Quantifying differences in bacterial abundance using mixed models

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Summary

Pro-inflammatory caspases play a role in sensing and responding to infection. The goal of the current study is to better understand the importance of caspases 1, 4, and 5 in humans and caspases 1 and 11 in mice in responding to infection by *Salmonella*. Experiments were conducted comparing bacterial abundance in wild-type and caspase-knockout human untransformed cells, human transformed cells, and mouse untransformed cells using confocal and fluorescence microscopy 7 hours after exposure to *Salmonella*. The statistical analyses account for the replicate study design and use appropriate data distribution models. The analyses also address issues of censoring and truncation that arise in some experiments.

Using mixed models, I estimate a higher rate of infection (RR: 1.67, $p = 0.02$) in count data and 138% higher bacterial fluorescence ($p = 0.0025$) in fluorescence data in caspase-4 knockout relative to wild type for human untransformed cells. For mouse untransformed cells, I estimate significantly higher odds of caspase-1 knockout ($p = 0.0025$) and caspase-1/11 double knockout ($p = 0.0038$) cells being highly infected relatively to wild-type cells, while caspase-11 knockout cells were not significantly different ($p = 0.69$). For human transformed cells, I estimate significantly higher odds of caspase-4 knockout cells being highly infected in clone 4.15 ($p = 0.0011$) but not in 4.13 ($p = 0.11$), though the direction of the estimated effect is the same. Caspase-1 knockout and caspase-5 knockout clones all either had significantly lower or not significantly different odds of being highly infected relative to wild-type cells. Overall, the results provide evidence that caspase-4, but not caspase-1 and caspase-5, has an important role in the immune response to *Salmonella* infection in human intestinal epithelial cells, while caspase-1, but not caspase-11, has a corresponding important role in mouse intestinal epithelial cells.

Scientific background

Inflammasomes are important in regulating the activation of pro-inflammatory caspases and inducing inflammation in response to pathogens. In humans, pro-inflammatory caspases include caspase-1 (CASP1), caspase-4 (CASP4), and caspase-5 (CASP5). In mice, pro-inflammatory caspases include CASP1 and caspase-11 (CASP11). Mouse CASP11 is a homolog of human CASP4 and CASP5. Production of pro-inflammatory cytokines IL-1β and IL-18 depends on caspases. Currently, the inflammasome pathways important for sensing and responding to infection are not well understood. Specifically, it is believed that some caspases are critical to the immune response in human intestinal epithelial cells, while others are dispensable.

Salmonella enterica is a bacterium that can cause gastrointestinal infections in humans. To better understand the inflammasome pathways responsible for sensing and responding to *Salmonella* infection, the current study compares *Salmonella* pathogenesis in untransformed human, transformed human, and mouse intestinal epithelial cells with and without various caspase knockouts. At present, all of the data for the study have been collected. Manuscript preparation is ongoing, and the analyses presented in this report represent a subset of the analyses that will be included in the final paper.

Research methods

You and your collaborators conducted a series of experiments with the goal of quantifying differences in intracellular bacteria in cells of different genotypes after infection by Salmonella. The experiments were conducted on untransformed (HIE5) and transformed (C2B) human intestinal epithelial cells and untransformed mouse intestinal epithelial cells (MIE). For HIE5, WT cells were compared with *CASP4* knockout cells (C4). For C2B, WT cells were compared with knockouts of *CASP1* (C1), *CASP4* (C4), and *CASP5* (C5). There were also two clones of each knockout. For MIE cells, WT cells were compared with knockouts of *CASP1* (C1), *CASP11* (C11), and both *CASP1* and *CASP11* (C1/11).

In each experiment, the cells were exposed to an inoculum of bacteria and confocal microscopy was used to determine counts of bacteria in each cell at 0, 1, and 7 hours post-exposure. In addition, for HIE5, fluorescence microscopy was also used quantify bacterial abundance per cell. Each experiment was replicated between 3 and 6 times. Quantification was not done at 0 hours and 1 hour for some replicates.

Statistical questions

You are interested in finding an appropriate statistical approach to quantifying differences in intracellular bacteria between WT cells and knockouts. Specifically, an appropriate analysis should account for the replicates in the study design – two cells within the same replicate may produce more similar results than two cells from different replicates, since cells within each replicate were infected by the same inoculum of bacteria. In addition, the

analysis should consider the discrete nature of the count data and the continuous but highly skewed nature of the fluorescence data.

