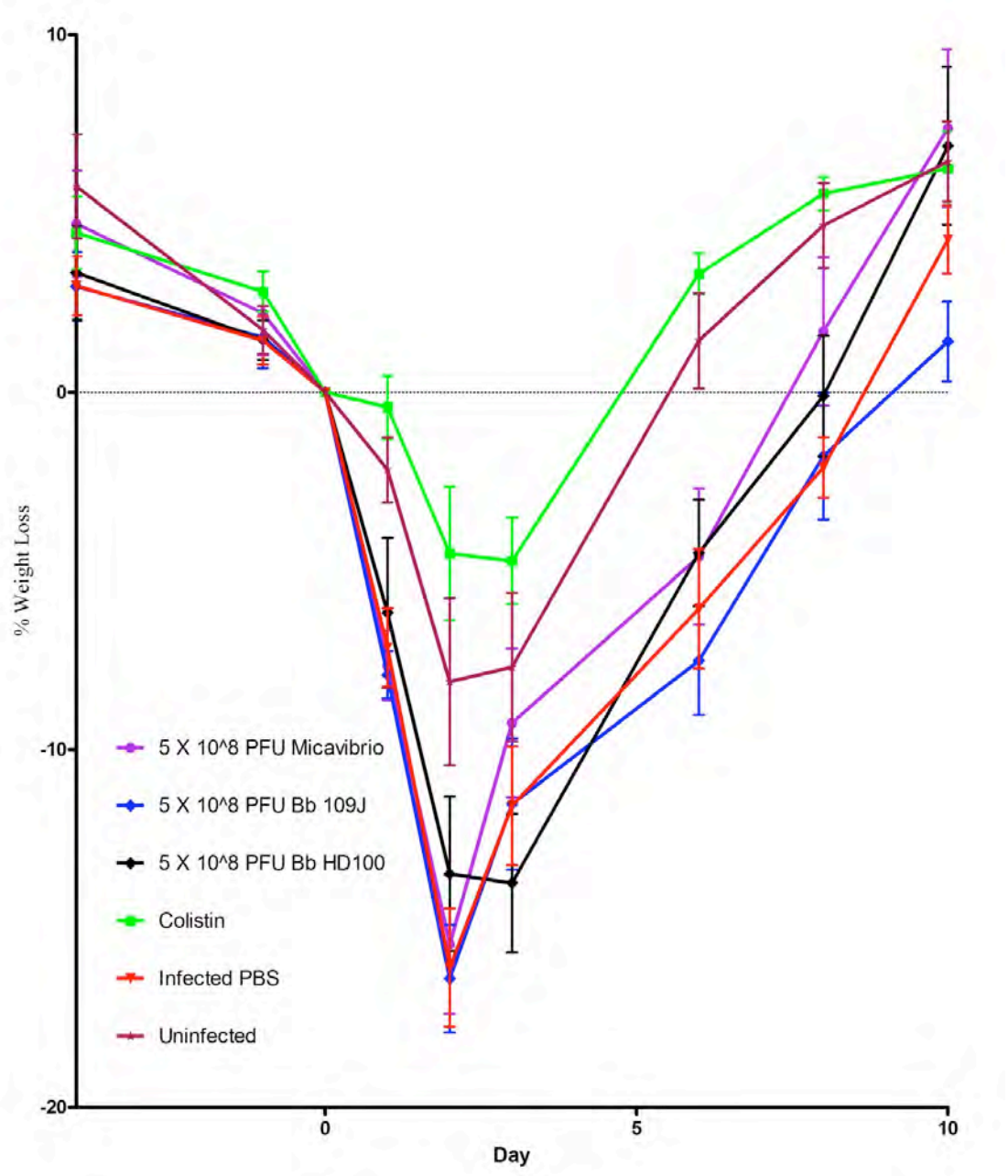
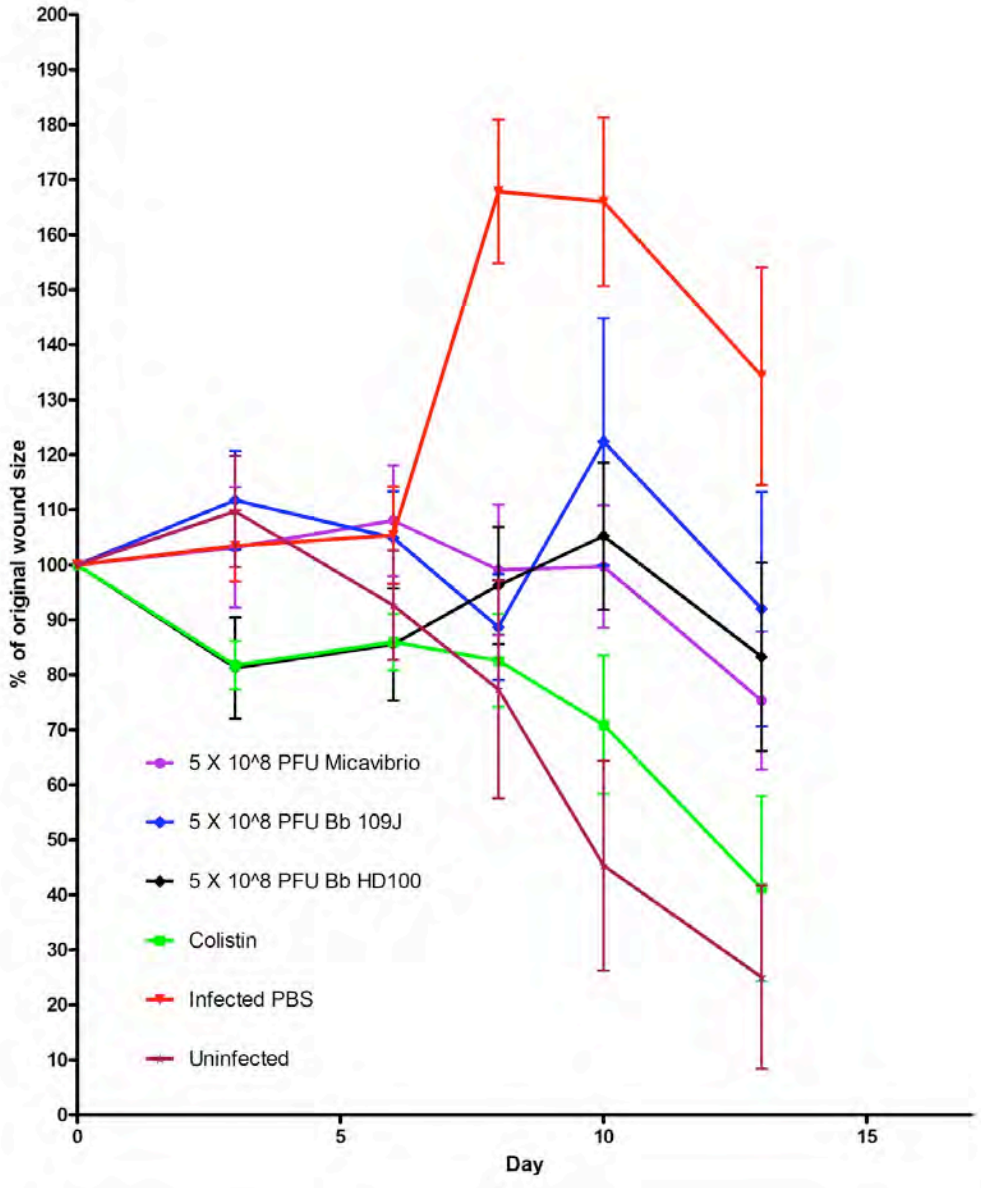


Figure 1 – Percentage weight loss in groups 1-5 over 10 days. Day (-4) & Day (-1) CP treatment, Day 0 – surgery, Day 0 treatment. Data for euthanized mice (if any) is not included.

There are no significant differences in mouse weights over time between the groups except the Colistin group and the mock infected. Colistin treatment seems to bring weight profile to normal

or above. The different treatments were non-identical in the time required for the wound to completely close. Differences in the healing process between the groups treated with *Micavibrio aeruginosavorus* & *Bd 109J/H100* and the rest of the groups in that experiment can be seen. Predatory bacteria seem to be partially effective in promoting wound closing – this is a favorable trend and thus we see it as a positive indicator. This experiment used 10X more *K. pneumoniae* cells in the inoculum.

Figure 2 – Percentage of wound size in groups 1-5 over 13 days. Data for euthanized mice (if any) is not included.



Experiment II conclusions.

Predatory bacteria are safe when applied topically to open wounds of CP treated mice. There might be an indication of a therapeutic potential using single treatment when using high inoculum of Kleb 4640. These conclusions are based on the measured effects of infection and known treatment (Colistin) or mock treatment (PBS) that were performed under the same conditions. The basis of our conclusion is the measurement of the following parameters: wound size, weight loss/gain.

Subtask 3.1. *Determining the **efficacy** of predatory bacteria in a mouse puncture wound model.*

Since we did not see any significant therapeutic potential using a single treatment of predatory bacteria to clear a wound infected with inoculum of Kleb 4640 we were interested to see if multiple dosing of predatory bacteria might be more effective.

Experiment I.

Objective: The primary objective of these experiments is to assess the efficacy of live *Bdellovibrio bacteriovorus* administration application on exposed wounds infected with *Klebsiella pneumoniae* strain 4640 in the mouse wound model. The primary measures of efficacy in these experiments are weight loss / gain of the mice over time, and wound size. These experiments use cyclophosphamide treated mice (immunocompromised model). Treatment was applied either as a single dose of predatory bacteria in PBS at 4 h post infection or as 5 consecutive treatments at the afternoon of the day of surgery and morning and afternoon treatments in the two following days. For Kb4640 the wounds were not covered with Tegaderm.

Results:

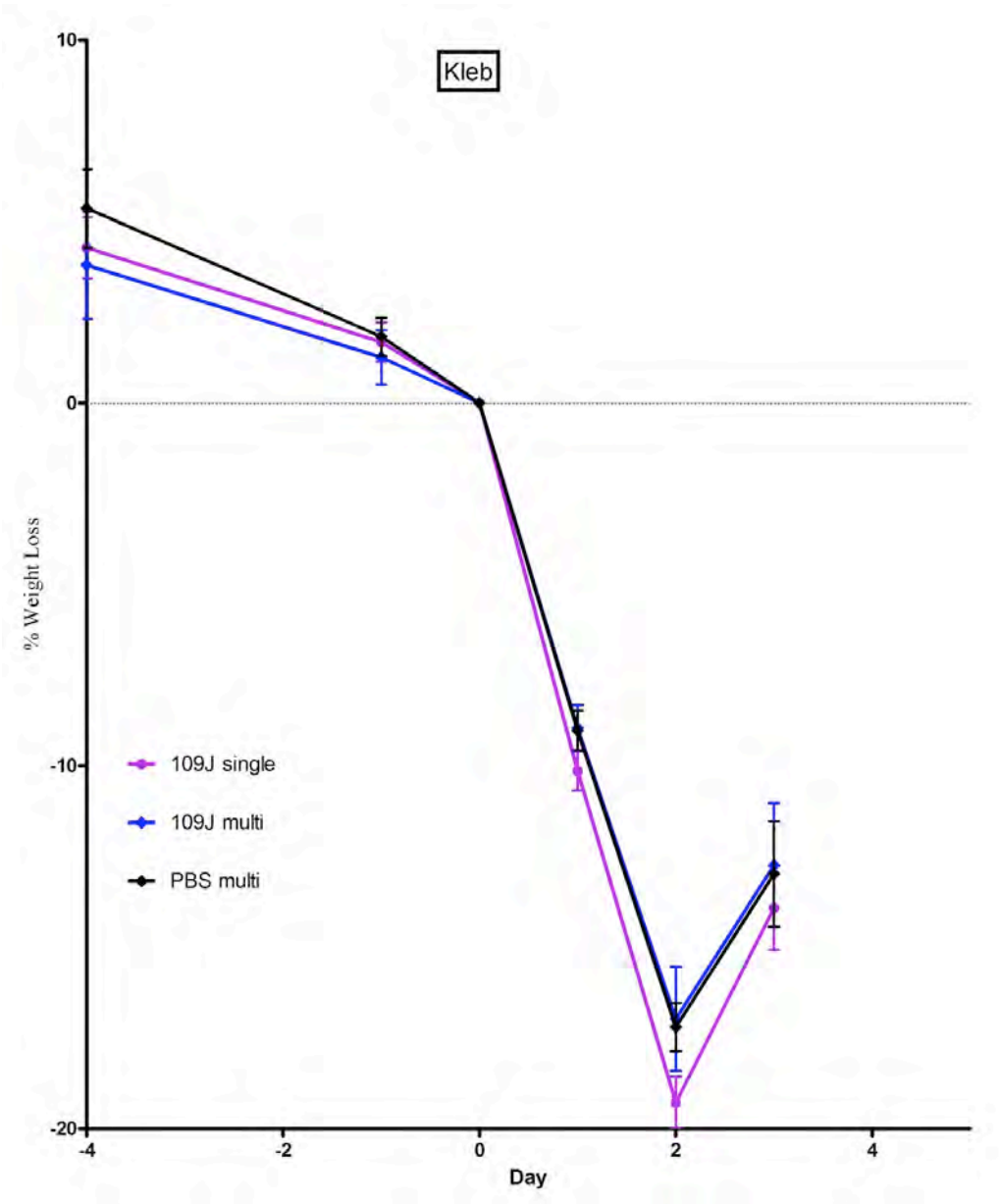
Not all mice survived the procedure and some succumbed to the infection. Deaths occurred in the first few days post-surgery. All other animals were on course to full recovery or fully recovered by the end of the experiment.

Clinical scores were similar in all groups during the first days until no clinical signs could be observed.

Weight loss trends from day (-4 or 0) to the last day that measurements were taken post operation are discussed hereon. For mice infected with KP4640 – the change in weight trends of the mock treated group overlap those of each of the groups treated with predatory bacteria (Figure 1). Similar trend is seen when examining the change in wound size over time (Figure 2). There seems to be no difference between treatment and control with respect to the effect on weight or wound size during the time course.

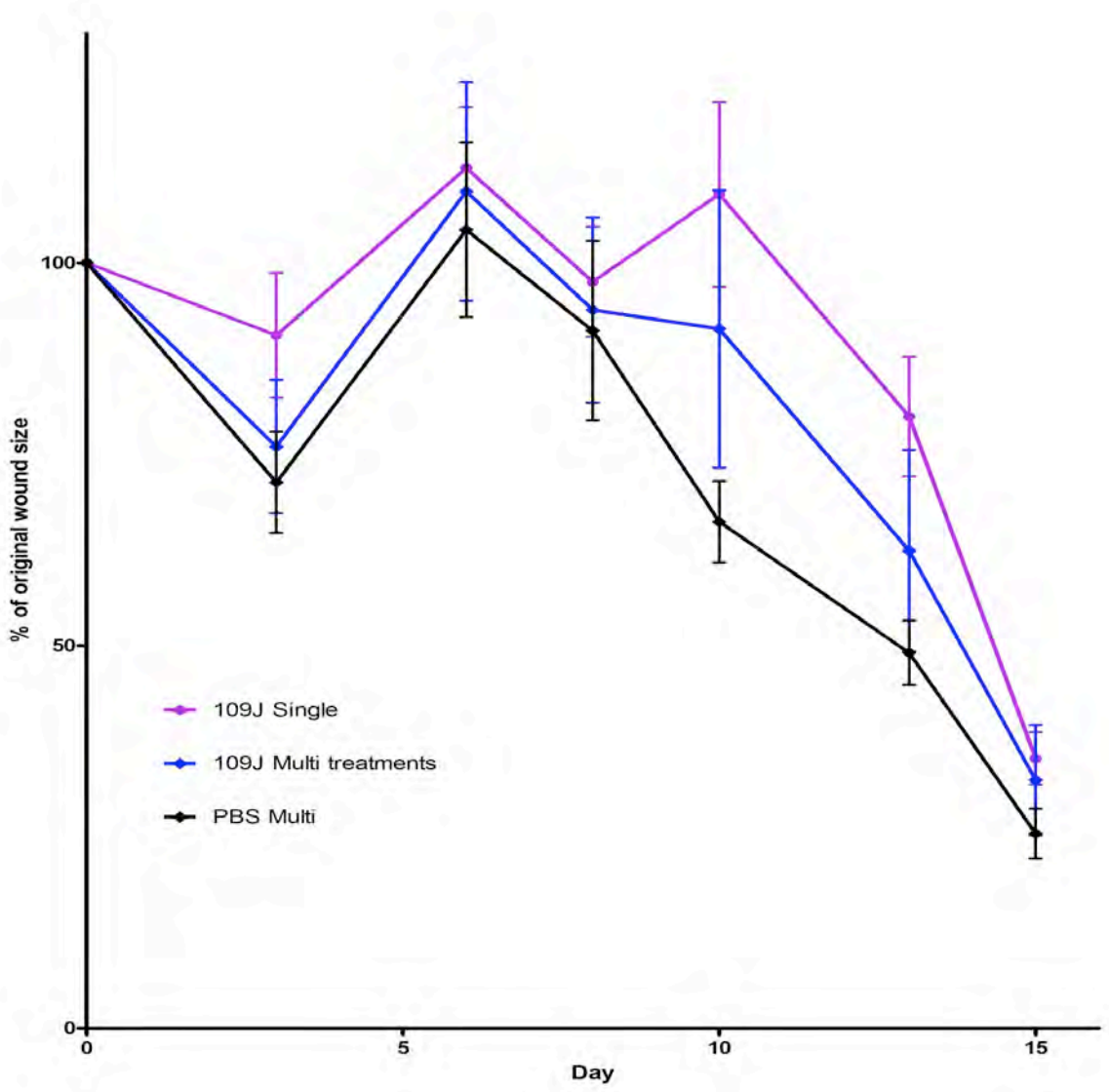
We decided to concentrate on a single predatory bacteria (109J) and compare multiple dosing to single dosing and multiple mock treatments to resolve Kleb 4640 infection. However, we did not observe a change in weight loss trends and a reduction in wound size during the first few days.

Figure 1 – Percentage weight loss in groups 1-3 over 7 days. Day (-4) & Day (-1) CP treatment, Day 0 – surgery, Day 0 treatment. Data for euthanized mice (if any) is not included.



The different treatments were almost identical in the time required for the wound to completely close.

Figure 2 – Percentage of wound size in groups 1-3 over 15 days. Data for euthanized mice (if any) is not included.



Experiment I conclusions.

Predatory bacteria are safe when applied topically to open wounds of CP treated mice. However, there was no indication of a therapeutic potential using Multiple or single treatments. The basis of our conclusion is the measurement of the following parameters: wound size, weight loss/gain.

Experiment II.

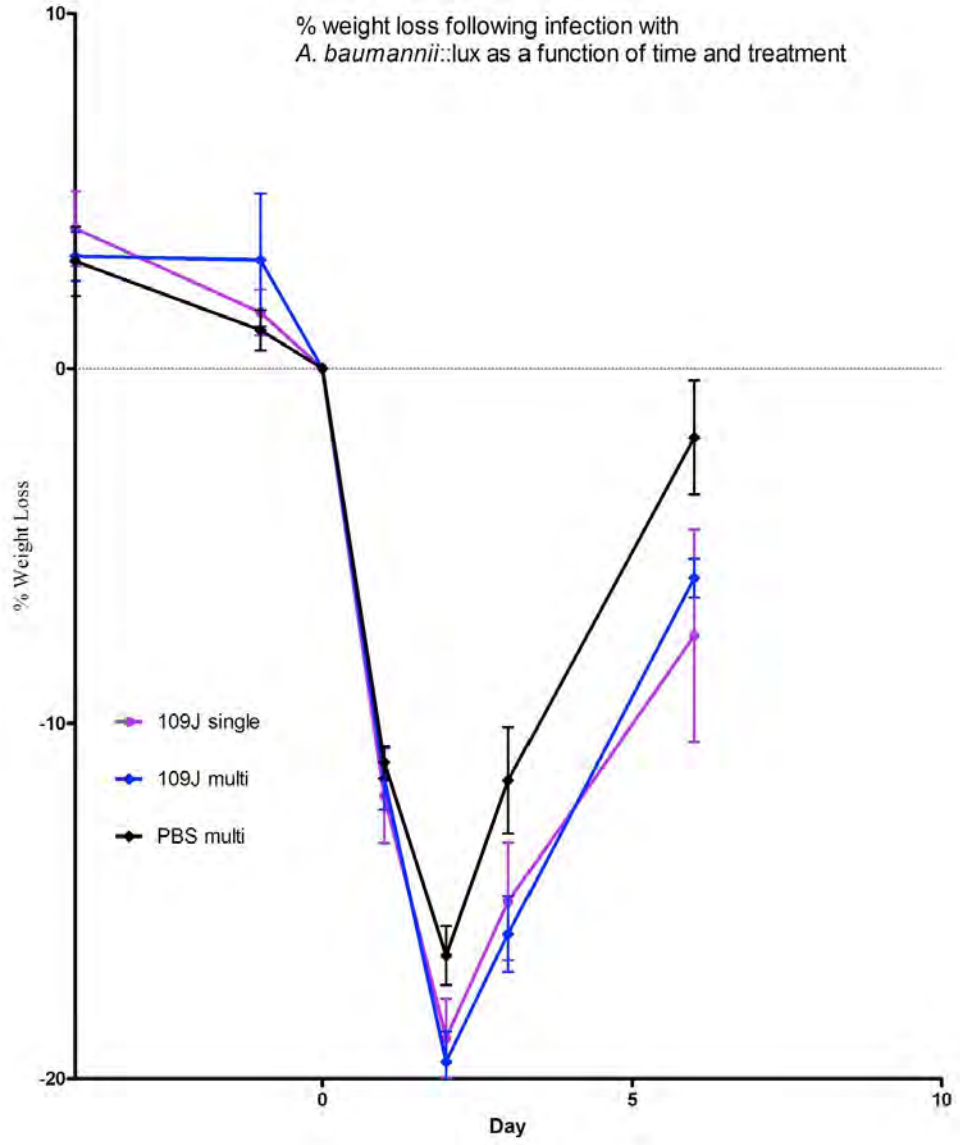
Objective: The primary objective of these experiments is to assess the efficacy of live *Bdellovibrio bacteriovorus* administration application on exposed wounds infected with *Acinetobacter baumannii* strain 5075::lux in the mouse wound model. The primary measures of efficacy in these experiments are weight loss / gain over time of the mice, mice wound size, and when appropriate bacterial loads estimate by luminescence. These experiments use cyclophosphamide treated mice (immunocompromised model). Treatment was applied either as a single dose of predatory bacteria in PBS at 4 h post infection or as 5 consecutive treatments at

the afternoon of the day of surgery and morning and afternoon treatments in the two following days. For Ab5075 infected wounds Tegaderm was applied to the wound immediately after inoculation of the wound and was taken off at day 6.

Results:

Not all mice survived the procedure and some succumbed to the infection. Deaths occurred in the first few days post-surgery. All other animals were on course to full recovery or fully recovered by the end of the experiment.
Clinical scores were similar in all groups during the first days until no clinical signs could be observed.
Weight loss trends from day (-4 or 0) to the last day that measurements were taken post operation are discussed hereon (Figure 1) and show small differences and application of the predatory bacteria seem to worsen the regain of weight somewhat. Similar trend is seen when examining the change in wound size over time (Figure 2). There seems to be no difference between treatment and control with respect to the effect on weight or wound size during the time course.
We decided to concentrate on a single predatory bacteria (109J) and compare multiple dosing to single dosing and multiple mock treatments to resolve AC5075 infection. However, we did not observe a change in weight loss trends (Figure 1), luminescence (Figure 3), and a possible slight reduction in wound size during the first few days (Figure 2).

Figure 1 – Percentage weight loss in groups 1-3 over 10 days. Day (-4) & Day (-1) CP treatment, Day 0 – surgery, Day 0 treatment. Data for euthanized mice (if any) is not included.


% weight loss following infection with
A. baumannii::lux as a function of time and treatment
% Weight Loss
10
0
-10
-20
0
5
10
Day
109J single
109J multi
PBS multi

The different treatments were almost identical in the time required for the wound to completely close. However, differences in the healing process between the Non-CP groups treated with *Bdellovibrio* and the rest of the groups in that experiment can be seen – this is a favorable trend and thus we see it as a positive indicator. Other minor differences in the time to close between treated with *Bdellovibrio* and the control PBS treated group can be observed in Figure 2.

Figure 2 – Percentage of wound size in groups 1-5 over 20 days. Data for euthanized mice (if any) is not included.

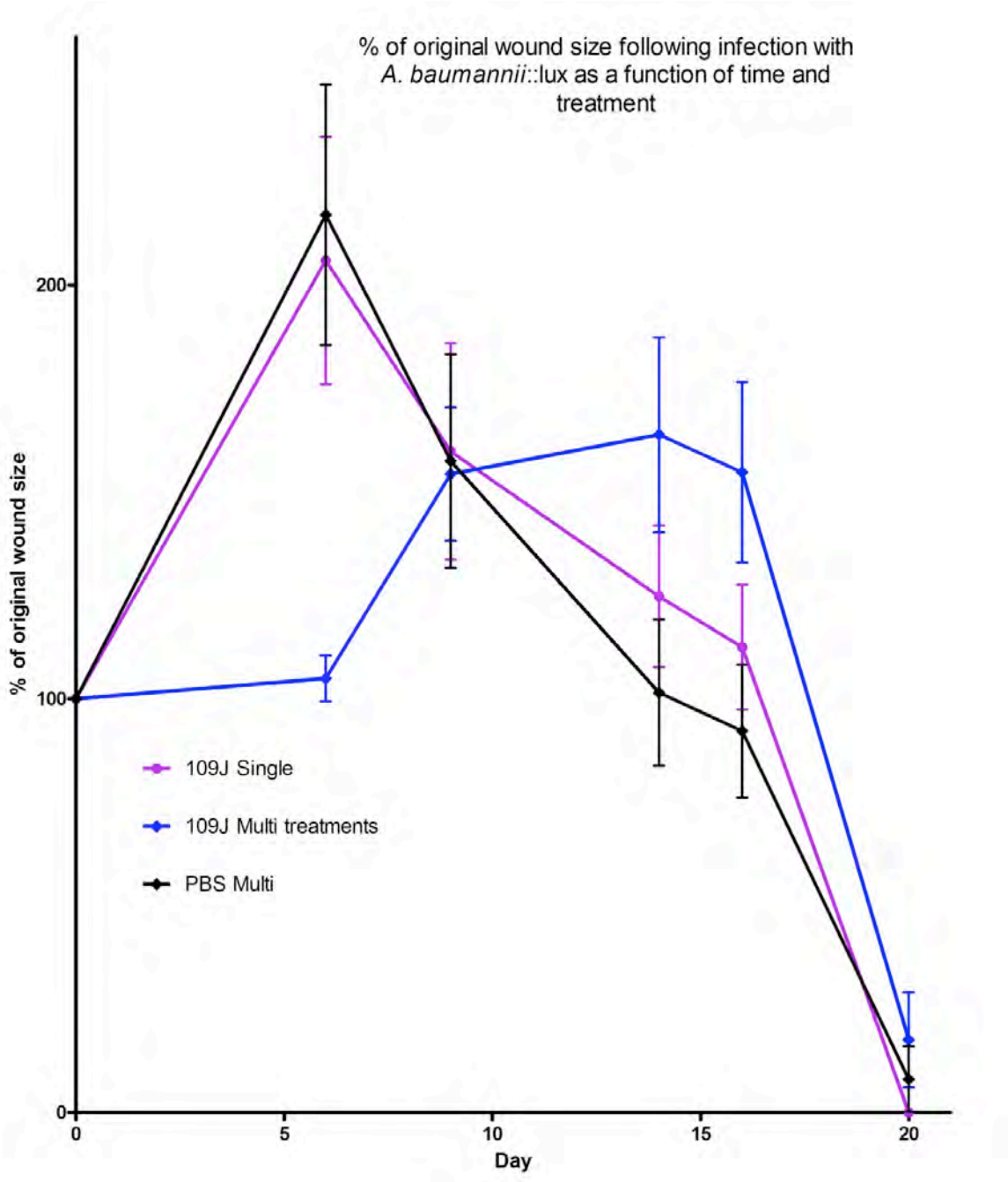
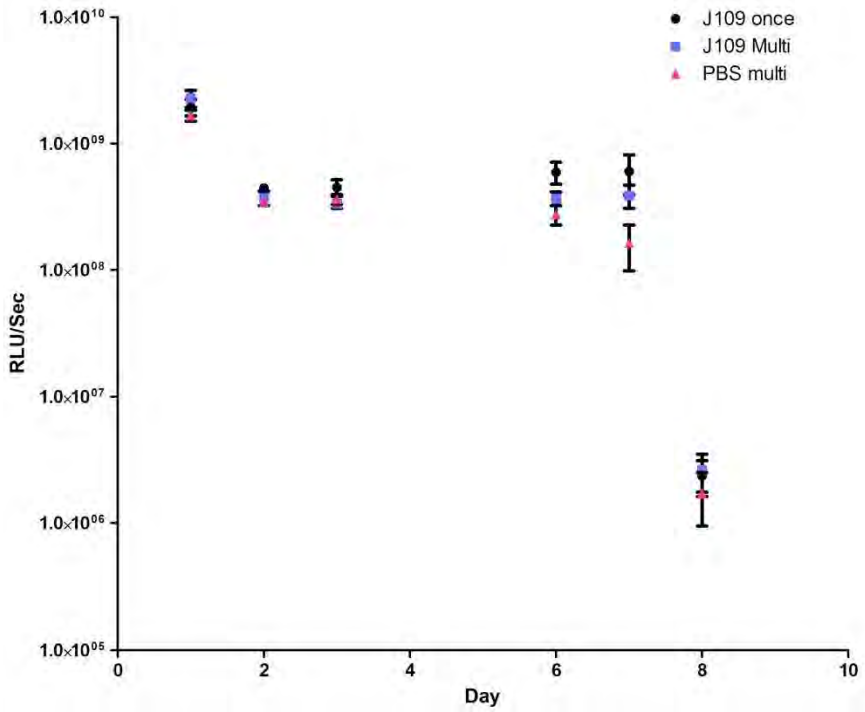


Figure 3 – Relative Light Units (RLU) in groups 1-3 over 8 days. Day (-4) & Day (-1) CP treatment, Day 0 – surgery, Day 0 treatment. At day 6 Tegaderm was removed and readings of the luminescence of the Tegaderm appear as the readings of Day 7. Data for euthanized mice (if any) is not included.



Key Research Accomplishments and Results.

Aim-1, Subtask 1.1. Development of genetically stabl e resistance to predation.

- Our data suggest that no genetically stable predation resistant phenotype developed in *K. pneumoniae* and *A. baumannii* following sequential predation by *B. bacteriovorus* 109J and *B. bacteriovorus* HD100.

- Our data suggest that no genetically stable predation resistant phenotype developed in *P. aeruginosa* Pa14 following sequential predation by *M. aeruginosavorus*.

- Our data suggest that host cells grown as a biofilm do not adapt to form predation resistant biofilms.

- *K. pneumoniae* ATCC 33495 prey cells which were randomly mutagenized by transposon insertion or UV radiation yield no stable resistance to predation by *B. bacteriovorus* 109J or *M. aeruginosavorus*.

Aim-1, Subtask 1.2. Investigating the ability of the predator to breach its host specificity and attack previously resistant bacteria.

- Our data suggest that *B. bacteriovorus* does not have an ability to breach its host specificity and attack previously resistant bacteria. This finding was verified using both Gram-negative and Gram-positive host.

- Our data suggest that *M. aeruginosavorus* does not have an ability to breach its host specificity and attack previously resistant bacteria. This finding was verified using both Gram-negative and Gram-positive host.

Subtask 1.3. Enriching for hyper predatory variants.

- Our data suggest that *B. bacteriovorus* 109J could become more virulent on a particular host. However, this increase in predation could develop even in the absence of the prey. Culturing the predator with the prey could slightly enhance predation. On the other hand, increased predation does not develop on all prey with some host bacteria maintaining their tolerance even after continuous culturing. Therefore, we can conclude that enhanced predation is specific and could develop on some host bacteria.

- Our data shows that after continuous predation cycles *M. aeruginosavorus* does not seem to develop an ability to become hyper virulent on *A. lwoffii*.

- Our data suggest that *Bdellovibrio* 109J and *Micavibrio* could be acclimated to prey at an elevated temperature of 39°C.

Aim II. Determining the effect of predatory bacteria on eukaryotic cells.

Subtask 2.1. *Determining the toxicity of predatory bacteria on mammalian cells.*

- Our data suggest that *B. bacteriovorus* 109J, HD100 and *Micavibrio* are non toxic to HaCAT human epidermal keratinocytes, Hs27 human skin fibroblasts and L929 murine fibroblasts and do not cause reduction in cell viability.

- Microscopic imaging of HaCAT and Hs27 cells exposed to the predators had revealed no change in cell morphology or detachment.

Subtask 2.2. *Determining the influence of predator exposure on cell cytokine profile.*

- Our data show that exposing HaCAT cells to high levels of predatory bacteria had caused no elevation in cytokine production in 11 out of the 13 examined cytokines. However, exposing Hs27 human skin fibroblasts to the predators did result in an increase in cytokines production.

Subtask 2.3. Cell attachment assay.

- Our finding demonstrates that predatory bacteria are capable of attaching to human cell lines. However, based on the fact that cell death was not observed in any of our experiments, we could conclude that the attachment does not lead to cell loss.

Aim III. Efficacy and toxicity in animal models.

Subtask 3.1. *Determining the toxicity of predatory bacteria in a mouse puncture wound model.*

The effect of applying predatory bacteria to open wounds was evaluated in both naïve and immunocompromised mice. Experiments were conducted using *B. bacteriovorus* 109J, HD100 and *M. aeruginosavorus*. Our findings show that when applied to open wound, predatory bacteria do not seem to cause any adverse effect to the animal. No significant difference with respect to weight loss, wound clinical score and wound time to close was detected in wound “infected” with the predator. No difference in the histology analysis was seen in mice inoculated with the predators.

Conclusions. Predatory bacteria are safe when applied topically to open wounds of naïve and immunocompromised mice. That said, we see no efficacy, i.e. no reduction of light units (RLU), which is reflective of bacterial numbers. We see no improvement of healing with respect to infected wounds.

Subtask 3.1. *Determining the efficacy of using predatory bacteria to control an active infection in a mouse puncture wound model.*

The effect of applying predatory bacteria to a wound infection was evaluated in immunocompromised mice. Experiments were conducted using a single dose of *B. bacteriovorus* 109J, HD100 or *M. aeruginosavorus*. Experiments were also conducted using multiple dosing of *B. bacteriovorus* 109J. The pathogens used to infect the wound were *Klebsiella pneumoniae* 4640 and *Acinetobacter baumannii* strain 5075::lux. As before, our findings indicate that predatory bacteria are safe when applied topically to open wounds of CP treated mice. However, we did not see any significant therapeutic potential using a single or multiple dosing treatments of predatory bacteria to clear a wound infected with inoculums *Klebsiella* or *Acinetobacter*. Conversely, in a few of our experiments, a slight reduction in wound size noted during the first few days. These conclusions are based on the measured effects of infection and known treatment (Colistin) or mock treatment (PBS) that were performed under the same conditions. The basis of our conclusion is the measurement of the following parameters: wound size, weight loss/gain.

Conclusion

Disease-causing microorganisms that have become resistant to drug therapy are an increasing cause of burn, wound, blast and bone infections, with many traditional antimicrobial agents becoming ineffective. Our main hypothesis is that predatory prokaryotes could serve as a novel therapeutic agent to control wound-related bacterial infections. In a previous study, we confirmed that predatory bacteria *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* are able to prey on a wide range of pathogens including bacteria isolated from Wounded Warriors. The aim of this proposal is to address key questions regarding the safety and efficacy of predatory bacteria and investigating predator prey interactions and resistance.

It was proposed that, unlike antibiotics or phage therapy, the selective pressure of predation does not generate genetically stable resistant variants in the host. In order to evaluate this hypothesis we conduct experiments aimed at increasing the selective pressure on the host and assessing if any genetically stable predation resistant phenotypes emerge. Using enrichment culturing techniques we have verified that no genetically stable predation resistant phenotype developed in *K. pneumoniae* and *A. baumannii* host cells following sequential predation by *B. bacteriovorus* 109J or HD100. Furthermore, sequential predation by *M. aeruginosavorus* also did not yield resistance in *P. aeruginosa*. As was seen with liquid cultures, *A. baumannii* host cells grown as a biofilm do not adapt to form predation resistant biofilms as a consequence of predation by *B. bacteriovorus* 109J or HD100.

We have previously conducted experiments aimed at investigating the host range of each predator. However, it could be speculated that during the predation process alterations might cause a change in host specificity. A breach in host specificity could be undesirable, as it might allow the predators to attack communal non-pathogenic Gram-negative bacteria. In order to examine if a breach or alteration in predator host specificity could develop, predation resistant bacteria were used, and an attempt was made to enrich for *Bdellovibrio* variants that could attack the previously resistant bacteria. We have used both a Gram-negative and Gram-positive bacteria for this study. The data obtained suggests that *B. bacteriovorus* does not have an ability to breach its host specificity and attack previously resistant bacteria. This was true for both *S. maltophilia* and *S. epidermidis* host cells. We also did not obtain any *M. aeruginosavorus* isolates that breached their host specificity and attacked previously resistant bacteria.

In an attempt to enrich for *B. bacteriovorus* hyper virulent isolates, we found that *B. bacteriovorus* 109J could become more virulent on particular host. However, this increase in predation could develop even in the absence of the prey. Culturing the predator with the prey could slightly enhance predation. On the other hand, increased predation does not develop on all prey with some host bacteria maintaining their tolerance even after continuous culturing. Therefore, we can conclude that enhanced predation is specific and could develop on some host bacteria and not others. Additionally, continuous predation cycles did not seem to increase the ability *M. aeruginosavorus* to reduce a predation tolerant host. Finally, we were able to enrich for predators that were acclimated to attack at elevated temperatures.

Although the effect of predation on prokaryotic Gram-negative cells is documented, limited data is available regarding predation on eukaryotic cells. To determine if predatory bacteria have an adverse affect on eukaryotic cells, three human cell lines were exposed to high concentrations of predatory bacteria. Our data show that subjecting human epidermal keratinocytes, human skin fibroblasts and murine fibroblasts to the predators have caused no measurable toxic effect as compared to the control. Further more no cell detachment or morphological changes were seen in cells which were incubated with the predators. When measuring the cytokine levels in cells that were exposed to the predators we have seen that predatory bacteria did not provoke an elevated inflammation response in human epidermal keratinocytes, however, it did provoke an inflammation response in fibroblasts. This might be explained by the ability of some cells to better regulate their inflammation response in the presence of LPS. However at this point we can only conclude that the inflammation response is cell specific and while some cells will be stimulated by the predators other cell lines might be provoked.

Finally, when administered in vivo, predatory bacteria had showed no negative effect on the wellbeing of wounded mice. This was also correct when immunocompromised mice were used. The introduction of the predators to open wound had caused no significant difference with respect to weight loss, wound clinical score and wound time to close as compared to the non-inoculated control. This is the first time to our knowledge that predatory bacteria are administered to wounded and immunocompromised animals. At this point of the study we verified that predatory bacteria are safe when applied topically to open wounds. The effect of applying predatory bacteria to a wounds infection was evaluated in immunocompromised mice. Experiments were conducted using a single dose of *B. bacteriovorus* 109J, HD100 or *M.*

aeruginosavorus. Experiments were also conducted using multiple dosing of *B. bacteriovorus* 109J. The pathogens used to infect the wound were *Klebsiella pneumoniae* 4640 and *Acinetobacter baumannii* strain 5075::lux. As before, our findings indicate that predatory bacteria are safe when applied topically to open wounds of CP treated mice. However, we did not see any significant therapeutic potential using a single or multiple dosing treatments of predatory bacteria to clear a wound infected with inoculums *Klebsiella* or *Acinetobacter*. Conversely, in a few of our experiments, a slight reduction in wound size noted during the first few days. These conclusions are based on the measured effects of infection and known treatment (Colistin) or mock treatment (PBS) that were performed under the same conditions. The basis of our conclusion is the measurement of the following parameters: wound size, weight loss/gain.

Reportable Outcome

Manuscripts.

During the lifetime of this proposal we have published several manuscripts. The manuscripts discuss the ability of predatory bacteria to attack defined drug resistant pathogens as well as to treat pathogens associated with eye infection. The work is within the scope of our long-term objective of using predator bacteria to treat human infection.

1. **Kadouri, E. D.,** To, K., Shanks, M. Q., and Doi, Y. 2013. Predatory Bacteria; A Potential Ally against Multidrug-Resistant Gram-Negative Pathogens. PLoS ONE. 8(5): e63397. doi:10.1371/journal.pone.0063397.

2. Shanks, M. Q., Davra, R. V., Romanowski, G. E., Brothers, M. K., Stella, A. N., Godboley, D., and **Kadouri. E. D.** 2013. An Eye to a Kill: Using Predatory Bacteria to Control Gram-Negative Pathogens Associated With Ocular Infections. PLoS ONE. PLoS ONE 8(6): e66723. doi:10.1371/journal.pone.0066723.

3. Shanks, R. M., and **Kadouri E. D.** 2014. Predatory prokaryotes wage war against eye infections. Future Microbiology. 9:429-432.

4. Shatzkes, K., Chae, R., Tang, C., Ramirez, G., Mukherjee, S., Connell, N., and **Kadouri, E. D.** 2015. Examining the safety of respiratory and intravenous inoculation of *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* in a mouse model. Scientific Reports. 5, 12899; doi: 10.1038/srep12899

Oral Presentations.

Several of the findings supported by this grant were presented by the PI at the following invited Presentations. (No abstracts were submitted).

1. **Kadouri, E. D.** Controlling Drug Resistant Bacteria. Department of Oral Biology seminar series. New Jersey Medical School. Newark, NJ. February. 2013.

2. **Kadouri, E. D.** Controlling Drug Resistant Bacteria- The Answer is Out There. Department of Biochemistry, Microbiology and Immunology. University of Ottawa, Faculty of Medicine. Ontario, Canada. April. 2013.

3. **Kadouri, E. D**. Bio-control of Drug Resistant Bacteria. Physiology, Ecology and Taxonomy (NEMPET) Meeting. Blue Mountain Lake, NY. June 2013.

4. **Kadouri, E. D**. The use of predatory bacteria to control select pathogens and treat respiratory infections, Grant kickoff meeting, DoD, Defense Advanced Research Project Agency (DARPA), Virginia. January 2014.

5. **Kadouri, E. D**. The use of predatory bacteria to control lung infections, Chemical & Biological Terrorism Defense, Gordon Research Conference, Ventura, CA. March 2015.

6. **Kadouri, E. D**. Predatory Bacteria, not a good way to kill a mouse. Physiology, Ecology and Taxonomy (NEMPET) Meeting. Blue Mountain Lake, NY. June 2015.

7. **Kadouri, E. D**. Predatory Bacteria. 28th Annual Biological Safety Symposium, Mid-Atlantic Biological Safety Association (MABSA) Meeting. Newark, NJ. June 2015.

8. **Kadouri, E. D**. Predatory Bacteria a New Ally in the Fight Against Infection. Rutgers School of Dental Medicine Research Seminar Series. Newark, NJ. September 2015.

Poster Presentations.

1. **Kadouri, E. D.,** and Godboley, D. The use of predatory prokaryotes to control human pathogens and biofilms. 4th ASM Conference on Beneficial Microbes. San Antonio, Texas, October 2012.

2. Davra, R., Mukherjee, S., Gancz, H., Zurawski, D., and **Kadouri, E. D.** The use of predatory bacteria to control select pathogens and treat respiratory infections. Military Health System Research Symposium (MHSRS). Fort Lauderdale, FL. August 2012.

3. Connell, N., Zurawski, D., and **Kadouri, E. D.** Predatory Bacteria, a New Ally in the Fight Against Human Infection. Institute for Infectious and Inflammatory Diseases Symposium, NJMS, Newark, NJ. September 2014.

Other achievements

Student research opportunities at Rutgers.

This project had given a few students the opportunity to conduct research and gather hands-on scientific experience.

Research Assistant at Rutgers.
Somdatta Mukherjee.
Shilpi Gupta.

Rotation Student at Rutgers.
Andrew Kim-Rotation M. S. student (GSBS-Rutgers).
Ameet Patheja- Rotation M. S. student (GSBS-Rutgers).
Vanessa Sahs- Rotation M. S. student (GSBS-Rutgers).
Gregory Ramirez- M.S. candidate (GSBS-Rutgers).
Kenneth Shatzkes- Ph. D candidate (GSBS-Rutgers).
Chi Tang- Rotation M. S. student (GSBS-Rutgers).
Fernanda Morais- Rotation M. S. student (GSBS-Rutgers).

Additional funding.
Information generated via this grant was used as preliminary data to secure the following grant:

1. ARO Agreement W911NF-14-2-0016

DoD, Defense Advanced Research Project Agency (DARPA)

The use of predatory bacteria to control select pathogens and treat respiratory

infections. 12/2013- 12/2014

*Kadouri PI

2. ARO Agreement W911NF-15-2-0036

DoD, Defense Advanced Research Project Agency (DARPA).

Predatory Bacteria, from Basic Research to Application.

2015- 2018

*Kadouri PI

* Zurawski (WRAIR). Co-investigator.

Appendices

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PLOS ONE

Predatory Bacteria: A Potential Ally against Multidrug-Resistant Gram-Negative Pathogens

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Abstract

Multidrug-resistant (MDR) Gram-negative bacteria have emerged as a serious threat to human and animal health. *Bdellovibrio* spp. and *Micavibrio* spp. are Gram-negative bacteria that prey on other Gram-negative bacteria. In this study, the ability of *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* to prey on MDR Gram-negative clinical strains was examined. Although the potential use of predatory bacteria to attack MDR pathogens has been suggested, the data supporting these claims is lacking. By conducting predation experiments we have established that predatory bacteria have the capacity to attack clinical strains of a variety of ß-lactamase-producing, MDR Gram-negative bacteria. Our observations indicate that predatory bacteria maintained their ability to prey on MDR bacteria regardless of their antimicrobial resistance, hence, might be used as therapeutic agents where other antimicrobial drugs fail.