In some experiments, counts above 30 or 100 bacteria were censored, since the person conducting the experiment could not reliably determine counts above this number. In this case, an analysis based on means would be biased, but an analysis using a dichotomized outcome can still provide valid results. Finally, the analysis should reflect that the focus of the study is on quantifying differences in bacterial abundance among *infected* cells. In fact, the data for uninfected cells (which have 0 bacteria count) were not collected in all experiments.

Statistical methods

For each experiment, the analysis involves regression of bacterial abundance at 7 hours on genotype. Mixed models use replicate-specific random effects to account for the correlated nature of cells within each replicate. The random effects are assumed to be independent.

In this report, the experiments are numbered according to the order of figures in your manuscript: 5b, 5c, 5e, 5g. For experiment 5b, a complete count of the number of bacteria in each cell was available. A two-part model (i.e., a hurdle model) was used to separately model whether a cell is infected or not and how many bacteria were in the cell. The first component involves a logistic mixed model for whether the count is 0. The second component involves a truncated negative binomial mixed model for the bacterial count of infected cells. The second component is most relevant to addressing the scientific aim and may be interpreted separately.

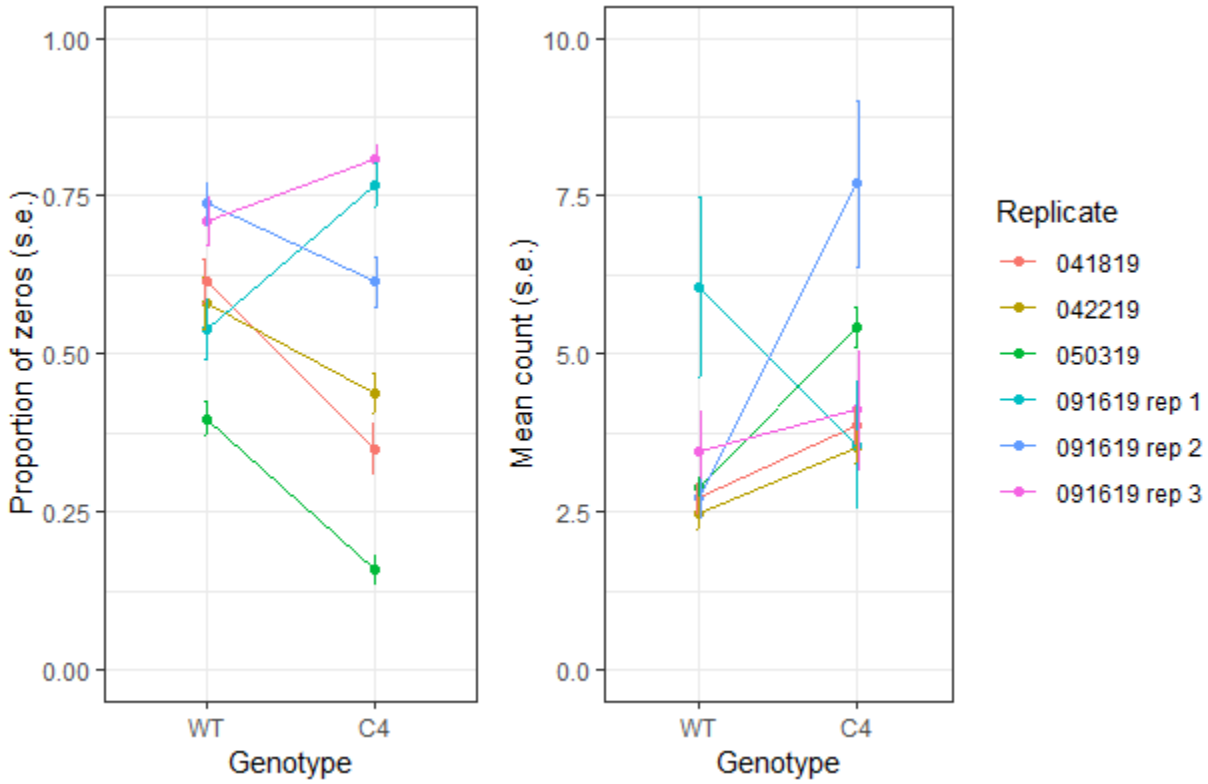
For experiment 5c, a linear mixed model was used to regress log-transformed fluorescence intensity on genotype. The log transformation reduces skewness and is commonly used with fluorescence data. Cells with zero fluorescence were considered uninfected and therefore excluded from the analysis.