Citation: Kadouri DE, To K, Shanks RMQ, Doi Y (2013) Predatory Bacteria: A Potential Ally against Multidrug-Resistant Gram-Negative Pathogens. PLoS ONE 8(5): e63397. doi:10.1371/journal.pone.0063397

Editor: Axel Cloeckaert, Institut National de la Recherche Agronomique, France

Received February 28, 2013; **Accepted** March 31, 2013; **Published** May 1, 2013

Funding: This work was partly supported by the Department of the ARMY USAMRAA #W81XWH-12-2-0067 and by UMDNJ departmental start-up fund to DEK. YD was supported in part through a career development award, the National Institutes of Health (K22AI080584). RMQS was supported by NIH grants AI085570, EY08098, and a Research to Prevent Blindness Career Development award. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Since antimicrobial drugs were first discovered they have saved countless lives. However, pathogenic multidrug-resistant (MDR) bacteria have emerged as a serious threat to human health. Of particular concern are MDR Gram-negative bacteria producing highly potent ß-lactamases such as the extended-spectrum ß-lactamase and KPC-type ß-lactamase [1]. It is estimated that in the United States alone nearly 2 million patients develop hospital-acquired infection yearly [2], many of which are caused by these MDR pathogens. The magnitude of the problem has highlighted the need to develop new ways to control infection.

An alternative approach to combat antimicrobial-resistant bacterial infections is the use of predatory bacteria to eliminate MDR pathogens. *Bdellovibrio* spp. and *Micavibrio* spp. are Gram-negative bacteria which belong to the delta and alpha subgroup of proteobacteria respectively [3,4]. The *Bdellovibrio* life cycle involves attack phase cell that seek, attach to, and invade a Gram-negative bacterial host, and a growth phase cell that develops within the host [5–7]. The *Micavibrio* life cycle also exhibits an attack phase cell that allows it to find its Gram-negative bacterial host and to attach to the prey's surface, followed by extracellular growth of the predator [8–10]. We have previously demonstrated that both *Bdellovibrio* and *Micavibrio* have the potential to prey on a wide range of human pathogens grown both planktonically and as a biofilm [11–13]. However, the majority of the studies utilized culture collection reference strains or clinical strains for which the antibiotic susceptibility data were lacking [11,13]. Therefore, the ability of predator bacteria to attack contemporary clinical strains of MDR bacteria has remained unclear. To address this question, we examined the capacity of the two predatory bacteria to prey on MDR Gram-negative clinical strains producing clinically relevant ß-lactamases and representing various opportunistic nosocomial pathogens.

Materials and Methods

A total of 14 MDR clinical strains isolated between 2005 and 2011 were tested, including *Acinetobacter baumannii* [2], *Escherichia coli* [5], *Klebsiella pneumoniae* [5], and *Pseudomonas* spp. [2]. They were selected to include species which are commonly encountered clinically, and to represent a variety of potent ß-lactamases, including extended-spectrum ß-lactamase (ESBL), KPC-type carbapenemase, AmpC-type ß-lactamase, and metallo-ß-lactamase. Antimicrobial susceptibility was tested using the disk diffusion method and interpreted according to the breakpoints endorsed by the Clinical and Laboratory Standards Institute (CLSI) (Table 1) [14]. The ß-lactamases produced were characterized previously [15,16] or otherwise determined by PCR and sequencing [17]. Three predatory bacteria were used in this study: *Bdellovibrio bacteriovorus* 109J (ATCC 43826), *B. bacteriovorus* HD100 and *Micavibrio aeruginosavorus* strain ARL-13 [5,10]. The predators were grown and maintained as described before [11]. Predator stock-lysates were made by co-culturing host cells with the predators in diluted nutrient broth (DNB) and allowing the co-culture to incubate at 30°C on a rotary shaker until the culture became clear. To culture the predators, co-cultures were prepared by adding 2 ml of washed host cells ($\sim 1 \times 10^9$ CFU/ml) to 2 ml of

Table 1. Host pathogens used in the study and their antibiotic susceptibility.

Bacteria and strain	Source	ß-lactamase gene	Antibiotic susceptibility
Acinetobacter baumannii			
AB276	Sputum	OXA-23	Ceftazidime (R); Cefotaxime (R); Cefepime (S); Imipenem (I); Meropenem (R); Gentamicin (R); Amikacin (R); Ciprofloxacin (R); Tetracycline (R); Trimethoprim-sulfamethoxazole (R)
AB285	Donor bronchus	OXA-40	Ceftazidime (R); Cefotaxime (R); Cefepime (R); Imipenem (R); Meropenem (R); Gentamicin (R); Amikacin (R); Ciprofloxacin (R); Tetracycline (R); Trimethoprim-sulfamethoxazole (R)
Escherichia coli			
YD429	Urine	CTX-M-15	Ceftazidime (I); Cefotaxime (R); Cefepime (S); Imipenem (S); Meropenem (S); Gentamicin (R); Amikacin (S); Ciprofloxacin (R); Tetracycline (R); Trimethoprim-sulfamethoxazole (R)
YD438	Blood	SHV-7	Ceftazidime (S); Cefotaxime (S); Cefepime (S); Imipenem (S); Meropenem (S); Gentamicin (S); Amikacin (S); Ciprofloxacin (R); Tetracycline (R); Trimethoprim-sulfamethoxazole (S)
YD446	Urine	CTX-M-14	Ceftazidime (S); Cefotaxime (R); Cefepime (S); Imipenem (S); Meropenem (S); Gentamicin (S); Amikacin (S); Ciprofloxacin (R); Tetracycline (R); Trimethoprim-sulfamethoxazole (R)
YDC354	Urine	KPC-2	Ceftazidime (R); Cefotaxime (R); Cefepime (S); Imipenem (R); Meropenem (R); Gentamicin (S); Amikacin (S); Ciprofloxacin (R); Tetracycline (R); Trimethoprim-sulfamethoxazole (R)
AZ1285	Blood	CMY-33	Ceftazidime (R); Cefotaxime (R); Cefepime (R); Imipenem (S); Meropenem (S); Gentamicin (S); Amikacin (S); Ciprofloxacin (R); Tetracycline (R); Trimethoprim-sulfamethoxazole (S)
Klebsiella pneumoniae			
YD466	Wound	KPC-2	Ceftazidime (R); Cefotaxime (R); Cefepime (R); Imipenem (R); Meropenem (R); Gentamicin (S); Amikacin (R); Ciprofloxacin (R); Tetracycline (S); Trimethoprim-sulfamethoxazole (R)
AZ1032	Blood	SHV-7	Ceftazidime (R); Cefotaxime (R); Cefepime (S); Imipenem (S); Meropenem (S); Gentamicin (R); Amikacin (S); Ciprofloxacin (S); Tetracycline (S); Trimethoprim-sulfamethoxazole (R)
AZ1093	Blood	SHV-5	Ceftazidime (R); Cefotaxime (R); Cefepime (I); Imipenem (S); Meropenem (S); Gentamicin (R); Amikacin (R); Ciprofloxacin (S); Tetracycline (S); Trimethoprim-sulfamethoxazole (R)
AZ1136	Blood	CTX-M-2	Ceftazidime (R); Cefotaxime (R); Cefepime (R); Imipenem (S); Meropenem (I); Gentamicin (R); Amikacin (R); Ciprofloxacin (R); Tetracycline (S); Trimethoprim-sulfamethoxazole (R)
AZ1169	Blood	SHV-12	Ceftazidime (R); Cefotaxime (R); Cefepime (S); Imipenem (S); Meropenem (S); Gentamicin (S); Amikacin (S); Ciprofloxacin (R); Tetracycline (S); Trimethoprim-sulfamethoxazole (R)
Pseudomonas aeruginosa			
GB771	Sputum	PME-1	Ceftazidime (R); Cefotaxime (R)[1]; Cefepime (R); Imipenem (R); Meropenem (R); Gentamicin (R); Amikacin (S); Ciprofloxacin (R); Tetracycline (R)[1]; Trimethoprim-sulfamethoxazole (R)[1]
Pseudomonas putida			
YA241	Sputum	VIM-1	Ceftazidime (R); Cefotaxime (R)[1]; Cefepime (R); Imipenem (R); Meropenem (R); Gentamicin (R); Amikacin (S); Ciprofloxacin (R); Tetracycline (I)[1]; Trimethoprim-sulfamethoxazole (R)[1]

(R) Resistant; (I) intermediate; (S) susceptible.

[1]Breakpoints are not defined for cefotaxime, tetracycline and trimethoprim-sulfamethoxazole by the CLSI; interpretation based on the breakpoints for *A. baumannii*.

doi:10.1371/journal.pone.0063397.t001

predatory bacteria stock-lysate in 20 ml of DNB. The co-cultures were incubated for 24 hrs until the predator reached a final concentration of $\sim 1\times10^8$ PFU/ml. Thereafter, the lysates were filtered through a 0.45-μm Millex pore-size filter (Millipore, Billerica, MA) in order to remove remaining host cells (predator filtered lysate). As a control, filtered sterilized lysate was prepared by passing the lysates through three 0.22 μm pore-size filters [12,13]. Predation experiments were conducted as described previously [11]. In brief, 5 ml of DNB co-cultures were made by adding to 0.5 ml of washed host cells to 0.5 ml of predator filtered lysate or predator-free control. The cultures were placed at 30°C on a rotary shaker for 48 hrs.

Results and Discussion

The ability of each predator to attack the host was measured by the reduction in host cell viability, determined by dilution plating and CFU enumeration, and compared to the initial host concentration and predator-free control. Cell viability was measured following 24 and 48 hrs of incubation. Each co-culture

Table 2. Change in host viability following predation.

Bacteria and strain	$Time_0$ (CFU/ml)	Control (Log_{10} change)	*B. bacteriovorus* 109J (Log_{10} change)	*B. bacteriovorus* HD100 (Log_{10} change)	*M. aeruginosavorus* ARL-13 (Log_{10} change)
Acinetobater baumannii					
AB276	3.38×10^8	+0.47±0.21	−3.92±0.27	−3.79±0.07	na
AB285	2.50×10^8	+0.12±0.28	−3.56±0.06	−2.75±0.11	na
Escherichia coli					
YD429	3.13×10^8	+0.03±0.02	−1.7±0.20	−3.68±0.11	na
YD438	1.38×10^8	+0.09±0.51	−3.55±0.20	−3.89±0.84	na
YD446	4.50×10^8	+0.07±0.03	−2.96±0.18	−3.2±0.3*	na
YDC354	4.25×10^8	+0.01±0.11	−0.1±0.12*$^{\Psi}$	−3.72±0.07	na
AZ1285	6.00×10^8	+0.80±1.13	−3.61±0.07	−3.8±0.84	na
Klebsiella pneumoniae					
YD466	3.63×10^8	+0.24±0.18	−3.99±0.36	−3.73±0.20	−2.91±0.19
AZ1032	4.38×10^8	+0.07±0.05	−2.75±0.10	−4.04±0.56	−0.05±0.07$^{\Psi}$
AZ1093	4.30×10^8	+0.28±0.23	−2.42±0.07	−4.09±0.39	−0.70±0.11$^{\Psi}$
AZ1136	4.63×10^8	+0.08±0.03	−3.54±0.36	−2.83±0.09	−3.01±0.19
AZ1169	4.61×10^8	−0.43±0.20	−4.51±0.55	−1.79±0.15	−2.85±0.05*
Pseudomonas aeruginosa					
GB771	2.53×10^8	+0.71±0.26	−3.96±0.32*	−3.07±0.68	−2.64±0.29
Pseudomonas putida					
YA241	6.25×10^6	+0.94±0.22	−2.4±0.14	−3.90±0.35	−1.41±0.35

Co-cultures were prepared by adding host cells to harvested predator cells ($\sim1\times10^7$ PFU) or predator free control. Values represent the maximum log_{10} change measured following 24 or 48 (*) hrs of incubation (compared to t_0). Each experiments was conducted in triplicates with value representing the mean and standard error.
n.a- not applicable.
Ψ = experiment was conducted twice yielding similar result.
Time 0- initial host concentration (CFU/ml).
+ = Increase in host numbers.
− = Decrease in host numbers.
doi:10.1371/journal.pone.0063397.t002

was performed in triplicate. The ability of the predators to attack each of the MDR pathogens is shown in Table 2. *B. bacteriovorus* HD100 was able to prey on all examined host bacteria with a greater than 2, 3 and 4 log_{10} reduction measured for 93%, 78% and 35% of the attacked strains, respectively. *B. bacteriovorus* 109J was able to prey on 13 of the 14 host bacteria (93%) with a greater than 2, 3 and 4 log_{10} reduction measured for 85%, 64% and 28% of the predation positive strains, respectively. Five out of the 7 (71%) examined host bacteria were reduced by *M. aeruginosavorus* ARL-13, with 80% and 40% of the predator-susceptible strains showing a 2 and 3 log_{10} reduction, respectively. In this study *Micavibrio* was examined only on *P. aeruginosa* and *K. pneumoniae* as previous study suggested that *M. aeruginosavorus* ARL-13 is most capable of preying on these pathogens [11,13]. The predators maintained their ability to prey on the host cells despite the MDR status. Furthermore, no clear patterns emerged when comparing the antibiotic susceptibility of the host cells to predation. The different host specificity observed for each predator, as well as the differential capacity of each predator strain to prey on certain stains within the same species, is well documented for both *Bdellovibrio* and *Micavibrio* [6,9,11,13,18–20]. However, as the mechanisms that govern host specificity are not fully understood, it is difficult to speculate on the reason way some host strains are consumed by the predators whereas others are not.

Conclusions

With the increased occurrence of MDR pathogens, many of which can no longer be treated adequately by conventional antimicrobial agents, becoming a major clinical concern, the concept of using predatory bacteria as live antimicrobials is gaining momentum [21–23]. Although the putative ability of predatory bacteria to attack MDR pathogens was hypothesized, it was never clearly demonstrated. Our data confirms that predatory bacteria maintained their ability to prey on MDR bacteria regardless of their antimicrobial resistance. This study further highlight the potential application of predatory bacteria as a biological control agent with the capability to prey on MDR Gram-negative pathogens which are currently found in clinical settings.

Acknowledgments

The authors thank Genesis Cano and Tiffany Aston for provided technical assistance.

Author Contributions

Conceived and designed the experiments: DEK RMQS YD. Performed the experiments: KT. Analyzed the data: DEK. Contributed reagents/materials/analysis tools: YD. Wrote the paper: DEK RMQS YD.

References

1. Kallen AJ, Srinivasan A (2010) Current epidemiology of multidrug-resistant gram-negative bacilli in the United States. Infect Control Hosp Epidemiol 31 Suppl 1: S51–54.
2. Klevens RM, Edwards JR, Richards CL Jr., Horan TC, Gaynes RP, et al. (2007) Estimating health care-associated infections and deaths in U.S. hospitals, 2002. Public Health Rep 122: 160–166.
3. Davidov Y, Huchon D, Koval SF, Jurkevitch E (2006) A new alpha-proteobacterial clade of *Bdellovibrio*-like predators: implications for the mitochondrial endosymbiotic theory. Environ Microbiol 8: 2179–2188.
4. Davidov Y, Jurkevitch E (2004) Diversity and evolution of *Bdellovibrio*-and-like organisms (BALOs), reclassification of *Bacteriovorax* starrii as Peredibacter starrii gen. nov., comb. nov., and description of the *Bacteriovorax*-Peredibacter clade as *Bacteriovoracaceae* fam. nov. Int J Syst Evol Microbiol 54: 1439–1452.
5. Rendulic S, Jagtap P, Rosinus A, Eppinger M, Baar C, et al. (2004) A predator unmasked: life cycle of *Bdellovibrio bacteriovorus* from a genomic perspective. Science 303: 689–692.
6. Stolp H, Starr MP (1963) *Bdellovibrio bacteriovorus* gen. et sp. n., a Predatory, Ectoparasitic, and Bacteriolytic Microorganism. Antonie Van Leeuwenhoek 29: 217–248.
7. Sockett RE (2009) Predatory lifestyle of *Bdellovibrio bacteriovorus*. Annu Rev Microbiol 63: 523–539.
8. Lambina VA, Afinogenova AV, Romai Penabad S, Konovalova SM, Pushkareva AP (1982) *Micavibrio admirandus* gen. et sp. nov. Mikrobiologiia 51: 114–117.
9. Lambina VA, Afinogenova AV, Romay Penobad Z, Konovalova SM, Andreev LV (1983) New species of exoparasitic bacteria of the genus Micavibrio infecting gram-positive bacteria. Mikrobiologiia 52: 777–780.
10. Wang Z, Kadouri DE, Wu M (2011) Genomic insights into an obligate epibiotic bacterial predator: *Micavibrio aeruginosavorus* ARL-13. BMC Genomics 12: 453.
11. Dashiff A, Junka RA, Libera M, Kadouri DE (2011) Predation of human pathogens by the predatory bacteria *Micavibrio aeruginosavorus* and *Bdellovibrio bacteriovorus*. J Appl Microbiol 110: 431–444.
12. Kadouri D, O'Toole GA (2005) Susceptibility of biofilms to *Bdellovibrio bacteriovorus* attack. Appl Environ Microbiol 71: 4044–4051.
13. Kadouri D, Venzon NC, O'Toole GA (2007) Vulnerability of pathogenic biofilms to *Micavibrio aeruginosavorus*. Appl Environ Microbiol 73: 605–614.
14. Clinical and Laboratory Standards Institute (2012) Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Second Informational Supplement. Wayne, PA.
15. Tian GB, Adams-Haduch JM, Bogdanovich T, Wang HN, Doi Y (2011) PME-1, an extended-spectrum b-lactamase identified in *Pseudomonas aeruginosa*. Antimicrob Agents Chemother 55: 2710–2713.
16. Doi Y, Paterson DL, Adams-Haduch JM, Sidjabat HE, O'Keefe A, et al. (2009) Reduced susceptibility to cefepime among *Escherichia coli* clinical isolates producing novel variants of CMY-2 b-lactamase. Antimicrob Agents Chemother 53: 3159–3161.
17. Kim YA, Qureshi ZA, Adams-Haduch JM, Park YS, Shutt KA, et al. (2012) Features of infections due to *Klebsiella pneumoniae* carbapenemase-producing *Escherichia coli*: emergence of sequence type 131. Clin Infect Dis 55: 224–231.
18. Jurkevitch E, Minz D, Ramati B, Barel G (2000) Prey range characterization, ribotyping, and diversity of soil and rhizosphere *Bdellovibrio* spp. isolated on phytopathogenic bacteria. Appl Environ Microbiol 66: 2365–2371.
19. Rogosky AM, Moak PL, Emmert EA (2006) Differential predation by *Bdellovibrio bacteriovorus* 109J. Curr Microbiol 52: 81–85.
20. Markelova NY (2010) Predacious bacteria, *Bdellovibrio* with potential for biocontrol. Int J Hyg Environ Health 213: 428–431.
21. Dwidar M, Monnappa AK, Mitchell RJ (2012) The dual probiotic and antibiotic nature of *Bdellovibrio bacteriovorus*. BMB Rep 45: 71–78.
22. Martin MO (2002) Predatory prokaryotes: an emerging research opportunity. J Mol Microbiol Biotechnol 4: 467–477.
23. Sockett RE, Lambert C (2004) *Bdellovibrio* as therapeutic agents: a predatory renaissance? Nat Rev Microbiol 2: 669–675.

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An Eye to a Kill: Using Predatory Bacteria to Control Gram-Negative Pathogens Associated with Ocular Infections

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Abstract

Ocular infections are a leading cause of vision loss. It has been previously suggested that predatory prokaryotes might be used as live antibiotics to control infections. In this study, *Pseudomonas aeruginosa* and *Serratia marcescens* ocular isolates were exposed to the predatory bacteria *Micavibrio aeruginosavorus* and *Bdellovibrio bacteriovorus*. All tested *S. marcescens* isolates were susceptible to predation by *B. bacteriovorus* strains 109J and HD100. Seven of the 10 *P. aeruginosa* isolates were susceptible to predation by *B. bacteriovorus* 109J with 80% being attacked by *M. aeruginosavorus*. All of the 19 tested isolates were found to be sensitive to at least one predator. To further investigate the effect of the predators on eukaryotic cells, human corneal-limbal epithelial (HCLE) cells were exposed to high concentrations of the predators. Cytotoxicity assays demonstrated that predatory bacteria do not damage ocular surface cells *in vitro* whereas the *P. aeruginosa* used as a positive control was highly toxic. Furthermore, no increase in the production of the proinflammatory cytokines IL-8 and TNF-alpha was measured in HCLE cells after exposure to the predators. Finally, injection of high concentration of predatory bacteria into the hemocoel of *Galleria mellonella*, an established model system used to study microbial pathogenesis, did not result in any measurable negative effect to the host. Our results suggest that predatory bacteria could be considered in the near future as a safe topical bio-control agent to treat ocular infections.

Citation: Shanks RMQ, Davra VR, Romanowski EG, Brothers KM, Stella NA, et al. (2013) An Eye to a Kill: Using Predatory Bacteria to Control Gram-Negative Pathogens Associated with Ocular Infections. PLoS ONE 8(6): e66723. doi:10.1371/journal.pone.0066723

Editor: Michael Hensel, University of Osnabrueck, Germany

Received April 3, 2013; **Accepted** May 10, 2013; **Published** June 18, 2013

Funding: This work was partly supported by the Department of the ARMY USAMRAA #W81XWH-12-2-0067 and by UMDNJ departmental start-up fund to DEK and RMQS was supported by National Institutes of Health (NIH) grant AI085570 and a Research to Prevent Blindness Career Development award. The authors thank the NIH EY08098 grant sponsored hybridoma module for providing HCLE cells. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

In an era of increasing antibiotic resistance among bacterial pathogens, the search for new antibiotics and novel treatments for infections caused by these organisms is a priority among researchers. One novel treatment is biological therapy using specific bacteriophage for controlling the infecting pathogen [1–3]. Another novel treatment that might hold the potential to treat antibiotic resistant infections are predatory bacteria [4]. Recently, several studies have highlighted the ability of predatory bacteria *Bdellovibrio* spp. and *Micavibrio* spp. to prey on Gram-negative pathogens. Among the pathogens which were evaluated were bacteria associated with oral infections [5–7], gastrointestinal infections [8], zoonotic infection [9], pathogens associated with food processing and spoilage [10–12], as well as bacteria linked to systemic infections, burns and wounds [13]. Although the data published so far supports the claim that predatory bacteria could be used to control human pathogens, there is still concern regarding the toxic effects of administering large numbers of Gram-negative bacteria as live antibiotics. Therefore, treatment of local infections where the pathogens are easily accessible to topical or locally injected treatment would be ideal candidates to demonstrate a "proof of concept" that infections can be successfully treated with predatory bacteria.

One such local bacterial infection that is treated by direct administration of antibiotic to the site of infection is keratitis, infection of the cornea. Bacterial keratitis can be caused by both Gram-positive and Gram-negative pathogens. Common Gram-negative pathogens associated with keratitis are *Pseudomonas aeruginosa* and *Serratia marcescens* [14–16]. Bacterial keratitis is usually localized to an area of the cornea and is treated with antibiotic solutions delivered topically to the eye.

The first step in demonstrating that predatory bacteria can successfully treat bacterial keratitis caused by Gram-negative bacteria is showing that the predatory bacteria can kill Gram-negative bacteria isolated from keratitis cases. Secondly, we must show that the predatory bacteria are non-toxic and non-inflammatory to human cells. In this study we tested whether predatory bacterial species *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* were able to kill keratitis isolates of *P. aeruginosa* and *S. marcescens*, including antibiotic-resistant isolates, *in vitro*. We also tested whether *B. bacteriovorus* and *M. aeruginosavorus* were cytotoxic and inflammatory to human corneal-limbal epithelial cells (HCLE)

in vitro. To further test whether these predatory bacteria were pathogenic, we used the *Galleria monella* pathogenesis model to determine whether *B. bacteriovorus* and *M. aeruginosavorus* reduced the viability of the *G. monella* larvae.

Materials and Methods

Bacterial strains, and growth conditions

The predatory bacteria used in the study were *Bdellovibrio bacteriovorus* strains HD100, 109J (ATCC 43826) and *Micavibrio aeruginosavorus* strain ARL-13 [17,18]. Ten *Pseudomonas aeruginosa* and nine *Serratia marcescens* isolates were examined in this study. All clinical isolates were isolated from keratitis patients by Dr. Ritterband at the New York Eye Infirmary and Regis Kowalski at the UPMC Eye Center. Many of these bacteria were fluoroquinolone resistant and previously used in antibiotic efficacy studies [19,20]. *Pseudomonas aeruginosa* and *Serratia marcescens* were grown with aeration and maintained in LB media. Predator stock-lysates were prepared by co-culturing the predators in the presence of host bacteria suspended in diluted nutrient broth (DNB), a 1:10 dilution of nutrient broth amended with 3 mM MgCl2 and 2 mM $CaCl_2$ [13]. *E. coli* ZK2686 and *P. aeruginosa* UCBPP-PA14 were used as host cells for *B. bacteriovorus* and *M. aeruginosavorus*, respectively. The co-cultures were incubated on a rotary shaker at 30C. Fresh predator cultures were prepared as previously described [13,21,22], in brief, 2 ml of overnight-grown host cells ($\sim 1\times10^9$ CFU/ml) were added to 2 ml of predatory bacteria taken from a stock-lysate and suspended in 20 ml DNB. The co-cultures were incubated for 24 hrs at 30°C to reach $\sim 1\times10^8$ PFU/ml predator's cells. At this point, the lysates were filtered through a 0.45 μm Millex-HV pore-size filter (Millipore, Billerica, MA) in order to remove any residual host cells (harvested predator).

Predation Experiments

Predation experiments were conducted as previously described [13]. Five ml co-cultures were prepared by adding 0.5 ml of washed host cells to 0.5 ml of freshly harvested predator bacteria in DNB media. The cultures were incubated at 30°C for 48 hrs. The capability of *B. bacteriovorus* and *M. aeruginosavorus* to prey was evaluated by the reduction in prey cell viability in the predator co-cultures. Cell viability was measured by dilution plating and CFU enumeration at 24 and 48 hrs. Each co-culture was conducted twice in triplicate.

Cytotoxicity assays

B. bacteriovorus and *M. aeruginosavorus* were prepared as described above using 5 ml of washed host cells and 5 ml of freshly harvested predator in 50 ml DNB media. The co-cultures were incubated for 24 and 36 hrs for *B. bacteriovorus* and *M. aeruginosavorus*, respectively. Thereafter, the lysates were filtered four times through a 0.45-μm Millex-HV pore-size filter to remove any residual host cells. The filtered harvested lysate was washed twice by centrifugation, 15,000 rpm for 30 min, and resuspended in 2 ml of DNB. Aliquots of the predator preparation was removed and plated on agar plates, to confirm that the samples are free from host cells. Samples were also taken to determine predator concentration using standard double-layered agar method [23]. Purifications were conducted on 3 and 4 separate occasions for *M. aeruginosavorus* and *B. bacteriovorus*, respectively.

Cytotoxicity assays were conducted as described [24] with some modifications, Human corneal-limbal epithelial (HCLE) [25] cells were cultured in 24-well plates until they were confluent. HCLE cells were grown in Keratinocyte serum-free medium (KSFM) with L-Glutamine, supplemented with 25 μg/ml BPE, 0.2 ng/ml EGF, and 1 mM $CaCl_2$. HCLE cells were seeded without antibiotics to prevent interference of antibiotics in subsequent assays. The plates were incubated in an incubator at 37°C with 5% CO_2. The wells were washed 3 times using PBS, pH 7.4 (Sigma-Aldrich, St. Louis, MO) and 450 μl of KSFM media was added to each well. Thereafter, wells were inoculated with 50 μl of each predator prep ($\sim 0.2–1.1\times10^9$ PFU/well for *B. bacteriovorus* strains and $\sim 2\times10^8$ PFU/ well for *M. aeruginosavorus*) or predator free DNB control for maximum viability. Other controls included 0.25% of triton X-100 to measure total killing and 50 μl of DNB washed *P. aeruginosa* PA14 ($\sim 2.5\times10^7$ CFU/well) as a positive control for bacterial cytotoxicity. Cell cultures were incubated for 4 and 24 hrs. After the incubation, aliquots of medium were removed from each well, centrifuged to remove bacteria, and stored at –20°C for subsequent pro-inflammatory cytokine analysis. The cells were then washed three times with PBS. Alamar Blue viability reagent (Invitrogen) in KSFM containing amikacin (10 μg/ml) was added to each well (500 μl/well) to assess cell viability. Fluorescence was measured after 1.5 hrs of incubation using a Synergy 2 microplate reader (Biotek) at 500/27 nm excitation and 620/40 nm emission wavelength. Experiments were conducted four times using *B. bacteriovorus* and three times using *M. aeruginosavorus*. Each experiment was conducted in quadruplicate (4 cell culture wells).

Cytokine analysis

HCLE supernatants were collected at 4 and 24 hrs post bacterial exposure. Four biological samples were used for ELISA and also tested on two different days with a different harvest sample set. IL-8 and TNFα ELISAs were run on the 4 and 24-hour sample sets according to manufacturer's instructions (for IL-8, R & D Systems®; for TNFα, Thermo Scientific Pierce Biotechnology). Upon completion of the assay, samples were read according to the manufacturer's instructions on a Synergy 2 plate reader (BioTek). Samples were graphed and statistical analysis was performed using GraphPad Prism 5 using one-way ANOVA with Tukey's post-hoc test.

Toxicity assay in *Galleria mellonella* invertebrate infection model

Viability experiments were conducted as described previously [26] with some modifications. *Galleria mellonella* larvae were obtained from New York worms (New York Worms, Glen Cove, NY). Larvae were in their final instar stages and had equal size and weight (330±30 mg). *B. bacteriovorus* strains, 109J, HD100, and *M. aeruginosavorus* ARL-13 were grown and concentrated as descried for the cytotoxicity assays. The predators were suspended in phosphate buffered saline (PBS), pH 7.4 (Sigma-Aldrich, St. Louis, MO). Final predator concentration 2×10^9 PFU/ml. Five micro-liters of each sample was injected into the hemocoel of each larva via the last left proleg using a Hamilton 25 μl syringe and 30.5-gauge needle. Prior to use, the syringes were sterilized using bleach. The syringes were cleaned with 70% alcohol and distilled water and the needles were changed between every sample. In addition to the predators, worms were also injected with 5 μl of PBS buffer (negative control) and 5 μl of 8×10^4 CFU/ml *P. aeruginosa* PA14 (positive control). After injection, the worms were incubated at 30C and the numbers of live larvae were scored for 11 days. Larvae were considered dead when they display no movement in response to gentle shaking of the dish or touching with a pipette tip. Six petri dishes containing 5 worms were assigned to each experimental and control groups (30 worms total for each sample).

Statistical Analysis

Graphpad Prism 5 software was used to perform statistical analysis. This analysis consisted of One-way ANOVA with Tukey's multiple comparison test.

Results

Predation by B. bacteriovorus and M. aeruginosavorus

When exposed to the predators, all of the isolates were found to be susceptible to at least one predator. All *S. marcescens* isolates were found to be susceptible to predation by both *B. bacteriovorus* 109J and HD100, with cell reduction ranging from 1.7 $\log_{10}$ to greater than 5 $\log_{10}$, compared to the initial cell concentration and the predator free control (Table 1). 100% of the tested *P. aeruginosa* isolates were reduced by *B. bacteriovorus* HD100. However, only 70% of the isolates were reduced by the 109J strain. Eight of the 10 *P. aeruginosa* isolates were reduced by *M. aeruginosavorus* with a greater than 2-$\log_{10}$ reduction measured for 87% of the predation positive strains (Table 1). It was previously shown that *M. aeruginosavorus* ARL-13 is able to use *P. aeruginosa* as prey; however, it is unable to utilize *S. marcescens* [13,22]. Therefore, in this study, *M. aeruginosavorus* was not tested on *S. marcescens*.

Cytotoxicity B. bacteriovorus and M. aeruginosavorus to HCLE cells

As a first step towards judging the suitability of predatory bacteria for ocular infections, we tested whether *B. bacteriovorus* and *M. aeruginosavorus* were cytotoxic to HCLE cells *in vitro*. Bacteria were co-incubated with HCLE cells at an MOI of ~100 with a known cytotoxic *P. aeruginosa* strain [27] as a positive control, and >800 for each of the predatory bacteria. Bacteria and HCLEs were co-incubated for 4 and 24 hours, then bacteria were removed and HCLE cells were tested for viability using the fluorescent vital stain alamar blue. Whereas *P. aeruginosa* was highly cytotoxic at both time points, the predatory bacteria were not significantly different from the mock at either 4 or 24 hrs ($p>0.05$, ANOVA, with Tukey's post-test) (Figure 1).

Production of pro-inflammatory cytokines following exposure to predatory bacteria

Because the predatory bacteria used in this study are Gram-negative bacteria, we predicted that they may cause adverse inflammatory effects upon exposure to ocular cells. Supernatants of HCLE cells co-incubated for four hrs with *B. bacteriovorus* and *M. aeruginosavorus* in the above noted cytotoxicity studies and were analyzed for proinflammatory cytokines IL-8 and TNF-a. These cytokines were chosen because they are expressed by ocular surface cells exposed to bacteria [28,29]. Whereas the positive

Table 1. Predation of *S. marcescens* and *P. aeruginosa* ocular isolates by predatory bacteria.

Bacteria and strain	Time$_0$ (CFU/ml)	Control ($\log_{10}$ change)	*B. bacteriovorus* 109J ($\log_{10}$ change)	*B. bacteriovorus* HD100 ($\log_{10}$ change)	*M. aeruginosavorus* ARL-13 ($\log_{10}$ change)
Serratia marcescens					
K912	1.25×10^8	+0.74±0.46	−1.7±0.15	−2.62±0.03	na
K1064	9.43×10^8	−0.04±0.06	−2.63±0.06	−4.55±0.10	na
K1097	4.32×10^8	+0.09±0.13	−3.56±0.07	−4.17±0.10	na
K1154	5.64×10^8	−0.09± 0.05	−3.91±0.06	−4.24± 0.01	na
K1885	3.48×10^8	+0.22±0.02	−3.7±0.24	−4.6±0.19	na
K1496	6.06×10^8	+0.07±0.10	−3.74±0.01	−5.28±0.08	na
K2093	3.91×10^8	+0.07±0.08	−2.88±0.06	−3.94±0.2	na
K2119	1.25×10^8	+0.24±0.11	−3.5±0.24	−5.48±0.06	na
K2282	1.28×10^8	+0.89±0.03	−4.39±1.13	−3.05±0.29	na
Pseudomonas aeruginosa					
PaA	3.56×10^8	+0.29±0.18	−4.97±0.13	−3.5±0.19	−1.10±0.50*
PaB	5.00×10^8	+0.23±0.03	−3.67±0.01	−2.74±0.22*	+0.12±0.08$^{\Psi}$
PaC	7.03×10^8	+0.07±0.06	−2.13±0.15	−3.91±0.03	−2.98±0.08
PaD	3.26×10^8	−0.69±0.02	−2.06±0.27	−3.66±0.16	−2.86±0.21*
Pa16	8.28×108	−0.07±0.03	−3.58 ±0.06	−2.18±0.24*	−0.19±0.09
K2418	4.91×10^8	+0.25±0.10	+0.18±0.06	−3.01±0.42*	−2.74±0.40
K2409	1.07×10^9	−0.08±0.03	−4.18±0.14	−4.48±0.06	−2.03±0.16*
K2222	8.51×10^8	−0.01±0.26	−2.78±0.11	−2.19±0.43	−2.85±0.04*
K2414	7.26×10^8	+0.16±0.08	−0.04±0.33$^{\Psi}$	−1.16±0.23	−2.85±0.10*
K2421	8.38×10^8	+0.29±0.10	−0.29±0.21	−2.61±0.22	−3.51±0.43

Co-cultures were prepared by adding host cells to harvested predator cells (~1×10^7 PFU final concentration) or predator free control. Values represent the maximum $\log_{10}$ change measured following 24 or 48 (*) hrs of incubation (compared to t_0). Each experiments was conducted twice in triplicate yielding similar results. Value representing the mean and standard error from one representative experiment.

n.a.- not applicable.

Time 0- initial host concentration (CFU/ml).

+ = Increase in host numbers.

− = Decrease in host numbers.

doi:10.1371/journal.pone.0066723.t001

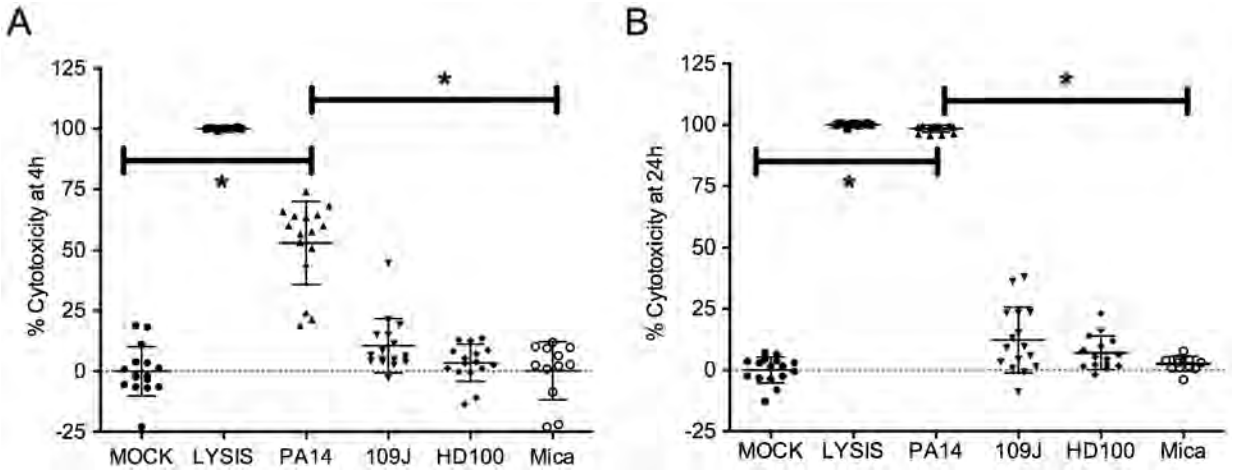

Figure 1. Cytotoxicity to human corneal-limbal epithelial cells *in vitro*. Alamar blue vital stain was used to measure cytotoxicity from positive control *P. aeruginosa* strain PA14 (average MOI = 111), detergent lysis (LYSIS), medium only negative control (MOCK), and experimental strains *B. bacteriovorus* strain 109J (average MOI = 4720), *B. bacteriovorus* strain HD100 (average MOI = 1039), and *M. aeruginosavorus* (Mica, average MOI = 853). HCLE viability was measured after 4 h (A) and 24 h (B) of exposure. Total independent data points from 4 experiments are shown. Asterisks indicate significant differences ($p<0.001$, ANOVA with Tukey's post-test). Only PA14 was significantly different than MOCK. Error bars indicate one standard deviation.
doi:10.1371/journal.pone.0066723.g001

control, *P. aeruginosa*, elicited a strong and significant induction of both cytokines, neither IL-8 nor TNF-a was found to be elevated above the mock negative control in HCLE supernatants co-incubated with any of the three predatory bacteria (Figure 2A–B). The same pattern was observed after co-incubation of the predatory and positive control bacteria at 24 hrs for IL-8; however, TNF-a levels were undetectable (data not shown).

In vivo effect of predatory bacteria on *G. mellonella*

G. mellonella was recently recognized as a suitable host model system to study microbial pathogenesis and innate immunity [30–34]. The *G. mellonella* system generally demonstrates a positive correlation between virulence factors found in mammals to those isolated in the insect, emphasizing the ability to utilize the system to bridge between in vitro studies and vertebrates [26]. In order to further evaluate the potential risk of using predatory bacteria *G. mellonella* worms were exposed to high concentrations of each predator. All of the worms injected with *P. aeruginosa* PA14 (4×10^2 CFU/worm) were nonviable 24 hrs post-injection. However, worms injected with PBS, *B. bacteriovorus* 109J, HD100 (1.1×10^7 PFU/worm) and *M. aeruginosavorus* ARL-13 (0.9×10^7 PFU/worm) were all viable 24 hrs post-injection. 11 days post-injection, the viability of the worms were 96.6%, 100%, 96.6% and 93.3% viability for the control, *B. bacteriovorus* 109J, HD100 and *M. aeruginosavorus* ARL-13, respectively. Furthermore, no change in larva pigmentation was observed in the predator-infected worms (Data not shown). In *G. mellonella* the change in color indicates melanization caused by the host immune response to the microbial

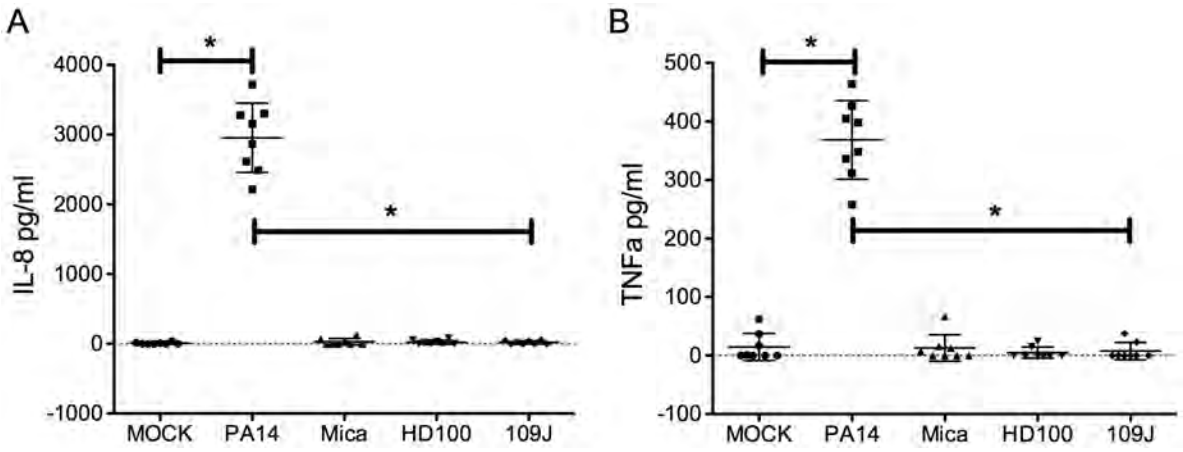

Figure 2. Inflammatory response of human corneal-limbal epithelial cells to predatory bacteria *in vitro*. Pro-inflammatory cytokines IL-8 (panel A) and TNF-α (panel B) were measured using ELISA assays. Cell supernatants taken from HCLE cells after 4 hrs of incubation with positive control *Pseudomonas aeruginosa* strain PA14 (average MOI = 111), detergent lysis (LYSIS), medium only negative control (DNB), and experimental strains *B. bacteriovorus* strain 109J (avgerage MOI = 4720), *B. bacteriovorus* strain HD100 (average MOI = 1039), and *M. aeruginosavorus* (Mica, average MOI = 853). Total independent data points from 2 experiments are shown. Asterisks indicate significant differences ($p<0.001$, ANOVA with Tukey's post-test). Only PA14 was significantly different than MOCK. Error bars indicate one standard deviation.
doi:10.1371/journal.pone.0066723.g002

challenge [26]. Thus, based on our finding it could be concluded, that unlike other pathogens, predatory bacteria do not provoke an aggressive innate immune response when injected.

Discussion

In this study, we have demonstrated that *M. aeruginosavorus* ARL-13 is able to prey on clinical isolates of *P. aeruginosa* isolated from ocular infections. This finding is in line with earlier reports regarding the host specificity of this predator and its ability to attack *P. aeruginosa* [13,22,35]. Our data also suggest that both *B. bacteriovorus* 109J and HD100 are capable of using *S. marcescens* as a host. This finding is in agreement with a study reporting a 3 $\log_{10}$ reduction in cell viability of a non clinical isolate of *S. marcescens* following predation by *B. bacteriovorus* 109J [13]. In addition, *B. bacteriovorus* 109J and HD100 were able to prey on *P. aeruginosa*. However, the ability of the HD100 strain to attack was broader than that of 109J, preying on 100% and 70% of the isolates, respectively. The narrower ability of *B. bacteriovorus* 109J to prey on *P. aeruginosa* is aligned with a recent report in which *B. bacteriovorus* 109J was able to reduce only 1 out of 4 *P. aeruginosa* examined strains [5], as wall as earlier findings showing a limited ability of some *B. bacteriovorus* to prey on *P. aeruginosa* [8,36]. The different host- and intra-species strain- specificity demonstrated by *Bdellovibrio* spp. and *Micavibrio* spp. is well documented. Furthermore, predation ability was found to be specific to the *B. bacteriovorus* strain used, with different *B. bacteriovorus* strains demonstrating unique host specificity [8,13,22,35–38]. As the mechanisms that define the predator's host specificity are not fully known, we could only speculate on the reason why certain bacteria are recognized as a host while others are not.

A major concern that needs to be addressed when evaluating the potential use of predatory bacteria as topical live-antibiotics are the risks associated with applying Gram-negative microorganisms to human cells. To this end, we have conducted cytotoxicity assays in which HCLE cells were exposed to high concentrations of the predators. Our data indicate that predatory bacteria are significantly less cytotoxic than the control *P. aeruginosa*. Low cytotoxicity was observed even after an extended exposure period. Although only a small number of studies regarding the safety of using predatory bacteria were conducted, the current data does support the claims that predatory bacteria could be considered safe. In a review article published by Dwider at al [4] the authors cited a study in which *Bdellovibrio* was injected into mice rabbits and guinea pigs and were found to be non pathogenic to the animals [39]. In a separate study conducted by Lenz and Hespell [40], the investigators attempted to grow the predatory bacteria *B. bacteriovorus* 109J, *Bacteriovorax stolpii* UKi2 and *Peredibacter starrii* A3.12 in the presence of eukaryotic cells. It was concluded that predatory bacteria are unable to grow on hamster kidney cells, mouse liver cells and bovine mammary gland cells. Furthermore, the predators did not grow within rabbit ova, following injection, nor were they able to grow in media containing rabbit ova extracts. Thus, it seems that mammalian cells could not be used by the predators as prey and could not support predator proliferation in the absent of a Gram-negative host. The inability of the predator to grow and establish itself in the intestinal microflora was shown in a study in which *Bdellovibrio* strain MS7 was fed to Channel catfish, northern leopard frogs, and mice. The predator viability also declined when inoculated into rabbit ileal loops. As *Bdellovibrio* could not proliferate *in vivo* it reduces the risk of permanent establishment within the mammalian host, rendering the predator, in the study, as nonpathogenic [41].

In a recent study conducted at the University of Nottingham [9], the *in vivo* effect of *Bdellovibrio* in a poultry vertebrate model was examined. It was found that *B. bacteriovorus* HD100, which was orally administered to chicks, caused no negative health effects on the birds. Furthermore, the authors were not able to recover viable *Bdellovibrio* from the gut flora, fecal matter or drinking water of the predator-inoculated birds, concluding that the risk of spreading predatory bacteria during treatment is low. *Bdellovibrio* treatment was also found to improve the well being of the birds colonized with *Salmonella* Enteritidis in the therapeutic trail.