For experiments 5e and 5g, cells containing more than 12 bacteria (5e) or 50 bacteria (5g) were categorized as highly infected. The choice of the cutoff was selected based on the distribution of bacterial counts at the 1-hour timepoint, when no cells were highly infected. Data on uninfected cells were not available for these experiments. Logistic mixed models were used to evaluate whether knockout cells had higher odds of being highly infected relative to WT cells.

Testing was done at the 0.05 significance level. Mixed effects modeling was done using the Generalized Linear Mixed Models using Template Model Builder (glmmTMB) package in R version 3.6.1.

Results

Figure 1. Proportion of uninfected HIE5 cells (left) and mean bacterial count for infected HIE5 cells (right). Error bars represent standard errors. Summarized data from a total of 2,355 cells are shown in the left panel and from a total of 1,082 infected cells are shown in the right panel.

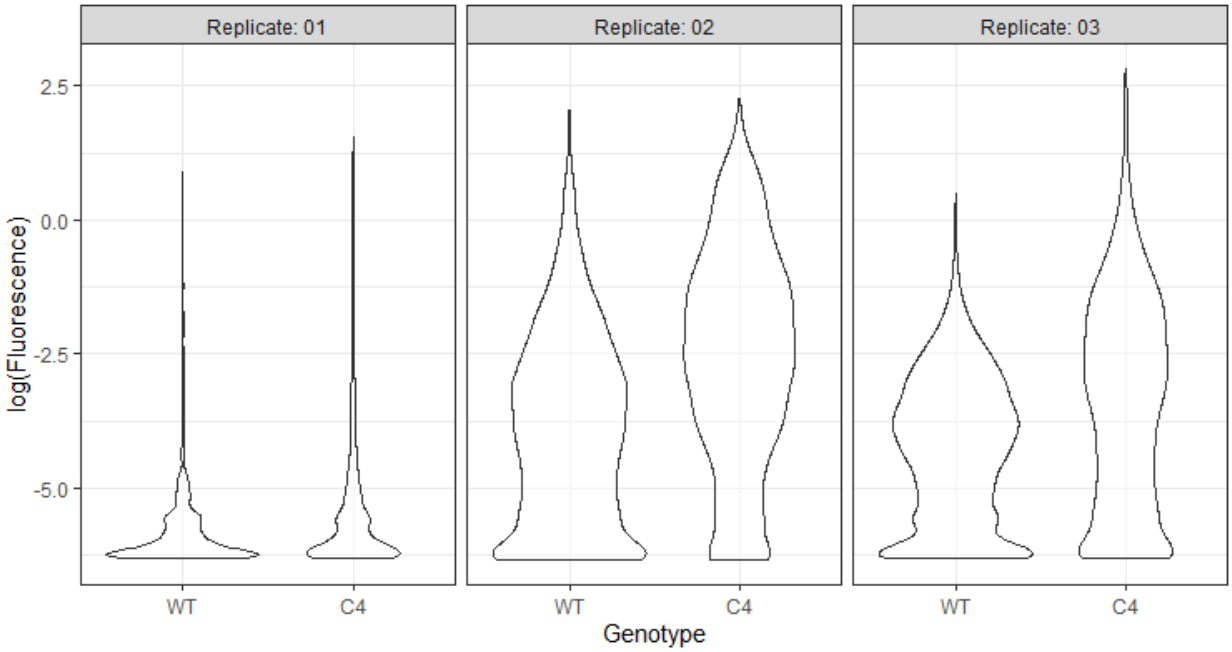


Six replicates were done on HIE5 cells in experiment 5b. The proportion of uninfected cells was lower for the C4 knockouts in four replicates and higher for two replicates (Figure 1, left panel). The mean bacterial count in infected cells was higher for the C4 knockouts in five replicates and lower in one replicate (Figure 1, right panel). In the hurdle model (Table 1), the logistic model did not find significant evidence of differences in zero-inflation between WT and C4 knockouts. Using a truncated negative binomial regression, I estimate a 67% higher rate of infection in C4 knockout cells relative to WT cells (RR: 1.67, p = 0.02).

Table 1. Hurdle model estimates. The first component involves a logistic mixed model, while the second component involves a truncated negative binomial mixed model.

1) Logistic	Odds ratio (95% CI)	P-value
Genotype		
WT (Reference)	–	–
C4	0.73 (0.37, 1.42)	0.35
2) Truncated negative binomial	Rate ratio (95% CI)	P-value
Genotype		
WT (Reference)	–	–
C4	1.67 (1.07, 2.60)	0.02

Figure 2. Distribution of log-transformed fluorescence intensity in MIE cells. Data from 38,315 cells with non-zero fluorescence are shown.



Three replicates were done on MIE cells in experiment 5c. In the first replicate, most likely due to a technical error, very little fluorescence was captured (Figure 2). While cells with zero fluorescence were presumed to be uninfected and therefore excluded from analysis, there exists a cluster of cells with near zero fluorescence in all replicates. This cluster may also represent uninfected cells. Nevertheless, using a linear mixed model on the log-transformed fluorescence intensity, I estimate 138% higher geometric mean fluorescence intensity in C4 knockouts relative to WT ($p = 0.0025$).

Table 2. Linear mixed model estimates. Estimates are calculated on the log scale and transformed back to the original scale for reporting.

	Log scale (Difference)	Linear scale (Ratio)	
	Point estimate (95%CI)	Point estimate (95%CI)	P-value
Genotype			
WT (Reference)	–	–	–
C4	0.87 (0.31, 1.43)	2.38 (1.36, 4.18)	0.0025

Four replicates were done on MIE cells in experiment 5e. Cells containing at least 30 bacteria were censored at 30, though these cells represent a small proportion of the total sample (Table 3). Furthermore, the censoring occurred only in knockout cells but not in WT cells, so any estimated positive effect of knockout can be considered a conservative estimate of the true effect. It can be seen that WT and C11 knockouts generally had lower bacterial abundance than C1 knockouts and C1/11 double knockouts across replicates (Figure 3).

Table 3. Characteristics of MIE data. Cells containing at least 30 bacteria were censored, and cells containing more than 12 bacteria were considered highly infected.

Replicate	Genotype	N	Censored	High count
1	WT	23	0 (0.0%)	0 (0.0%)
	C1	22	3 (13.6%)	5 (22.7%)
	C11	37	0 (0.0%)	2 (5.4%)
	C1/11	48	14 (29.2%)	29 (60.4%)
2	WT	49	0 (0.0%)	4 (8.2%)
	C1	70	1 (1.4%)	9 (12.9%)
	C11	27	0 (0.0%)	0 (0.0%)
	C1/11	95	4 (4.2%)	35 (36.8%)
3	WT	33	0 (0.0%)	0 (0.0%)
	C1	77	1 (1.3%)	11 (14.3%)
	C11	34	0 (0.0%	0 (0.0%)
	C1/11	86	7 (8.1%)	11 (12.8%)
4	WT	35	0 (0.0%)	0 (0.0%)
	C1	75	2 (2.7%)	7 (9.3%)
	C11	0	–	–
	C1/11	47	2 (4.3%)	2 (4.3%)

Figure 3. Distribution of bacterial counts for infected MIE cells. Counts above 30 are censored and shown as 30. Counts are slightly jittered for visual clarity.