An additional concern of applying live predatory bacteria is the risk of inadvertently causing inflammation which in could inhibit wound healing and increase the risk of tissue damage. In this study we have demonstrated that exposure to high doses of the predators did not elevate the production of the proinflammatory cytokines IL-8 and TNF-alpha by HCLE cells. Experimental evidence supports that neutrophils attracted by the bacteria-induced inflammation are a major cause of scarring and tissue damage associated with vision loss in keratitis [42]. The low cytotoxic activity of *B. bacteriovorus* HD100 LPS and its reduced ability to induce TNF-alpha and IL-6, compared to an *E. coli* control, was previously reported in a study using a human macrophage cell line [43]. The authors attributed their findings to the unique structure of the *B. bacteriovorus* LPS Lipid A molecule. Unlike Lipid A from many Gram-negative bacteria that contain negatively charged phosphate groups, the *B. bacteriovorus* Lipid A molecule has α-D-mannose residues which reduced its affinity to LPS receptors thereby lowering inflammation. Our data confirm that *B. bacteriovorus* HD100 and 109J do not enhance proinflammatory cytokines production. Our data also demonstrate, for the first time, that as reported for *B. bacteriovorus o*, *M. aeruginosavorus* also does not enhance inflammation.

Although, our data show that predatory bacteria have little or no adverse effect when applied to human cell cultures, we were interested to evaluate the effect of predatory bacteria *in vivo*. To address whether predatory bacteria a tolerated by eukaryotic cells *in vivo*, a *G. mellonella* microbial pathogenesis model was selected. Although, we did not measure the viability of the predators within the worm over time, we might still conclude that injecting relatively high doses of predatory bacteria do not provoke any measurable toxic or harmful effect to the worm. *G. mellonella* is recognized as a suitable host model system to study the pathogenesis of both bacteria and yeast and was used to examine pathogenic attributes of many human pathogens including pathogens associated with eye infections [26,30,32]. These studies established the use of *G. mellonella* for a variety of applications such as: examining microbial pathogenicity and lethality, evaluating microbial growth and proliferation, isolating virulence factors, and inspecting putative virulence mechanisms [30,32-34,44]. Since the innate immune systems of mammals and insects have several features in common [31], *G. mellonella* could also be used as a model system for studying the host innate immune response to microbial infection as well as identifying microbial virulence factors that mediate the immune response [33,45].

The potential use of predatory bacteria as a bio-control agent to treat eye infections was first suggested some 40 years ago. In a study conducted in 1972 [46] the "pro-biotic" ability of *E. coli* and *B. bacteriovorus* to impact the pathogenesis of *Shigella flexneri* in animal modules was examined. It was shown that the *B. bacteriovorus* was able to reduce the severity of keratoconjunctivitis induced by *S. flexneri* in a rabbit keratoconjunctivitis model. The simultaneous inoculation of *Bdellovibrio* with *S. flexneri* was able to prevent the development of the infection. The rate of development of typical keratoconjunctivitis was also decreased when *Bdellovibrio*

was administered within 48 hrs of the initial *S. flexneri* infection. In a more recent study, the ability of *B. bacteriovorus* 109J to inhibit growth of and reduce the adherence of *Moraxella bovis* to Madin-Darby bovine kidney (MDBK) cells, used to mimic bovine keratoconjunctivitis, was confirmed [47]. The ability of *Bdellovibrio* to survive and prey in human fluids was also demonstrated in an experiment in which *B. bacteriovorus* 109J was abele to significantly reduce biofilms of *Aggregatibacter actinomycetemcomitans* in the presence of human saliva that contains many of the same antimicrobial compounds as do tears [5].

In conclusion, our work demonstrates that predatory bacteria have the ability to attack "real-life" Gram-negative human pathogens associated with ocular infection. Furthermore, *in vitro* studies had revealed that the presence of high concentrations of predatory bacteria don't appear to be harmful to human cells. These findings, coupled with the ability of predator bacteria to prey in conditions that might be encountered in the eye, emphasize the potential use of applying predator bacteria as a topical agent to treat eye infections caused by pathogens which are resistant to traditional antimicrobials. The efficacy of predatory bacteria to control infection using *in vivo* models of ocular infection should be the focus of future studies. The long-term goal is to develop a topical predator bacteria product, which might include a single or multispecies predator bacteria mix and could be used alone or in concert with traditional antimicrobial therapies.

Acknowledgments

Kevin To, Genesis Cano and Tiffany Aston provided technical assistance.

Author Contributions

Conceived and designed the experiments: DEK RMQS. Performed the experiments: VRD EGR KMB DG DEK. Analyzed the data: DEK RMQS NAS. Contributed reagents/materials/analysis tools: RMQS DEK. Wrote the paper: RMQS DEK NAS.

References

1. Fukuda K, Ishida W, Uchiyama J, Rashel M, Kato S, et al. (2012) *Pseudomonas aeruginosa* keratitis in mice: effects of topical bacteriophage KPP12 administration. PLoS One 7: e47742.
2. Gorski A, Targonska M, Borysowski J, Weber-Dabrowska B (2009) The potential of phage therapy in bacterial infections of the eye. Ophthalmologica 223: 162–165.
3. Santos TM, Ledbetter EC, Caixeta LS, Bicalho ML, Bicalho RC (2011) Isolation and characterization of two bacteriophages with strong in vitro antimicrobial activity against *Pseudomonas aeruginosa* isolated from dogs with ocular infections. Am J Vet Res 72: 1079–1086.
4. Dwidar M, Monnappa AK, Mitchell RJ (2012) The dual probiotic and antibiotic nature of *Bdellovibrio bacteriovorus*. BMB Rep 45: 71–78.
5. Dashiff A, Kadouri DE (2011) Predation of oral pathogens by *Bdellovibrio bacteriovorus* 109J. Mol Oral Microbiol 26: 19–34.
6. Van Essche M, Quirynen M, Sliepen I, Loozen G, Boon N, et al. (2011) Killing of anaerobic pathogens by predatory bacteria. Mol Oral Microbiol 26: 52–61.
7. Van Essche M, Quirynen M, Sliepen I, Van Eldere J, Teughels W (2009) *Bdellovibrio bacteriovorus* attacks *Aggregatibacter actinomycetemcomitans*. J Dent Res 88: 182–186.
8. Markelova NY (2010) Predacious bacteria, *Bdellovibrio* with potential for biocontrol. Int J Hyg Environ Health 213: 428–431.
9. Atterbury RJ, Hobley L, Till R, Lambert C, Capeness MJ, et al. (2011) Effects of orally administered *Bdellovibrio bacteriovorus* on the well-being and *Salmonella* colonization of young chicks. Appl Environ Microbiol 77: 5794–5803.
10. Lu F, Cai J (2010) The protective effect of *Bdellovibrio*-and-like organisms (BALO) on tilapia fish fillets against *Salmonella enterica* ssp. enterica serovar Typhimurium. Lett Appl Microbiol 51: 625–631.
11. Fratamico PM, Whiting RC (1995) Ability of *Bdellovibrio-bacteriovorus* 109J to Lyse Gram-Negative Food-Borne Pathogenic and Spoilage Bacteria. J Food Protect 58: 160–164.
12. Fratamico PM, Whiting RC (1996) Isolation of *Bdellovibrios* that prey on *Escherichia coli* O157:H7 and *Salmonella* species and application for removal of prey from stainless steel surfaces. J Food Safety 16: 161–173.
13. Dashiff A, Junka RA, Libera M, Kadouri DE (2011) Predation of human pathogens by the predatory bacteria *Micavibrio aeruginosavorus* and *Bdellovibrio bacteriovorus*. J Appl Microbiol 110: 431–444.
14. Das S, Sheorey H, Taylor HR, Vajpayee RB (2007) Association between cultures of contact lens and corneal scraping in contact lens related microbial keratitis. Arch Ophthalmol 125: 1182–1185.
15. Hume EB, Willcox MD (2004) Emergence of *Serratia marcescens* as an ocular surface pathogen. Arch Soc Esp Oftalmol 79: 475–477.
16. Varaprasathan G, Miller K, Lietman T, Whitcher JP, Cevallos V, et al. (2004) Trends in the etiology of infectious corneal ulcers at the F. I. Proctor Foundation. Cornea 23: 360–364.
17. Rendulic S, Jagtap P, Rosinus A, Eppinger M, Baar C, et al. (2004) A predator unmasked: life cycle of *Bdellovibrio bacteriovorus* from a genomic perspective. Science 303: 689–692.
18. Wang Z, Kadouri DE, Wu M (2011) Genomic insights into an obligate epibiotic bacterial predator: *Micavibrio aeruginosavorus* ARL-13. BMC Genomics 12: 453.
19. Kowalski RP, Pandya AN, Karenchak LM, Romanowski EG, Husted RC, et al. (2001) An in vitro resistance study of levofloxacin, ciprofloxacin, and ofloxacin using keratitis isolates of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Ophthalmology 108: 1826–1829.
20. Rhee MK, Kowalski RP, Romanowski EG, Mah FS, Ritterband DC, et al. (2004) A laboratory evaluation of antibiotic therapy for ciprofloxacin-resistant *Pseudomonas aeruginosa*. Am J Ophthalmol 138: 226–230.
21. Kadouri D, O'Toole GA (2005) Susceptibility of biofilms to *Bdellovibrio bacteriovorus* attack. Appl Environ Microbiol 71: 4044–4051.
22. Kadouri D, Venzon NC, O'Toole GA (2007) Vulnerability of pathogenic biofilms to *Micavibrio aeruginosavorus*. Appl Environ Microbiol 73: 605–614.
23. Shilo M, Bruff B (1965) Lysis of Gram-negative bacteria by host-independent ectoparasitic *Bdellovibrio bacteriovorus* isolates. J Gen Microbiol 40: 317–328.
24. Wingard JB, Romanowski EG, Kowalski RP, Mah FS, Ling Y, et al. (2011) A novel cell-associated protection assay demonstrates the ability of certain antibiotics to protect ocular surface cell lines from subsequent clinical *Staphylococcus aureus* challenge. Antimicrob Agents Chemother 55: 3788–3794.
25. Gipson IK, Spurr-Michaud S, Argueso P, Tisdale A, Ng TF, et al. (2003) Mucin gene expression in immortalized human corneal-limbal and conjunctival epithelial cell lines. Invest Ophthalmol Vis Sci 44: 2496–2506.
26. Jander G, Rahme LG, Ausubel FM (2000) Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. J Bacteriol 182: 3843–3845.
27. Ramirez JC, Fleiszig SM, Sullivan AB, Tam C, Borazjani R, et al. (2012) Traversal of multilayered corneal epithelia by cytotoxic *Pseudomonas aeruginosa* requires the phospholipase domain of exoU. Invest Ophthalmol Vis Sci 53: 448–453.
28. Willcox MD (2007) Pseudomonas aeruginosa infection and inflammation during contact lens wear: a review. Optom Vis Sci 84: 273–278.
29. Zhang J, Wu XY, Yu FS (2005) Inflammatory responses of corneal epithelial cells to *Pseudomonas aeruginosa* infection. Curr Eye Res 30: 527–534.
30. Garcia-Lara J, Needham AJ, Foster SJ (2005) Invertebrates as animal models for *Staphylococcus aureus* pathogenesis: a window into host-pathogen interaction. FEMS Immunol Med Microbiol 43: 311–323.
31. Kavanagh K, Reeves EP (2004) Exploiting the potential of insects for in vivo pathogenicity testing of microbial pathogens. FEMS Microbiol Rev 28: 101–112.
32. Miyata S, Casey M, Frank DW, Ausubel FM, Drenkard E (2003) Use of the *Galleria mellonella* caterpillar as a model host to study the role of the type III secretion system in *Pseudomonas aeruginosa* pathogenesis. Infect Immun 71: 2404–2413.
33. Peleg AY, Jara S, Monga D, Eliopoulos GM, Moellering RC Jr, et al. (2009) *Galleria mellonella* as a model system to study *Acinetobacter baumannii* pathogenesis and therapeutics. Antimicrob Agents Chemother 53: 2605–2609.
34. Seed KD, Dennis JJ (2008) Development of *Galleria mellonella* as an alternative infection model for the *Burkholderia cepacia* complex. Infect Immun 76: 1267–1275.
35. Lambina VA, Afinogenova AV, Romay Penobad Z, Konovalova SM, Andreev LV (1983) New species of exoparasitic bacteria of the genus Micavibrio infecting gram-positive bacteria. Mikrobiologiia 52: 777–780.
36. Stolp H, Starr MP (1963) *Bdellovibrio Bacteriovorus* gen. et sp. n., a Predatory, Ectoparasitic, and Bacteriolytic Microorganism. Antonie Van Leeuwenhoek 29: 217–248.
37. Jurkevitch E, Minz D, Ramati B, Barel G (2000) Prey range characterization, ribotyping, and diversity of soil and rhizosphere *Bdellovibrio* spp. isolated on phytopathogenic bacteria. Appl Environ Microbiol 66: 2365–2371.
38. Rogosky AM, Moak PL, Emmert EA (2006) Differential predation by *Bdellovibrio bacteriovorus* 109J. Curr Microbiol 52: 81–85.
39. Verklova ZS (1973) Study of the virulence, toxicity and immunogenicity of different strains of *Bdellovibrio bacteriovorus*. Gig Sanit 38: 10–13.
40. Lenz RW, Hespell RB (1978) Attempts to Grow *Bdellovibrios* Micurgically-Injected into Animal-Cells. Arch Microbio 119: 245–248.
41. Westergaard JM, Kramer TT (1977) *Bdellovibrio* and the intestinal flora of vertebrates. Appl Environ Microbiol 34: 506–511.

42. Hazlett LD (2005) Role of innate and adaptive immunity in the pathogenesis of keratitis. Ocul Immunol Inflamm 13: 133–138.
43. Schwudke D, Linscheid M, Strauch E, Appel B, Zahringer U, et al. (2003) The obligate predatory *Bdellovibrio bacteriovorus* possesses a neutral lipid A containing alpha-D-Mannoses that replace phosphate residues: similarities and differences between the lipid As and the lipopolysaccharides of the wild type strain *B. bacteriovorus* HD100 and its host-independent derivative HI100. J Biol Chem 278: 27502–27512.
44. Andrejko M, Mizerska-Dudka M, Jakubowicz T (2009) Antibacterial activity in vivo and in vitro in the hemolymph of *Galleria mellonella* infected with *Pseudomonas aeruginosa*. Comp Biochem Physiol B Biochem Mol Biol 152: 118–123.
45. Dubovskiy IM, Krukova NA, Glupov VV (2008) Phagocytic activity and encapsulation rate of *Galleria mellonella* larval haemocytes during bacterial infection by *Bacillus thuringiensis*. J Invertebr Pathol 98: 360–362.
46. Nakamura M (1972) Alteration of *Shigella pathogenicity* by other bacteria. Am J Clin Nutr 25: 1441–1451.
47. Boileau MJ, Clinkenbeard KD, Iandolo JJ (2011) Assessment of *Bdellovibrio bacteriovorus* 109J killing of *Moraxella bovis* in an in vitro model of infectious bovine keratoconjunctivitis. Can J Vet Res 75: 285–291.

Future MICROBIOLOGY

EDITORIAL

Predatory prokaryotes wage war against eye infections

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Predatory bacteria

Predatory bacteria, bacteria that prey upon other bacteria, are gaining interest as a potential therapeutic tool to combat infections. Recent reviews cover what is known about the biology and potential application of these organisms [1–3]. The focus of this article is to discuss the potential use of predatory bacteria to control ocular infections. The most studied predatory bacterium to date is *Bdellovibrio bacteriovorus*, a small, highly-motile, Gram-negative bacterium that preys on other Gram-negative bacteria. This bacterium has an extraordinary life cycle in which it collides with its prey, burrows through the bacterial membrane, divides into several daughter cells, and lyses the host bacteria to start a new round of predation [3]. Other predatory bacteria, such as *Micavibrio aeruginosavorus* may also have medical potential [4–6]; however, *B. bacteriovorus* will be discussed here as it is the most thoroughly studied.

Efficacy against major pathogens

Recent studies have shown that *B. bacteriovorus* is capable of killing a broad range of human pathogens and opportunistic pathogens [4] including multidrug-resistant hospital isolates of *Acinetobacter baumannii*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* [5]. Predatory bacteria are able to effectively prey upon bacteria in biofilms [7], which are notoriously difficult to treat due to antibiotic tolerance.

Question of safety

It is easy to imagine using these organisms to treat unwanted pathogens in aquatic and industrial settings; however, the use of these predators to treat human and animal infections is another question. Are these organisms safe? The strongest evidence to support safety in humans are findings reported by Iebba *et al.*, in which *B. bacteriovorus* was found in the gut of healthy individuals [8]. Earlier work highlighted the fact that *Bdellovibrio* had a unique lipid A portion of its lipopolysaccharide structure that was much less immunogenic than typical lipopolysaccharide [9]. A recent review by the Mitchell group covers what is known regarding safety of these predatory bacteria [1], which demonstrated that multiple studies failed to detect deleterious effects following topical application, ingestion or injection of *B. bacteriovorus* into vertebrates. This includes a recent study conducted at the University of Nottingham (UK) that documented no negative health effects on chickens, which received oral treatments of *B. bacteriovorus* [10]. While these studies are limited, they do positively support the notion that predatory bacteria may be part of future therapeutic strategies.

Potential use on the ocular surface

It would seem that predatory bacteria would have its greatest success as an antimicrobial or adjuvant to be used alongside

KEYWORDS
• antibiotic resistance • antimicrobial • *Bdellovibrio bacteriovorus* • conjunctivitis • *Micavibrio aeruginosavorus* • predatory bacteria • topical therapy

"Predatory bacteria are able to effectively prey upon bacteria in biofilms, which are notoriously difficult to treat due to antibiotic tolerance."

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antibiotics for topical infections such as those of the skin or other mucosal surfaces. Ocular surface infections such as bacterial conjunctivitis and microbial keratitis have a large cost to society and are a major source of vision loss. These infections are often caused by Gram-negative bacteria such as *Haemophilus influenzae*, *P. aeruginosa*, and *Serratia marcescens*; the latter two have been verified as being susceptible to predation by *B. bacteriovorus* [6].

B. bacteriovorus has been reported to be resistant to β-lactam antibiotics, so it is conceivable that combination therapy using both predatory bacteria and antibiotics could be used [2]. In addition to antibiotics, the combination of using predatory bacteria with biofilm-degrading enzymes or phage was also brought forward [11,12]. Unlike antibiotics or the use of bacteriophage to kill bacteria, no stable genetic resistance of host bacteria to *B. bacteriovorus* has been identified, despite attempts to isolate resistance [13]. Additional attempts to enrich for prey-resistant phenotypes by culture enrichment or mutagenesis also failed [Kadouri DE, Pers. Comm.]. An important theoretical advantage of predatory prokaryotes over antibiotics is that if host bacteria did evolve resistance, then there will be equivalent selective pressure for predatory bacteria to evolve mechanisms to overcome any resistance mechanisms acquired by its host bacteria.

Review of the literature

The original paper relating to the potential of *B. bacteriovorus* in treating eye infections dates from 1972 and remains the only paper to test *B. bacteriovorus* in an ocular model *in vivo* [14]. In this paper, Nakamura showed that *Shigella flexneri* eye infections could be prevented, by co-inoculation with *B. bacteriovorus* [14]. Instillation of *B. bacteriovorus* at 12, 48 and 72 h after *S. flexneri* inoculation was tested and time-dependent prevention of keratoconjunctivitis was observed, with apparent full protection at 12 h and little protection by 72 h. At first glance, this suggests that the predatory bacteria efficiently killed the *S. flexneri* bacteria; however, in the same study, the co-inoculation of nonpredatory bacteria *Escherichia coli* was equally as effective in preventing *S. flexneri* infections. Therefore, it is not clear whether predation had any impact on the reduced virulence of *S. flexneri* in the study. An important outcome of this study was that no adverse affect was described when 10^9 *B. bacteriovorus* were applied topically to rabbit eyes, supporting that they do not induce a strong inflammatory response.

The second paper aligning predatory bacteria and ocular infections, used an "*in vitro* model of infectious keratoconjunctivitis to assess *Moraxella bovis* pathogenesis" [15]. The use of 'keratoconjunctivitis' could be considered to be an overstatement as the mammalian cells used in the model were bovine kidney cells and the study was performed *in vitro*. Nevertheless, *B. bacteriovorus* provided some protection to the mammalian cells against *Mycobacterium bovis* under the conditions used in this study.

A third study used two strains of *B. bacteriovorus* and one *Micavibrio* strain to test whether they could prey upon common ocular bacterial pathogens and to determine whether predatory bacteria have deleterious effects to a human corneal cell line. This *in vitro* study demonstrated that predatory bacteria can kill human ocular pathogens *in vitro*, are not cytotoxic to human corneal limbal epithelial cells, and do not induce proinflammatory markers (IL-8 and TNF-α) from human corneal limbal epithelial cells [5].

Challenges

Potential limitations to the use of predatory bacteria for ocular infections include: limited host range; susceptibility to host defense and antibiotics; and potential allergic reactions. Reported predatory bacteria cannot prey upon Gram-positive bacteria, which is a major problem as Gram-positive bacteria are a major cause of ocular infections [16]. In one recent publication, Monnappa *et al.* showed that *B. bacteriovorus* proteolytic enzymes can prevent biofilm formation by *Staphylococcus aureus* [12]. While it is unlikely feasible to treat infections with strains that overexpress proteases as it might have a negative effect on the surrounding tissue, this study serves as a proof of principle that the native enzymes produced by *B. bacteriovorus* could be manipulated for biocontrol of Gram-positive bacteria. In fact, the genomes of predatory bacteria are replete with genes predicted to code for hydrolytic enzymes evolved to digest its bacterial host [17,18]. Another drawback of using predatory bacteria to control infection is, like other predators in nature, the predator never eradicates all of its prey from its environment. Although this might be seen as a major obstacle, the predator might still be able to clear the bulk of the infection allowing the immune system to deal with the residual

"Another drawback of using predatory bacteria to control infection is, like other predators in nature, the predator never eradicates all of its prey from its environment."

pathogens. Predatory bacteria could also be used in sync with other antimicrobial therapies such as phage or antibiotics, rendering them more efficient and allowing total removal of the infection. An additional obstacle is that the human ocular surface is an inhospitable place for microbes due to innate immune defenses [19]; therefore, the use of predatory bacteria as a probiotic might not be feasible. This is because the predatory bacteria may be killed by the immune system and because there is a limited food supply for predatory bacteria on the ocular surface [20]. However, as the relationship between predatory bacteria and the immune system was not investigated, one could argue that a poorly inflammatory *Bdellovibrio* wound not provoke an immune response and will not be rapidly cleared from the site. Lastly, it is conceivable that certain patients would develop allergies to predatory bacteria that could impact their widespread use as therapeutics.

Conclusion

The existing literature on the use of predatory bacteria to treat ocular infections, or indeed any infections, is lacking *in vivo* studies specifically designed to evaluate the ability of predatory bacteria to treat ocular infections without toxicity and excessive inflammation. Despite the weaknesses of predatory bacteria, the lack of stable resistance by opportunistic pathogens to *B. bacteriovorus* makes them an attractive potential therapeutic.