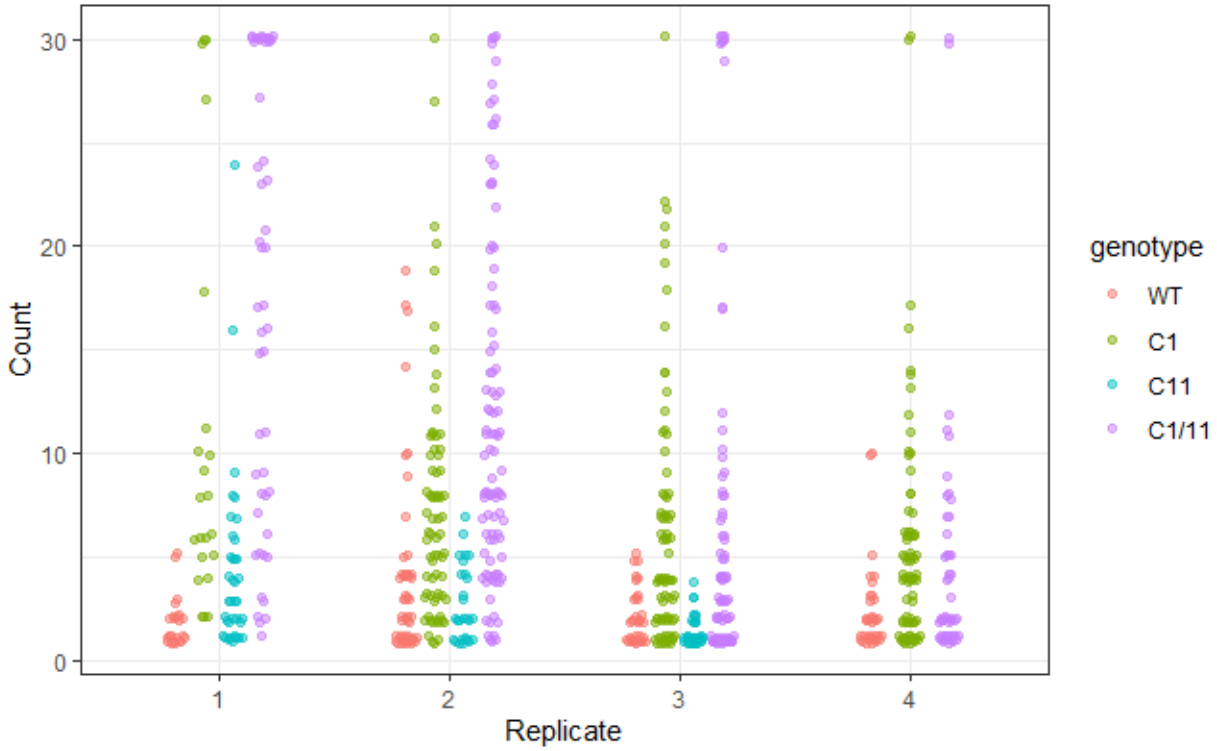


Table 4. Truncated negative binomial mixed model estimates.

	Rate ratio (95% CI)	P-value
Genotype		
WT (Reference)	–	–
C1	3.77 (2.64, 5.37)	2.6e-13
C11	0.77 (0.32, 1.88)	0.57
C1/11	4.79 (2.97, 7.74)	1.5e-10

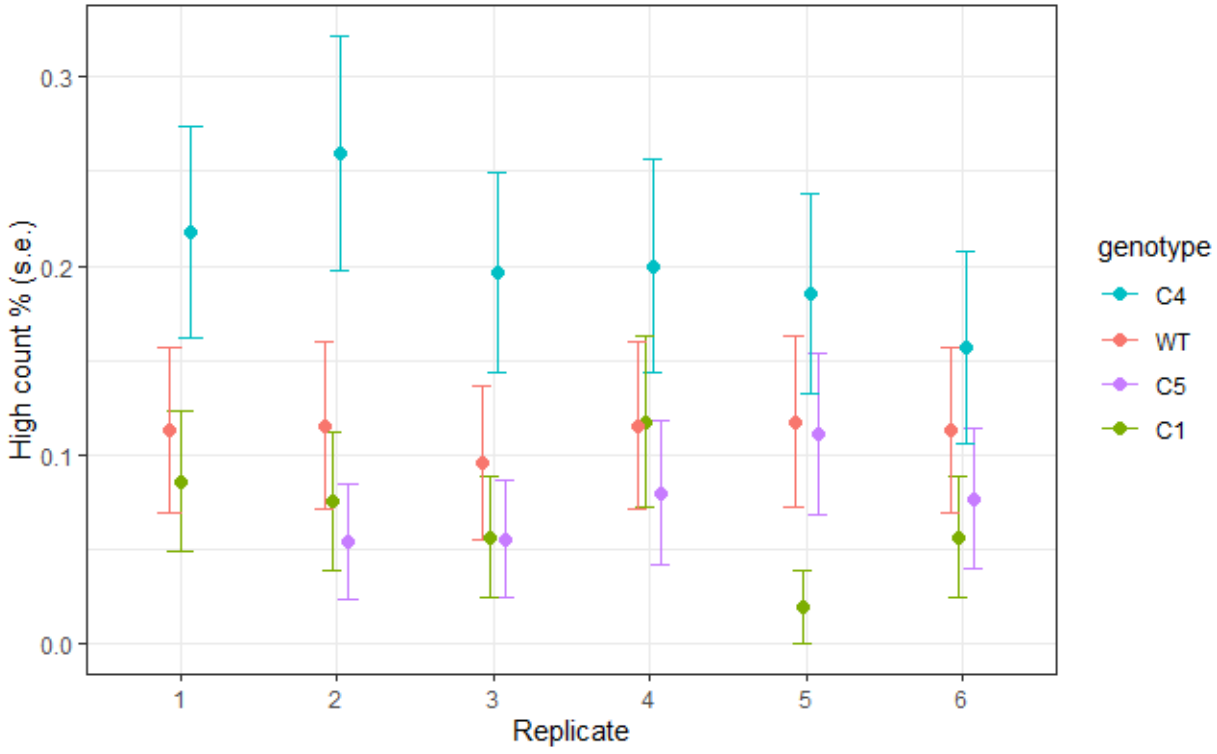
Using a truncated negative binomial mixed model (Table 4), I estimate a 277% higher rate of infection in C1 knockout relative to WT ($p < 0.0001$) and 379% higher rate of infection in C1/11 double knockout relative to WT ($p < 0.0001$), while C11 and WT cells were not significantly different ($p = 0.57$).

For comparison, I also used a logistic mixed model on the dichotomized outcome (Table 5). Here, I estimate 430% higher odds of a C1 knockout cell being highly infected compared to a WT cell (p = 0.0025), and 909% higher odds of a C1/11 double knockout cell being highly infected compared to a WT cell (p = 0.0038). While the effect sizes, p-values, and interpretations change between the negative binomial model and the logistic model, both models suggest that CASP1 plays an important role in the response to *Salmonella* infection in MIE.

Table 5. Logistic mixed model estimates.

	Odds ratio (95% CI)	P-value
Genotype		
WT (Reference)	–	–
C1	5.30 (1.80, 15.64)	2.5e-03
C11	0.58 (0.04, 8.19)	0.69
C1/11	10.09 (2.11, 48.23)	3.8e-03

Figure 4. Proportion of infected C2B cells with high bacterial counts. Cells containing more than 50 bacteria were considered highly infected. Replicates 1-4 use clones 1.5, 4.15, and 5.14, while replicates 5 and 6 use clones 1.13, 4.13, and 5.26 for genotypes C1, C4, and C5, respectively.



For C2B cells, six replicates of the experiment (5g) were done, including two replicates using one set of clones (1.13, 4.13, 5.26) and four replicates using another set of clones (1.5, 4.15, 5.14). For the analysis, different clones are considered to be different genotypes. A noticeably higher proportion of C4 knockout cells are highly infected (Figure 4), relative to WT and the other knockouts. Using a mixed effects logistic regression model, I estimate 64% higher odds of a C4-4.13 cell having high bacterial count relative to WT ($p = 0.11$) and 121% higher odds of a C4-4.15 cell having high bacterial count relative to WT ($p = 0.0011$). The odds ratio estimates for both C1 and both C5 clones are below 1, suggesting that knocking out CASP1 and CASP5 do not result in increased bacterial abundance.

Table 6. Logistic mixed model estimates.

	Odds ratio (95% CI)	P-value
Genotype		
WT (Reference)	–	–
C1, 1.13	0.32 (0.11, 0.92)	0.03
C1, 1.5	0.73 (0.40, 1.32)	0.29
C4, 4.13	1.64 (0.89, 3.05)	0.11
C4, 4.15	2.21 (1.37, 3.58)	1.1e-03
C5, 5.14	0.53 (0.26, 1.11)	0.09
C5, 5.26	0.83 (0.39, 1.73)	0.62

Final comments

Overall, the results suggest that CASP4 plays an important role in the human immune response to *Salmonella* infection while CASP1 and CASP5 may be dispensable. Similarly, it appears that CASP1 has a corresponding important role in the mouse immune response, while CASP11 may be dispensable. The strengths of the present analysis lie in using mixed models to account for correlated data and in using appropriate distributions to model the data so that valid statistical inferences can be made. The analyses also address the issues of censoring (of counts above 30) and truncation (of zero count cells) in appropriate ways.

One limitation of the analyses is the use of a dichotomized outcome to circumvent the censoring issue. This results in a loss of information relative to the original data. The initial choice of cutoff for “highly infected” was also determined from the distribution of bacterial counts at the 7-hour time point, although a cutoff based on scientific reasoning would be preferable. The current version of the analysis bases the cutoff on the distribution of bacterial counts at the 1-hour time point, which represents a population of cells that are not “highly infected”.

A second limitation of the analyses is the lower reliability of mixed models when the number of replicates is small. In this scenario, the model may not be able to accurately capture the variability between replicates. However, mixed models provide easily interpretable results and perform better than alternative methods that account for correlated data in this scenario.

Currently, I am working on revising the analyses for a few additional experiments based on our previous discussion. I am also checking over the other statistical analyses presented in the manuscript.