Future perspective

As the medical community is facing a new area of drug-resistant pathogens, the need for new therapeutics and approaches to control infection is never more urgent. In the last few years, substantial progress in our understanding of the biology and genetics of predatory bacteria has been made. Additionally, our understanding of the ability of predatory bacteria to control human pathogens and biofilms *in vitro* has improved. We believe that in the next few years, work will be conducted with the aim of better understanding the mechanisms involved in predation and, most importantly, what governs prey specificity. *In vivo* studies will be conducted with the goal of assuring that predatory bacteria are harmless to the host and to test the efficacy of using predatory bacteria to control infection. Finally, we speculate that for predatory bacteria to be successfully used as live antibiotics, they will need to be coupled with other therapeutics to ensure that resistance does not develop and to enhance their effectiveness as suitable adjuvants for our limited arsenal of antibiotics.

> "...for predatory bacteria to be successfully used as live antibiotics, they will need to be coupled with other therapeutics to ensure that resistance does not develop..."

Acknowledgements

The authors thank Kristin Hunt for critical reading of the manuscript.

Financial & competing interests disclosure

RMQ Shanks was supported by NIH grant AI085570, EY08098, the Eye and Ear Foundation of Pittsburgh, and unrestricted funds from Research to Prevent Blindness. DE Kadouri was supported in part by Department of the ARMY USAMRAA #W81XWH-12-2-0067. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

References

Papers of special note have been highlighted as:
• of interest

1 Dwidar M, Monnappa AK, Mitchell RJ. The dual probiotic and antibiotic nature of *Bdellovibrio bacteriovorus*. *BMB Rep.* 45(2), 71–78 (2012).

• **Excellent review summarizing the potential applications of predatory bacteria.**

2 Pasternak Z, Njagi M, Shani Y *et al.* In and out: an analysis of epibiotic vs periplasmic bacterial predators. *ISME J.* 8, 625–635 (2013).

3 Sockett Re. Predatory lifestyle of *Bdellovibrio bacteriovorus*. *Annu. Rev. Microbiol.* 63, 523–539 (2009).

4 Dashiff A, Junka RA, Libera M, Kadouri DE. Predation of human pathogens by the predatory bacteria *Micavibrio aeruginosavorus* and *Bdellovibrio bacteriovorus*. *J. Appl. Microbiol.* 110(2), 431–444 (2011).

• **Thorough study showing predation by predatory bacteria on key human pathogens and biofilms.**

5 Kadouri De, To K, Shanks Rm, Doi Y. Predatory bacteria: a potential ally against multidrug-resistant Gram-negative pathogens. *PLoS ONE* 8(5), e63397 (2013).

6 Shanks RM, Davra VR, Romanowski EG *et al.* An eye to a kill: using predatory bacteria to control Gram-negative pathogens associated with ocular infections. *PLoS ONE* 8(6), e66723 (2013).

• **Recent paper demonstrating the ability of predatory bacteria to kill ocular pathogens and their inability to damage ocular surface cells.**

7 Kadouri D, O'Toole GA. Susceptibility of biofilms to *Bdellovibrio bacteriovorus* attack. *Appl. Environ. Microbiol.* 71(7), 4044–4051 (2005).

8 Iebba V, Santangelo F, Totino V *et al.* Higher prevalence and abundance of *Bdellovibrio bacteriovorus* in the human gut of healthy subjects. *PLoS ONE* 8(4), e61608 (2013).

• **Provides evidence that *Bdellovibrio bacteriovorus* may be a human commensal.**

9 Schwudke D, Linscheid M, Strauch E *et al.* The obligate predatory *Bdellovibrio*

bacteriovorus possesses a neutral lipid A containing alpha-D-mannoses that replace phosphate residues: similarities and differences between the lipid as and the lipopolysaccharides of the wild type strain B. bacteriovorus HD100 and its host-independent derivative HI100. *J. Biol. Chem.* 278(30), 27502–27512 (2003).

10 Atterbury RJ, Hobley L, Till R *et al.* Effects of orally administered *Bdellovibrio bacteriovorus* on the well-being and *Salmonella* colonization of young chicks. *Appl. Environ. Microbiol.* 77(16), 5794–5803 (2011).

- ***In vivo* study to evaluate the saftey, efficacy, and environmental impact of a veterinary application of predatory bacteria.**

11 Chen H, Williams HN. Sharing of prey: coinfection of a bacterium by a virus and a prokaryotic predator. *MBio* 3(2), e00051–e00012 (2012).

12 Monnappa AK, Dwidar M, Seo JK, Hur JH, Mitchell RJ. *Bdellovibrio bacteriovorus* inhibits *Staphylococcus aureus* biofilm formation and invasion into human epithelial cells. *Sci. Rep.* 4, 3811 (2014).

13 Shemesh Y, Jurkevitch E. Plastic phenotypic resistance to predation by *Bdellovibrio* and like organisms in bacterial prey. *Environ. Microbiol.* 6(1), 12–18 (2004).

14 Nakamura M. Alteration of *Shigella* pathogenicity by other bacteria. *Am. J. Clin. Nutr.* 25(12), 1441–1451 (1972).

15 Boileau MJ, Clinkenbeard KD, Iandolo JJ. Assessment of *Bdellovibrio bacteriovorus* 109J killing of *Moraxella bovis* in an *in vitro* model of infectious bovine keratoconjunctivitis. *Can. J. Vet. Res.* 75(4), 285–291 (2011).

16 Kowalski RP, Kowalski TA, Shanks RM, Romanowski EG, Karenchak LM, Mah FS. *In vitro* comparison of combination and monotherapy for the empiric and optimal coverage of bacterial keratitis based on incidence of infection. *Cornea* 32(6), 830–834 (2013).

17 Rendulic S, Jagtap P, Rosinus A *et al.* A predator unmasked: life cycle of *Bdellovibrio bacteriovorus* from a genomic perspective. *Science* 303(5658), 689–692 (2004).

- **First reported genome sequence of a *Bdellovibrio bacteriovorus*.**

18 Wang Z, Kadouri DE, Wu M. Genomic insights into an obligate epibiotic bacterial predator: *Micavibrio aeruginosavorus* ARL-13. *BMC Genomics* 12, 453 (2011).

19 McDermott AM. Antimicrobial compounds in tears. *Exp. Eye Res.* 117, 53–61 (2013).

20 Graham JE, Moore JE, Jiru X *et al.* Ocular pathogen or commensal: a PCR-based study of surface bacterial flora in normal and dry eyes. *Invest. Ophthalmol. Vis. Sci.* 48(12), 5616–5623 (2007).

SCIENTIFIC REPORTS

OPEN

Received: 27 March 2015
Accepted: 30 June 2015
Published: 07 August 2015

Examining the safety of respiratory and intravenous inoculation of *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* in a mouse model

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***Bdellovibrio* spp. and *Micavibrio* spp. are Gram-negative predators that feed on other Gram-negative bacteria, making predatory bacteria potential alternatives to antibiotics for treating multi-drug resistant infections. While the ability of predatory bacteria to control bacterial infections *in vitro* is well documented, the *in vivo* effect of predators on a living host has yet to be extensively examined. In this study, respiratory and intravenous inoculations were used to determine the effects of predatory bacteria in mice. We found no reduction in mouse viability after intranasal or intravenous inoculation of *B. bacteriovorus* 109J, HD100 or *M. aeruginosavorus*. Introducing predators into the respiratory tract of mice provoked a modest inflammatory response at 1 hour post-exposure, but was not sustained at 24 hours, as measured by RT-qPCR and ELISA. Intravenous injection caused an increase of IL-6 in the kidney and spleen, TNF in the liver and CXCL-1/KC in the blood at 3 hours post-exposure, returning to baseline levels by 18 hours. Histological analysis of tissues showed no pathological changes due to predatory bacteria. Furthermore, qPCR detected predators were cleared from the host quickly and efficiently. This work addresses some of the safety concerns regarding the potential use of predatory bacteria as a live antibiotic.**

Bdellovibrio bacteriovorus and *Micavibrio aeruginosavorus* are small, highly motile, uniflagellate Gram-negative bacteria that prey naturally on other Gram-negative bacteria[1,2]. Recently, predatory bacteria have been considered as potential alternatives to traditional antibiotics for treating multi-drug resistant (MDR) Gram-negative bacterial infections. *B. bacteriovorus* have a predatory lifestyle where they attach to and enter the prey periplasm, multiply by exhausting the nutrients, lyse the cell, and then continue to seek out more prey to invade[1,3,4]. *Micavibrio* spp., in contrast, attach to, grow and kill prey at the surface of the prey cell in a 'vampire'-like fashion[2,5,6].

Bdellovibrio-and-like organisms (BALOs) are a promising potential novel agent against bacterial pathogens and present several advantages when considering their use for controlling infection[7]. Previous studies have confirmed the ability of predatory bacteria to control a broad range of important human pathogens *in vitro*, including MDR bacteria[8], grown both planktonically and in biofilms[9–11]. In addition,

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Treatment	#of Mice	%Viable on Day 5	%Viable on Day 50*
Control (PBS)	5	100%	100%
B. bacteriovorus 109J	5	100%	100%
B. bacteriovorus 109J (HK)	5	100%	100%
B. bacteriovorus HD100	5	100%	100%
B. bacteriovorus HD100 (HK)	5	100%	100%
M. aeruginosavorus ARL-13	5	100%	100%

Table 1. Host viability of intranasal inoculation of predatory bacteria. *2 mice from each treatment group from the '5 Day' experiment were visually assessed for up to 50 days. HK: heat-killed.

BALOs appear to have no negative effect on human cells when challenged *in vitro*[12]. Recent studies have presented evidence that BALOs might be native commensals of the human gut and might even play a role in maintaining healthy gut flora[13]. In addition, development of genetically stable predation-resistance in a normally susceptible species has yet to be confirmed[14], a major advantage over current available antibiotic therapies. To date, the majority of the studies dealing with predatory bacteria have been performed *in vitro*; the *in vivo* effect of predatory bacteria in a mammalian host is still not well understood.

Early animal studies found *B. bacteriovorus* to be non-pathogenic when injected into mice, rabbits and guinea pigs[7,15], while another study demonstrated that *B. bacteriovorus* could not survive in the gastrointestinal tracts of fish, mice and frogs[16]. A more recent study showed predatory bacteria are non-toxic when fed to young chicks[17]. To our knowledge, these studies were limited to observation of the animal host, with no examination of the host immunological response to predatory bacteria inoculation *in vivo*.

Before predatory bacteria can be used clinically, their safety in a mammalian host must be confirmed. In this study, respiratory and intravenous inoculation mouse models were used to demonstrate the effects of predatory bacteria. The work presented here highlights the potential use of predatory bacteria as a future biological-based agent for controlling infection.

Results

Effect of Respiratory Introduction of Predatory Bacteria

Host viability and histology. To examine the effect of respiratory exposure of predatory bacteria on host survival, we administered intranasally 1×10^9 PFU/mouse of *B. bacteriovorus* 109J, HD100 or 1×10^6 PFU/mouse of *M. aeruginosavorus* ARL-13 to three groups of C57BL/6 mice (5 mice per group) and monitored animals for any signs of infection, illness or discomfort. To measure the effect of predatory bacteria cell particles, 1×10^9 PFU/mouse of non-viable heat-killed *B. bacteriovorus* 109J or HD100 were also administered to two other groups of mice. Phosphate buffered saline (PBS) was used as a negative control. At five days post-inoculation, all 25 mice inoculated with viable or heat-killed predatory bacteria were healthy with no visual adverse effects or change in behavior (Table 1). At this point, three mice were sacrificed for further evaluation and two mice from each group were kept and visually assessed for up to 50 days. As before, no visual signs of infection were seen, with all inoculated mice remaining viable and healthy 50 days post-inoculation (Table 1).

Forty-eight hours post-inoculation, histological examination of the lungs and spleens of mice inoculated with *Bdellovibrio* or *Micavibrio* revealed no pathology compared to the control mice, treated with

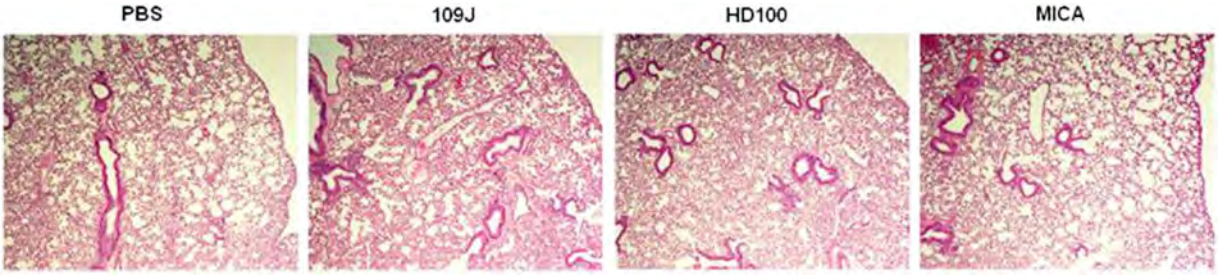


Figure 1. Histological examination of mouse lungs after respiratory introduction of predatory bacteria. Mice were inoculated intranasally with *B. bacteriovorus* 109J, HD100 or *M. aeruginosavorus* ARL-13. Histological examination of lungs exposed to *B. bacteriovorus* and *M. aeruginosavorus* revealed no pathology compared to the control mice treated with PBS. All images are representative micrographs that were taken at 48 hours post-intranasal inoculation and at 40X total magnification.

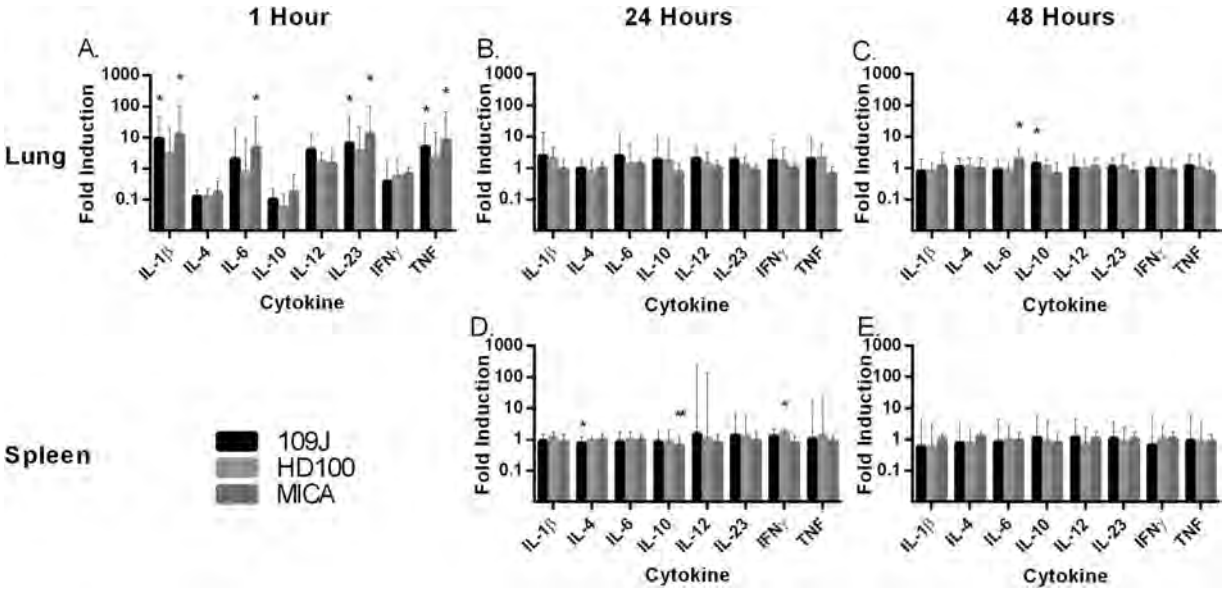


Figure 2. Inflammatory cytokine profile in response to respiratory introduction of predatory bacteria. qPCR analysis of IL-1β, IL-4, IL-6, IL-10, IL-12, IL-23, IFNγ, and TNF in response to intranasal inoculation of predatory bacteria relative to PBS control. Mice were inoculated intranasally with *B. bacteriovorus* 109J, HD100 or *M. aeruginosavorus* ARL-13. Expression of cytokines was assessed in the lung at (**A**) 1, (**B**) 24, and (**C**) 48 hours post-inoculation. Expression of cytokines was assessed in the spleen at (**D**) 24 and (**E**) 48 hours post-inoculation. Fold induction was calculated using the $\Delta\Delta C_t$ by approximation method using an endogenous calibrator (β-actin). For the one hour experiment, 6 mice per predatory bacterial strain (and PBS) were used. Twelve mice per strain (and PBS) were used at each of the 24 and 48 hour time points, with the exception of the Lung/24 hour experiment, where 6 mice were used. Data for the one hour time point is from one experiment; data for the 24 and 48 hour time points are from two independent experiments. Data represent mean ± standard deviation. Student's *t*-test was performed to compare each treated sample to their respective control sample, $^{*}P < 0.05$; $^{**}P < 0.01$.

PBS (Fig. 1). Lung parenchyma showed normal appearance and was well preserved in most of the sections. Some of the sections from both groups (inoculated and control) showed increased cellularity in some areas, predominantly mononuclear cells (lymphocytes and macrophages), but no neutrophils. It is most likely these changes resulted from removing and processing the tissue.

Host immune response to predatory bacteria. To examine the effects of introduction of predatory bacteria via the respiratory tract on the host immune response, we introduced each predator through intranasal inoculation into the respiratory tract of mice. Mice were visually monitored for signs of illness or discomfort, and euthanized at 1, 24 or 48 hours post-inoculation when organs and blood were harvested.

For the one hour time point experiment, 6 mice per predatory bacterial strain were exposed to 4×10^9 PFU/mouse of *B. bacteriovorus* 109J, 7×10^9 PFU/mouse of *B. bacteriovorus* HD100 or 5×10^8 PFU/mouse of *M. aeruginosavorus* ARL-13. At the 24 and 48 hour time points, 12 mice per strain were exposed to an inoculation dose of 1×10^9 PFU/mouse of both *B. bacteriovorus* 109J, HD100 or 1×10^{11} PFU/mouse of *M. aeruginosavorus* ARL-13. Total RNA was extracted from the lungs and spleen, and expression of inflammatory cytokines was measured using RT-qPCR.

As before, none of the total 90 mice that were inoculated with predatory bacteria exhibited any visual adverse effects and all were healthy at the time of sacrifice. At one hour post-inoculation, we observed an increase of IL-1β (9.0- and 12.3-fold), IL-23 (6.3- and 12.6-fold) and TNF (5.0- and 7.9-fold) in the lungs of mice exposed to *B. bacteriovorus* 109J or *M. aeruginosavorus*, respectively (Fig. 2A). However, this increased expression was not sustained at 24 or 48 hours post-inoculation (Fig. 2B,C). Conversely, none of the mice exposed to *B. bacteriovorus* HD100 exhibited a substantial (5-fold or higher) increase in expression of any inflammatory marker gene in the lung or spleen at any time point (Fig. 2). Furthermore, no inflammatory response was detected in the spleens of mice inoculated with either *B. bacteriovorus* 109J or *M. aeruginosavorus* at 24 or 48 hours (Fig. 2D,E). Inflammatory protein levels in the lungs of inoculated mice were measured with ELISA to confirm the results obtained from qPCR. We did observe a 4.7-fold increase in CXCL-1/KC protein expression due to *B. bacteriovorus* 109J at 24 hours. However, no other inflammatory protein showed a substantial fold induction (5-fold or higher) due to inoculation with any of the predatory bacteria (Fig. 3). Additionally, mice examined at five days post-inoculation still exhibited no substantial increases in proinflammatory marker gene expression (data not shown).

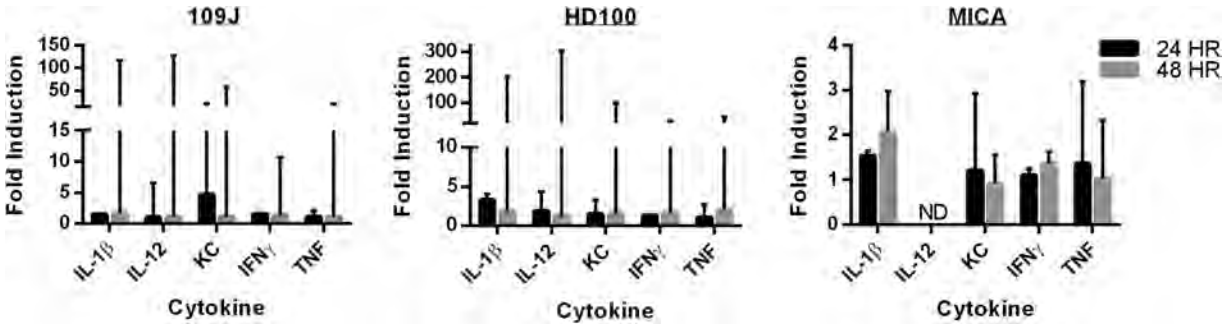


Figure 3. Inflammatory protein profile of the lung in response to intranasal inoculation of predatory bacteria. ELISA analysis of IL-1β, IL-12, CXCL-1/KC, IFNγ, and TNF in response to intranasal inoculation of predatory bacteria relative to PBS control. Mice were inoculated intranasally with *B. bacteriovorus* 109J, HD100 or *M. aeruginosavorus* ARL-13. Inflammatory proteins were assessed in the lung at 24 and 48 hours post-inoculation of (**A**) *B. bacteriovorus* 109J, (**B**) HD100, and (**C**) *M. aeruginosavorus* strain ARL-13. 12 mice per treatment group were used at each time point. Data from two independent experiments. Data represent mean ± standard error of the mean. Student's *t*-test was performed to compare each treated sample to their respective control sample, $*P < 0.05$; $**P < 0.01$.

As before, all mice inoculated with the predators were healthy with no visual adverse effects at any of the examined time points.

To assess the change in cytokine levels in the host's response to a known respiratory bacterial pathogen, we introduced 1.2×10^9 CFU/mouse of *K. pneumoniae* to mice (n = 2) through intranasal inoculation. As expected, we observed a 2260- and 80-fold induction of IL-6 in the lungs and spleen, respectively, as well as a 21-fold induction of TNF in the lungs of the host at 24 hours post-infection. Our data are consistent with previously published studies that demonstrate *K. pneumoniae* to elicit a strong cytokine response in the mouse lung[18,19]. In comparison, mice exposed to *B. bacteriovorus* 109J or *M. aeruginosavorus* showed only a 1.9- and 4.7-fold induction of IL-6, respectively at 1 hour post-inoculation (Fig. 2A), reflecting a much stronger immune response to *K. pneumoniae*. Collectively, our data indicate that when inhaled, predatory bacteria do not provoke an elevated, sustained immune response in mice.

Effect of Intravenous Introduction of Predatory Bacteria

Host viability and histology. The effect of predatory bacteria introduced via the intravenous route was also investigated. To this end, 1×10^8 PFU/mouse of *B. bacteriovorus* 109J or PBS control were injected into the tail vein of mice (5 mice per group). At 20-days post-inoculation, all mice injected with predatory bacteria were viable and healthy (Table 2). To model a multiple bacteremia event, a group of 5 mice were re-injected with 1×10^8 PFU/mouse of *B. bacteriovorus* 109J at 10-days post-initial injection. Again, we did not observe any reduction in mouse viability due to re-injection of predatory bacteria (Table 2). Histological examination, taken 20 days following injection, of the liver, kidney, and spleen revealed no pathology or signs of inflammation compared to the control mice inoculated with PBS (Fig. 4). Micrographs of the liver showed normal hepatic cells in both predator-infected mice and unexposed controls. Kidneys also showed normal structure with glomeruli and tubules. No pathology was detected in the spleen, with well-preserved red (presence of erythrocytes) and white (tightly packed lymphocytes) pulp.

Blood profiling. To determine the effect of intravenous inoculation of predatory bacteria on host blood cell profile, 100 µl of blood was removed from each mouse at 3 and 18 hours post-exposure. White blood cell (WBC) counts were performed and the levels of individual cell types determined (Fig. 5). Surprisingly, total WBC counts decreased at 3 and 18 hours post-injection compared to the control.

Treatment	#of Mice	%Viable on Day 20
Control (PBS)	5	100%
– Re-inject at 10 days	5	100%
B. bacteriovorus 109J	5	100%
– Re-inject at 10 days	5	100%

Table 2. Host viability after intravenous injection of predatory bacteria.

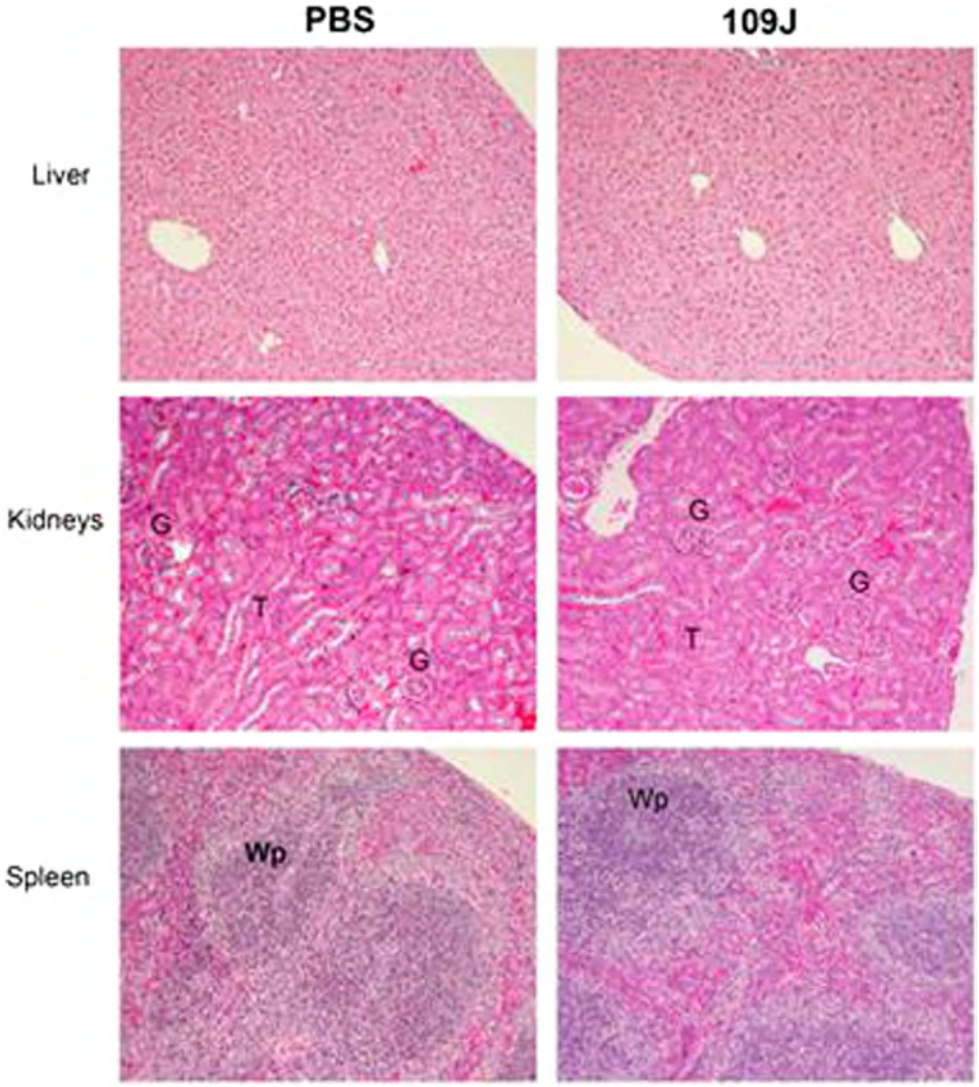


Figure 4. Histological examination of mice after intravenous injection of *B. bacteriovorus* 109J. Histological examination of mice injected through the tail vein with *B. bacteriovorus* 109J revealed no pathology compared to the control mice treated with PBS. All images are representative micrographs that were taken at 20 days post-tail vein injection and at 100X total magnification. G–glomeruli; T–tubules; Wp–white pulp.

A 3.5- fold increase in the percentage of neutrophils in the blood was seen at 3 hours post-injection in mice inoculated with *B. bacteriovorus* 109J. However, the level of neutrophils in the blood returned to comparable levels seen in control animals by 18 hours post-exposure. In contrast, the percentage of monocytes in the blood remained elevated by 4.7-fold at 18 hours post-injection. Decreased percentages of lymphocytes in the blood were seen in conjunction with the observed increases of neutrophils and monocytes resulting from inoculation with predatory bacteria. Eosinophils were found at comparably low levels in both the control and treated mice.

Host immune response to B. bacteriovorus 109J. To examine the effects of intravenous introduction of predatory bacteria on the host immune system, we injected 1×10^8 PFU/mouse of *B. bacteriovorus* 109J into the tail vein of mice (5 mice per treatment group). Mice were visually monitored for signs of illness or discomfort, and euthanized at either 3, 18 hours or 20 days post-injection, when organs and blood were harvested. To model a multiple bacteremia event, a group of mice were re-injected with 1×10^8 PFU/mouse of *B. bacteriovorus* 109J at 10 days post- initial injection. The kidney, liver and spleen were harvested to measure inflammatory cytokines (Fig. 6).

As we observed in the respiratory inoculation model, none of the 20 mice that were injected with predatory bacteria exhibited any observable adverse effects. At 3 hours post injection, we detected an increase in inflammatory cytokines TNF (9.0-fold) in the liver (Fig. 6B) and IL-6 (18- and 13-fold) in

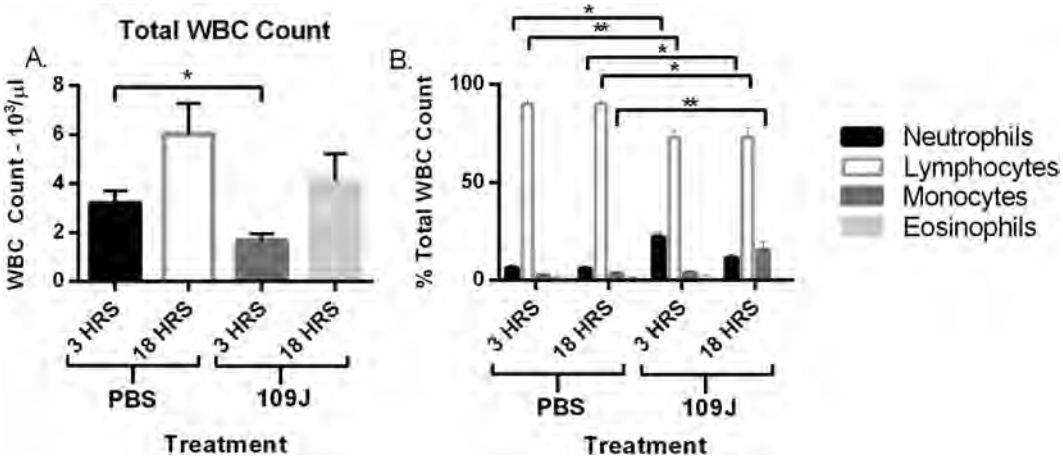


Figure 5. Inflammatory cell response to intravenous injection of *B. bacteriovorus* 109J. To profile the host cell response in the blood, mice were injected through the tail vein with *B. bacteriovorus* 109J. Profile of (**A**) total white blood cell (WBC) counts and (**B**) inflammatory cells after tail vein injection of *B. bacteriovorus* 109J. Blood was assessed at 3 and 18 hours post-injection. Blood profiles were performed by ANTECH Diagnostics (New Hyde Park, NY, USA). Data represent mean ± standard error of the mean. Student's *t*-test was performed to compare each treated sample to their respective control sample, $^{*}P < 0.05$; $^{**}P < 0.01$.

the kidney and spleen (Fig. 6A,C, respectively) relative to control. However, as with our results obtained from respiratory introduction, this increased expression was not sustained by 18 hours post-injection (Fig. 6). An ELISA using whole blood from inoculated mice revealed increases in inflammatory proteins, including IL-1β (13-fold), IL-6 (18-fold), IL-10 (13-fold), CXCL-1/KC (53-fold), IFNγ (27-fold) and TNF (8.7-fold), at 3 hours post-injection, but returned to baseline levels by 18 hours post-injection (Fig. 6D). Taken together, the data suggest that intravenous injection of *B. bacteriovorus* 109J does not provoke a sustained inflammatory response. Our data reflect the host's response to and efficient clearance of the invading organism.

Bacterial Dissemination within the Host. In order to examine predatory bacterial dissemination and migration following inoculation, we utilized primers targeting the 16S ribosomal RNA region for each of *B. bacteriovorus* 109J, HD100, or *M. aeruginosavorus*. Total RNA from organ samples collected from the previously described respiratory and intravenous mouse experiments were probed for detectable levels of predators using RT-qPCR (Fig. 7). For the one hour time point, an inoculation dose of 4×10^9 PFU/mouse of *B. bacteriovorus* 109J, 7×10^9 PFU/mouse of *B. bacteriovorus* HD100 or 5×10^8 PFU/mouse of *M. aeruginosavorus* ARL-13 was used. For each of the 24 and 48 hour time points, an inoculation dose of 1×10^9 PFU/mouse of both *B. bacteriovorus* 109J or HD100, or 1×10^{11} PFU/mouse of *M. aeruginosavorus* ARL-13 was introduced.

In the respiratory model, at one hour post-inoculation, *B. bacteriovorus* 109J was detected in the lungs in 6 out of the 6 mice examined (ranging from 10^5 to 10^{10} gene copy numbers), HD100 in 6/6 mice (10^4–10^{10}), and *M. aeruginosavorus* in 5/6 mice (10^4–10^6), (Fig. 7A). However, the number of predatory bacteria detected in the lungs dropped substantially by 24 and 48 hours post-inoculation with all tested strains. No predators were detected in the spleens of mice at either 24 or 48 hours post-inoculation (Fig. 7B).

In the intravenous model, *B. bacteriovorus* 109J was detected at high levels (10^3–10^8) in 5 out of the 5 injected mice at 3 hours post-injection (Fig. 7C). A modest drop in detectable 109J was seen at 18 hours post-injection, with complete clearance of the predators in all mice observed at 20 days post-injection. In addition, no predators were detected 10 days post-second injection in the multiple bacteremia model, suggesting complete clearance of the predators by 10 days post-injection (Fig. 7C). Altogether, our data indicate that *B. bacteriovorus* 109J bacteria are quickly and efficiently cleared from the tissue of mice exposed either intranasally or intravenously.

Discussion

The antibiotic-resistance crisis has inspired researchers in recent years to look for new approaches to treat life-threatening bacterial infections. One biologically-based microbial control strategy is the use of predatory bacteria[7]. The ability of predatory bacteria to prey efficiently on Gram-negative bacteria suggests a promising, novel way to combat infection. However, while efficacy has been shown *in vitro*, the effects of predatory bacteria *in vivo* have not been extensively examined.

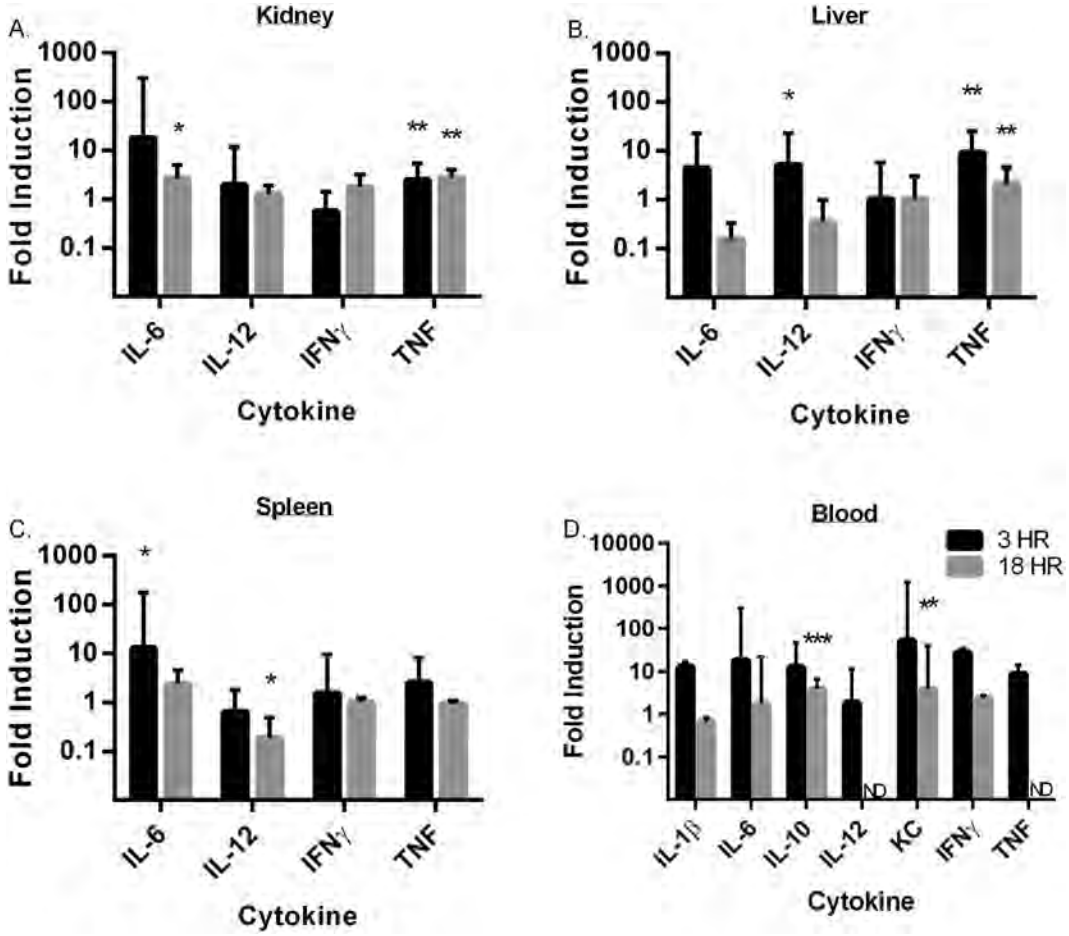


Figure 6. Inflammatory cytokine profile in response to intravenous injection of *B. bacteriovorus* 109J. (**A**–**C**) qPCR analysis of IL-6, IL-12, IFNγ, and TNF in response to tail vein injection of *B. bacteriovorus* 109J relative to PBS control. Expression of cytokines was assessed in the (**A**) kidney, (**B**) liver and (**C**) spleen at 3 and 18 hours post-injection. Fold induction was calculated using the $\Delta\Delta C_t$ by approximation method using an endogenous calibrator (β-actin). Five mice per treatment group were used at each time point. Data represent mean ± standard deviation. (**D**) ELISA analysis of IL-1β, IL-6, IL-10, IL-12, CXCL-1/KC, IFNγ, and TNF in response to tail vein injection of *B. bacteriovorus* 109J relative to PBS control. Inflammatory proteins were assessed in the blood at 3 and 18 hours post-injection. Five mice per treatment group were used at each time point. Data represent mean ± standard error of the mean. Student's *t*-test was performed to compare each treated sample to their respective control sample, $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

In this study, we assessed the effect of predatory bacteria exposure in the mouse. To verify that the results are not strain-specific, we used two strains of *B. bacteriovorus*; we are not aware of additional *M. aeruginosavorus* strains other than the ARL-13 strain. We administered high doses of predatory bacteria to mice via the respiratory and intravenous routes and examined the effect on host viability and immune response. Across the entire study, a total of 105 mice were inoculated intranasally and 20 mice were intravenously injected with predatory bacteria. In both models, we observed neither reduction in host viability nor adverse effects when administering high concentrations of predatory bacteria. A multiple bacteremia model also showed no effect on mouse viability after repeat exposure to predators. Furthermore, histological examination of tissue revealed no pathology in any of the organs tested, suggesting predatory bacteria have no visible negative effects on the overall health of the mice. To reduce the number of animals being sacrificed in the study, only *B. bacteriovorus* 109J was used in the intravenous model. Future studies should involve additional isolates to confirm the results.

Our results align with the findings reported in other animal models which found predatory bacteria non-toxic[15–17]. One such study evaluated the effects of *B. bacteriovorus* HD100 when orally administered to young chicks[17]. Surprisingly, *B. bacteriovorus* HD100 was found to be adaptable and was able to survive in the anaerobic conditions and higher body temperatures of the chick gut. While oral administration of the predators altered the chick's normal gut microbiota, there were no other visual adverse effects on their well-being. However, the study did not assess the chick's immune response to predatory bacteria,

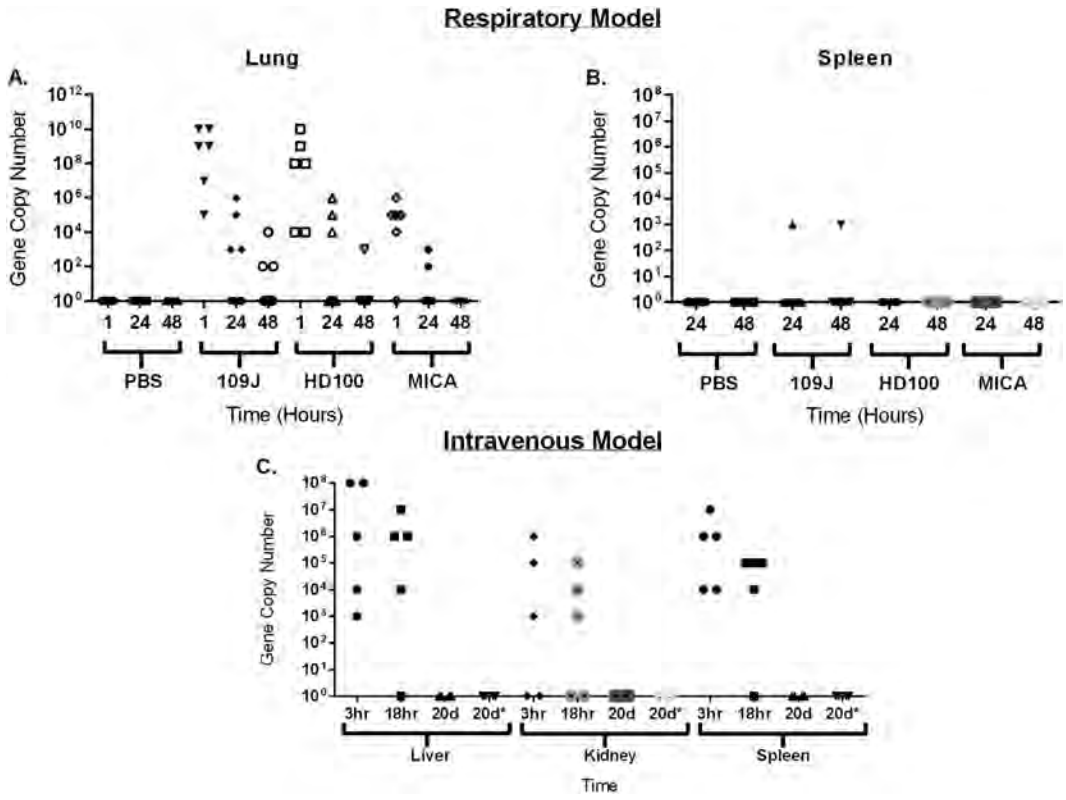


Figure 7. Predatory bacterial dissemination within host. qPCR detection of predatory bacteria within the host. For the respiratory model, the (**A**) lung and (**B**) spleen were probed for *B. bacteriovorus* 109J, HD100, and *M. aeruginosavorus*. In the intravenous model (**C**), the liver, kidney and spleen were probed for only *B. bacteriovorus* 109J. In the respiratory model, 6 mice per predatory bacterial strain (and PBS) were used at the one hour time point; 12 mice per treatment group were used at the 24 and 48 hour time points. Five mice per treatment group at each time point were used in the intravenous model. Each data point represents a single mouse's bacterial load. 20d* - mice re-injected with *B. bacteriovorus* 109J or PBS control at 10 days post-initial-injection (to model multiple bacteremia event).

and combined with the lack of adverse effects on the host, this left questions as to the immunogenicity of predatory bacteria in a living host.

We next looked to profile the host immune response to predatory bacteria introduction. We detected a modest immune response to predatory bacteria in both the respiratory and intravenous models. An increase in specific proinflammatory cytokines and chemokines was detected (namely IL-1β, IL-6, IL-23, CXCL-1/KC, IFNγ, and TNF). However, the response paled in comparison to the response caused by a known respiratory bacterial pathogen, *K. pneumoniae*. The initial increase in proinflammatory cytokines was not sustained, and cytokine levels were back to baseline levels by 24 and 18 hours post-inoculation, for the respiratory and intravenous models, respectively. Furthermore, bacterial dissemination experiments showed predatory bacteria were efficiently cleared from the host in both models. Although all mice were initially inoculated intranasally with 4×10^9 PFU/mouse, qPCR was able to detect 10^{10} gene copies of *Bdellovibrio* in three of the mice. qPCR has been found to slightly overestimate quantities of bacteria compared to standard microbiological plating methods, as there is no discrimination in amplification between viable and dead cells[20]; this could account for the slight difference in the numbers we observed. We detected no predators in the spleen at either 24 or 48 hours, possibly due to the predators being cleared from the host before reaching the spleen. The intravenous model showed complete clearance of the predators by 20 days post-injection and also determined that *B. bacteriovorus* 109J bacteria inoculated by repeated injections were just as efficiently cleared.

The proinflammatory cytokines that were induced by exposure to predatory bacteria represent hallmarks of activation of the innate immune response. Furthermore, profiling of mice exposed to *B. bacteriovorus* 109J saw a 3.5-fold increase in the percentage of neutrophils present in the blood at 3 hours post-injection. Neutrophils are key players in the innate immune response and constitute the first line of defense against invading pathogens[21]. The increase in neutrophils in the blood correlates with the large increase of CXCL-1/KC (53-fold) and IFNγ (27-fold) expression in the blood at 3 hours, as analyzed through ELISA. CXCL-1 is expressed by macrophages, neutrophils and epithelial cells. Both CXCL-1 and IFNγ have been found to have neutrophil chemoattractant activity[22,23]. Thus, we suspect that *B.*

bacteriovorus 109J is being cleared from the blood by neutrophils recruited to the site through a chemotactic gradient of expressed cytokines and chemokines.

While this limited immune response to predatory bacteria exposure may surprise some, it is important to note that predatory bacteria may be inherently non-pathogenic to mammalian hosts. A study looking at the effects of non-pathogenic Gram-negative bacteria on the immune response in the gut found similar patterns in cytokine expression levels when challenging with non-pathogenic strains of *Escherichia coli*, as well as an increase in TLR-4 expression[24]. Toll like receptors are a family of pattern recognition receptors that play a key role in innate immunity. It has been reported that *B. bacteriovorus* contains a unique lipopolysaccharide (LPS) possessing neutral lipid As containing α-D-mannoses that replace the usual negatively-charged phosphate residues found in the LPS on pathogenic bacteria[25]. The same study showed that this unique LPS provokes a weak immunogenic response from human mononuclear cells *in vitro*. Similar to our observations in our *in vivo* model, they detected smaller inductions of TNF and IL-6 as compared to that induced by pathogenic *E. coli*. As TLR-4 is responsible for detecting LPS expressed on or released from the surface of Gram-negative bacteria to activate the innate immune response, the neutral-charged LPS on *B. bacteriovorus* prevents a more robust response and thus results in less inflammation. This may also explain the slightly larger induction of cytokines observed in the lungs when inoculating the host with *M. aeruginosavorus* as compared to the two *B. bacteriovorus* strains. The LPS of *M. aeruginosavorus* has not been characterized. However, a previous study focusing on the use of predatory bacteria to control ocular infections found that *B. bacteriovorus* and *M. aeruginosavorus* both induced weak expression of IL-8 and TNF in human corneal-limbal epithelial cells *in vitro*[12], signaling that *M. aeruginosavorus* may contain an altered LPS as well. Further analysis of the LPS of *M. aeruginosavorus* must be done to confirm these results.

In conclusion, our results demonstrate that predatory bacteria *B. bacteriovorus* 109J, HD100 and *M. aeruginosavorus* ARL-13 are non-pathogenic in a mammalian host, do not induce a robust or sustained immune response, and are efficiently cleared from the host. Future studies should focus on assessing the efficacy of predatory bacteria to prey on Gram-negative pathogens *in vivo*.

Methods

Bacteria, strains and growth conditions. The predatory bacteria used in this study were *Bdellovibrio bacteriovorus* 109J (ATCC 43826), *B. bacteriovorus* HD100[3] and *Micavibrio aeruginosavorus* strain ARL-13[6]. *Klebsiella pneumoniae* ATCC 43816 was used and grown in LB media. Predatory bacteria were cultured and processed as previously described[9,12]. Predator stock-lysates were prepared by co-culturing the predators with host cells in diluted nutrient broth (DNB) (a 1:10 dilution of nutrient broth supplemented with 3 mM $MgCl_2$ and 2 mM $CaCl_2$). The co-cultures were incubated at 30°C until the culture cleared (stock-lysates). To cultivate high concentrations of *Bdellovibrio* for inoculation experiments, 10 ml of washed overnight culture host cells ($\sim 1 \times 10^9$ CFU/ml) were re-suspended in 90 ml of DNB containing 10 ml of predatory bacteria from the stock-lysates. *Micavibrio* was initially cultured in the same manner. To obtain higher predator concentrations, *Micavibrio* co-cultures were prepared in 200 ml of DNB containing 20 ml of the host and 20 ml of *Micavibrio* stock-lysates. *Bdellovibrio* were incubated on a rotary shaker for 24 hours. *Micavibrio* were incubated on a rotary shaker for 48 hours for the initial experiment. After the initial experiment, a 72 hour incubation period was used to reach higher concentrations of the predator. To purify and concentrate the predators, co-cultures were passed three times through a 0.45-μm Millex pore-size filter (Millipore) to remove residual prey and cell debris (filtered lysate). To further purify and concentrate predator samples, filtered lysate was pelleted three times by centrifugation at 29,000 g for 45 min using a Sorvall LYNX 4000 centrifuge (Thermo Fisher Scientific Inc). Each time, the pellet was washed and re- suspended in 50 ml of phosphate buffered saline (PBS). For the last wash, the predator pellet was re-suspended in 1-2 ml of PBS solution to reach a final optical absorbance of ~0.3–0.4 at 600 nm. Predator cell concentrations were determined each time using the standard double-layered agar method[26]. To confirm that the samples were free of host cells, 50 μl aliquots of the predator samples were removed and plated on LB agar TSB-blood plates. Since the predatory bacteria were used directly after isolation, the actual viable predator dose was known only a few days after each experiment, as the plaque-forming unit (PFU) appeared. Therefore, in some experiments, mainly involving *M. aeruginosavorus,* the inoculation size varied somewhat. Actual predator inoculation doses are specified for each experiment.

Mice. Wild type C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD, USA). The mice were housed under pathogen-free conditions at the Rutgers New Jersey Medical School animal facility. All experiments were performed in accordance with the protocols approved by the Institutional Animal Care and Use Committee of Rutgers New Jersey Medical School (protocol #13112A1) and the Animal Care and Use Review Office of the U.S. Army Medical Research and Material Command.

Respiratory inoculation model. Predatory bacteria were introduced by intranasal inoculation of C57BL/6 mice to model a respiratory infection. Animals were lightly anaesthetized with 3% isoflurane in oxygen for four minutes using an isoflurane vaporizer. Twenty-five μl of purified bacterial suspension were gently applied at both nostrils. Mice were inoculated with either PBS, *B. bacteriovorus* strain 109J,

B. bacteriovorus strain HD100, or *M. aeruginosavorus* strain ARL-13. After initial inoculation, animals were observed for the following five days and visually assessed for signs of infection, illness and discomfort. Two mice from each treatment group were kept and visually assessed for up to 50 days. To assess the immune response, lung, serum, liver, and spleen samples were collected at 1, 24, and 48 hours post-exposure. Organs for RNA extraction were stored in 1.0 ml of Trizol at −80 °C. Organs for ELISA were stored at −80 °C in 1.0 ml of PBS containing protease inhibitor. Samples for histology were stored in 1.0 ml of paraformaldehyde at 4 °C.

Intravenous inoculation model. Twenty-five μl of purified *B. bacteriovorus* strain 109J were introduced by tail vein injection to evaluate the effects of an acute bacteremia event on mouse viability and predator clearance. C57BL/6 mice were injected with either PBS or 1×10^8 PFU/mouse *B. bacteriovorus* strain 109J. After initial inoculation, animals were observed for up to 20 days and visually assessed for signs of infection, illness and discomfort. To model a multiple bacteremia event, groups of mice were re-injected with either PBS or 1×10^8 PFU/mouse *B. bacteriovorus* strain 109J at 10 days post-initial injection. To assess the host immune response, mice were kept for 3 or 18 hours post-exposure, when they were euthanized and lung, serum, liver, kidney and spleen samples collected.

RNA extraction. Samples were prepared as previously described[27]. Organs were homogenized in bead beater tubes. To extract total RNA, liquefied samples were centrifuged at 13,200 RPM for 20 minutes at 4 °C to remove tissue debris, and the supernatants were transferred to a new tube. Two-hundred μl of chloroform were added, and centrifuged again at 11,600 RPM for 15 minutes. The aqueous phase was transferred to a new tube and combined with equal volume of isopropanol to precipitate the RNA. Samples were then centrifuged at 11,600 RPM for 15 minutes, and remaining isopropanol removed. Pellets were washed twice with 500 ml of ice-cold 70% ethanol, removing the ethanol from the sample after centrifugation. The pellets were then re-suspended in 30 μl of nuclease-free water. Total RNA was then purified using the 'RNA Cleanup' protocol in the RNeasy® Mini Kit (Qiagen), and stored at −80 °C.

Host immune response profiling (qPCR). Samples were prepared as previously described[27]. cDNA synthesis was performed on total RNA isolated using the High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems) according to manufacturer's instructions. To profile the host immune response, TaqMan® probes targeting selected cytokines and an endogenous calibrator (β-actin) were utilized for qPCR. Samples were tested in duplicate, with each reaction containing: template (2.0 μl of synthesized cDNA), TaqMan® Gene Expression Master Mix (Applied Biosystems), TaqMan® probe for selected cytokine (Applied Biosystems), and nuclease-free water to 10 μl. A 7900HT Fast Real-Time PCR System (Applied Biosystems) was used with the following protocol: 50 °C for 2 min (1X), 95 °C for 10 min (1X), 95 °C for 15 sec/60 °C for 1 min (40X). Relative quantification of cytokines was performed using the $\Delta\Delta C_t$ by approximation method (As described in Reference[28] and[29]). Relative fold expression compared to control was calculated as $2^{-\Delta\Delta Ct}$, where $\Delta C_t = C_t$ (gene of interest)—C_t (normalizer = β-actin) and the $\Delta\Delta C_t = \Delta C_t$ (sample)—ΔC_t (calibrator). Calibrator was total RNA from mice inoculated with PBS.

ELISA. Organ samples were homogenized in bead beater tubes. Liquefied tissues were spun down at 12,000 RPM for 10 minutes at 4 °C. Resulting supernatant was filtered through a 0.22 μm filter at $12 \times g$ RCF for 4 minutes. Cytokines were measured using a V-Plex Proinflammatory Panel1 (mouse) Kit (K15048D-1; Meso Scale Discovery) according to manufacturer's instructions, and read on a SECTOR Imager 2400 (Meso Scale Discovery).

Blood profiling. One hundred μl of blood samples were removed from mice at 3 and 18 hours post-injection with *B. bacteriovorus* 109J and sent to ANTECH Diagnostics (New Hyde Park, NY, USA) for blood cell profiling.

Bacterial dissemination. To detect predatory bacterial dissemination within the host, primers targeting the 16S rRNA gene of each predatory bacterial strain were synthesized: *B. bacteriovorus* HD100 (Forward): 5′-GGAGGCAGCAGTAGGGAATA-3′, (Reverse): 5′-GCTAGGATCCCTCGTCTTACC-3′[30]; 109J (Forward): 5′-ACACGGTCCAGACTCCTACG-3′, (Reverse): 5′-ACGCTAGGATCCCTCGTCTT-3′; *M. aeruginosavorus* strain ARL-13 (Forward): 5′-GGCTTCACTTTGTCCAGAGC-3′; (Reverse): 5′-CAGAAAAACGCGAAATCCTC-3′. Samples were tested in triplicates, with each reaction containing: template (1.0 μl of cDNA synthesized above), SYBR Green PCR Master Mix (Applied Biosystems), and 500 nM (for 109J and *Micavibrio*) or 900 nM (for HD100) of each primer (synthesized at the Rutgers New Jersey Medical School Molecular Resource Facility). A 7900HT Fast Real-Time PCR System (Applied Biosystems) was used: 50 °C for 2 min (1X), 95 °C for 10 min (1X), 95 °C for 15 sec/60 °C for 1 min (40X), 95 °C for 15 sec/60 °C for 15 sec/95 °C for 15 sec (1X). For each qPCR run, a 10-fold dilution series of the standard (purified DNA from each predatory strain) was assessed in triplicate to validate qPCR performance and facilitate quantification. In addition, each qPCR run included negative controls (no template). Gene copy number was calculated using the 'Calculator for determining the number of copies of a template,' by URI Genomics & Sequencing Center (http://cels.uri.edu/gsc/cndna.html)[31].

Histology. All histopathological examination was performed by a pathologist that was not blinded to each specimen's treatment group. Paraformaldehyde-fixed organ segments from infected mice were paraffin-embedded and stained with hematoxylin and eosin (H&E) for cellular composition as previously described[32]. Stained sections were analyzed and photographed using a Nikon Microphot-FX photomicrographic system with NIS-Elements F3.0 software (Nikon Instruments Inc, Melville, NY).

Statistical analysis. qPCR data are presented as mean ± standard deviation. ELISA data are presented as mean ± standard error of the mean. Significant differences between the treated sample compared to respective control were examined using independent-samples student's *t*-tests. A P value of <0.05 was considered significant.

References

1. Stolp, H. & Starr, M. P. *BDELLOVIBRIO BACTERIOVORUS* GEN. ET SP. N., A PREDATORY, ECTOPARASITIC, AND BACTERIOLYTIC MICROORGANISM. *Antonie Van Leeuwenhoek* **29,** 217–248 (1963).
2. Lambina, V. A., Afinogenova, A. V., Romay Penobad, Z., Konovalova, S. M. & Andreev, L. V. [New species of exoparasitic bacteria of the genus *Micavibrio* infecting gram-positive bacteria]. *Mikrobiologiia* **52,** 777–780 (1983).
3. Rendulic, S. *et al.* A predator unmasked: life cycle of *Bdellovibrio bacteriovorus* from a genomic perspective. *Science* **303,** 689–692, doi: 10.1126/science.1093027 (2004).
4. Sockett, R. E. Predatory Lifestyle of *Bdellovibrio bacteriovorus*. *Annu. Rev. Microbiol.* **63,** 523–539, doi: 10.1146/annurev.micro.091208.073346 (2009).
5. Lambina, V. A., Afinogenova, A. V., Romai Penabad, S., Konovalova, S. M. & Pushkareva, A. P. [*Micavibrio admirandus* gen. et sp. nov]. *Mikrobiologiia* **51,** 114–117 (1982).
6. Wang, Z., Kadouri, D. E. & Wu, M. Genomic insights into an obligate epibiotic bacterial predator: *Micavibrio aeruginosavorus* ARL-13. *BMC Genomics* **12,** 453, doi: 10.1186/1471-2164-12-453 (2011).
7. Dwidar, M., Monnappa, A. K. & Mitchell, R. J. The dual probiotic and antibiotic nature of *Bdellovibrio bacteriovorus*. *BMB Rep* **45,** 71–78, doi: 10.5483/BMBRep.2012.45.2.71 (2012).
8. Kadouri, D. E., To, K., Shanks, R. M. & Doi, Y. Predatory bacteria: a potential ally against multidrug-resistant Gram-negative pathogens. *PLoS One* **8,** e63397, doi: 10.1371/journal.pone.0063397 (2013).
9. Dashiff, A., Junka, R. A., Libera, M. & Kadouri, D. E. Predation of human pathogens by the predatory bacteria *Micavibrio aeruginosavorus* and *Bdellovibrio bacteriovorus*. *J. Appl. Microbiol.* **110,** 431–444, doi: 10.1111/j.1365-2672.2010.04900.x (2011).
10. Kadouri, D. & O'Toole, G. A. Susceptibility of biofilms to *Bdellovibrio bacteriovorus* attack. *Appl. Environ. Microbiol.* **71,** 4044–4051, doi: 10.1128/aem.71.7.4044-4051.2005 (2005).
11. Kadouri, D., Venzon, N. C. & O'Toole, G. A. Vulnerability of pathogenic biofilms to *Micavibrio aeruginosavorus*. *Appl. Environ. Microbiol.* **73,** 605–614, doi: 10.1128/aem.01893-06 (2007).
12. Shanks, R. M. *et al.* An Eye to a Kill: Using Predatory Bacteria to Control Gram-Negative Pathogens Associated with Ocular Infections. *PLoS One* **8,** e66723, doi: 10.1371/journal.pone.0066723 (2013).
13. Iebba, V. *et al.* Higher prevalence and abundance of *Bdellovibrio bacteriovorus* in the human gut of healthy subjects. *PLoS One* **8,** e61608, doi: 10.1371/journal.pone.0061608 (2013).
14. Shemesh, Y. & Jurkevitch, E. Plastic phenotypic resistance to predation by *Bdellovibrio* and like organisms in bacterial prey. *Environ. Microbiol.* **6,** 12–18 (2004).
15. Verklova, Z. S. [Study of the virulence, toxicity and immunogenicity of different strains of *Bdellovibrio bacteriovorus*]. *Gig. Sanit.* **38,** 10–13 (1973).
16. Westergaard, J. M. & Kramer, T. T. *Bdellovibrio* and the intestinal flora of vertebrates. *Appl. Environ. Microbiol.* **34,** 506–511 (1977).
17. Atterbury, R. J. *et al.* Effects of orally administered *Bdellovibrio bacteriovorus* on the well-being and *Salmonella* colonization of young chicks. *Appl. Environ. Microbiol.* **77,** 5794–5803, doi: 10.1128/aem.00426-11 (2011).
18. Greenberger, M. J. *et al.* IL-12 gene therapy protects mice in lethal *Klebsiella pneumonia*. *J. Immunol.* **157,** 3006–3012 (1996).
19. Bartlett, K. H., McCray, P. B. & Thorne, P. S. Novispirin G10-Induced Lung Toxicity in a *Klebsiella pneumoniae* Infection Model. *Antimicrob. Agents Chemother.* **47,** 3901–3906, doi: 10.1128/AAC.47.12.3901-3906.2003 (2003).
20. Castillo, M. *et al.* Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR. *Vet. Microbiol.* **114,** 165–170, doi: 10.1016/j.vetmic.2005.11.055 (2006).
21. Kobayashi, S. D., Voyich, J. M., Burlak, C. & DeLeo, F. R. Neutrophils in the innate immune response. *Arch. Immunol. Ther. Exp. (Warsz.)* **53,** 505–517 (2005).
22. Bonville, C. A. *et al.* Interferon-gamma coordinates CCL3-mediated neutrophil recruitment *in vivo*. *BMC Immunol.* **10,** 14, doi: 10.1186/1471-2172-10-14 (2009).
23. De Filippo, K. *et al.* Mast cell and macrophage chemokines CXCL1/CXCL2 control the early stage of neutrophil recruitment during tissue inflammation. *Blood* **121,** 4930–4937, doi: 10.1182/blood-2013-02-486217 (2013).
24. Dogi, C. A., Galdeano, C. M. & Perdigon, G. Gut immune stimulation by non pathogenic Gram(+) and Gram(−) bacteria. Comparison with a probiotic strain. *Cytokine* **41,** 223–231, doi: 10.1016/j.cyto.2007.11.014 (2008).
25. Schwudke, D. *et al.* The obligate predatory *Bdellovibrio bacteriovorus* possesses a neutral lipid A containing alpha-D-Mannoses that replace phosphate residues: similarities and differences between the lipid As and the lipopolysaccharides of the wild type strain *B. bacteriovorus* HD100 and its host-independent derivative HI100. *J. Biol. Chem.* **278,** 27502–27512, doi: 10.1074/jbc.M303012200 (2003).
26. Shilo, M. & Bruff, B. Lysis of Gram-negative bacteria by host-independent ectoparasitic *Bdellovibrio bacteriovorus* isolates. *J. Gen. Microbiol.* **40,** 317–328 (1965).
27. Bhatt, K., Kim, A., Kim, A., Mathur, S. & Salgame, P. Equivalent functions for B7.1 and B7.2 costimulation in mediating host resistance to *Mycobacterium tuberculosis*. *Cell. Immunol.* **285,** 69–75, doi: 10.1016/j.cellimm.2013.09.004 (2013).
28. Nolan, T., Hands, R. E. & Bustin, S. A. Quantification of mRNA using real-time RT-PCR. *Nat. Protoc.* **1,** 1559–1582, doi: 10.1038/nprot.2006.236 (2006).
29. Applied Biosystems. Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR. (Waltham, MA, 2008).
30. Van Essche, M. *et al.* Development and performance of a quantitative PCR for the enumeration of *Bdellovibrionaceae*. *Environ. Microbiol. Rep.* **1,** 228–233, doi: 10.1111/j.1758-2229.2009.00034.x (2009).
31. Preiser, V. *et al.* The development of a multiplex real-time PCR to quantify *Fusarium* DNA of trichothecene and fumonisin producing strains in maize. *Analytical Methods*, doi: 10.1039/C4AY02581D (2015).

32. Koo, M. S. *et al.* Phosphodiesterase 4 inhibition reduces innate immunity and improves isoniazid clearance of *Mycobacterium tuberculosis* in the lungs of infected mice. *PLoS One* **6,** e17091, doi: 10.1371/journal.pone.0017091 (2011).

Acknowledgments

We thank Kamlesh Bhatt (Rutgers New Jersey School of Medicine) for her direction in multiple methods involved in the study. This work was supported by the Department of the ARMY USAMRAA grant number W81XWH-12-2-0067 to D.E.K. and by the U.S. Army Research Office and the Defense Advanced Research Projects Agency under Cooperative Agreement Number W911NF-14-2- 0016 to D.E.K. and N.D.C. The views and conclusions contained in this document are those of the authors and should not be interpreted as representing the official policies, either expressed or implied, of the Army Research Office, DARPA, or the U.S. Government.

Author Contributions

N.D.C., D.E.K and K.S conceived and designed the experiments. K.S., R.C., C.T., G.C.R. and S.M. performed the experiments. K.S., R.C., and L.T. analyzed the data. K.S. drafted the manuscript. N.D.C. and D.E.K. supervised the study and revised the manuscript. All authors reviewed the manuscript.

Additional Information

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Shatzkes, K. *et al.* Examining the safety of respiratory and intravenous inoculation of *Bdellovibrio bacteriovorus* and *Micavibrio aeruginosavorus* in a mouse model. *Sci. Rep.* **5**, 12899; doi: 10.1038/srep12899 (2015).

The Potential Application and Risks Associated with the Use of Predatory Bacteria as a Bio-control Agent Against Wound Infections

Grant number: DM110015
Cooperative Agreement number: W81XWH-12-2-0067

PI & Collaborator: Kadouri & Zurawski **Org**: University of Medicine and Dentistry of New Jersey & Walter Reed Army Institute of Research (WRAIR)

Total Award Amount: $968,171

Study/Product Aim(s) and Approach

•The objective of this study is to examine the impact and possible risks associated with using predatory bacteria as live antibiotics.

Aim I. Investigating predator prey interactions and resistance. We will conduct experiments aimed at examining the possible emergence of genetically stable resistance in bacterial host and the ability of the predator to breach its host specificity and attack previously resistant bacteria.

Aim II. Determining the effect of predatory bacteria on eukaryotic cells. We will examine the impact of predatory bacteria on non-microbial cells and assess whether predatory bacteria have an adverse or toxic affect on eukaryotic cells.

Aim III. Efficacy and toxicity in animal models. We will examine the toxicity and the immune response of the predator bacteria in established animal models of wound infection.

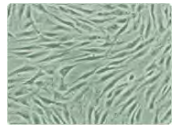
Timeline and Cost

Activities	Year 1	Year 2	Year 3-4
Aim 1. Investigating predator prey interactions and resistance.			
Aim 2. Determining the effect of predatory bacteria on eukaryotic cells.			
Aim 3. Efficacy and toxicity in animal models.			
Estimated Budget ($K)	296	445	214

Updated: 8/31/2015

I. Conducting experiments aimed at assuring that host bacteria don't become resistant to predation and that the predator maintains its host specificity.

II. Conducting experiments aimed at assuring that the predators don't have an adverse or toxic effect on eukaryotic cells.



III. Conducting experiments aimed at assuring the safety and efficacy of predatory bacteria in established animal models of wound infection.

We have verified that no genetically stable predation resistant phenotype developed in host cells following sequential predation. Our data also confirmed that the predators do not breach their host specificity and attack previously resistant bacteria. Toxicity experiments indicate that predatory bacteria are non toxic to eukaryotic cells and wounded mice. At this point we have seen no statistically significant indication of enhance wound heeling in infected animals treated with the predators based on wound size, and weight change.

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Goals/Milestones

CY13 Goals – Investigating predator prey interactions and resistance.

☑ Development of genetically stable resistance to predation.
☑ Examining the ability of the predator to breach its host specificity.
☑ Enriching for hyper predatory variants.

CY14-15 –Determining the effect of predatory bacteria on eukaryotic cells.

☑Determining the toxicity of predatory bacteria on mammalian cells.
☑Determine the influence of predator exposure on cell cytokine profile- In progress.
☑Cell attachment and invasion assays.

CY14-15– Efficacy and toxicity in animal models.

☑Determining the toxicity/efficacy of predatory bacteria in a mouse puncture model.
☑Determine the influence of predator exposure to mice by evaluating cytokine profile and histopathology.
☐Determine the influence of predator exposure to pigs by evaluating histopathology-Under evaluation.