

Aerobic Exposure Induced Viable but Nonculturable (VBNC) State in *Campylobacter jejuni*

Noah Sherman, Ximin Zeng, Wei Wang, Xinge Hu, Jun Lin
Department of Animal Science, The University of Tennessee, Knoxville, TN

Abstract

Campylobacter jejuni is a leading foodborne bacterial pathogen in the United States and developed countries. *C. jejuni* is considered a microaerophilic bacterium. However, under aerobic stresses, it can enter into a viable but non culturable state (VBNC), which makes *C. jejuni* persist in environments, and difficult to detect and control. To study development of the VBNC state, *C. jejuni* strain NCTC 11168 was incubated in MH broth in a shaking incubator (100 rpm) at 25°C (aerobic condition). At different time points, samples were collected for culturability assessment using the plating method (on days 0, 1, 2, 5, 12, and 19) and live/dead cell ratio measurement using the BacLight™ Bacterial Viability Kit on days 1, 5, 12, and 19. It's observed that the culturability continued to decrease and no colonies formed after day 5, while the live/dead cell ratio decreased but stayed at a relatively high level, indicating a significant portion of *C. jejuni* cells were in VBNC state. A chicken study was performed to explore the possible resuscitation under *in vivo* conditions. Different doses (10^8 , 10^9 , and 10^{10} cells/mL) of VBNC *C. jejuni* 11168 were inoculated into 3-day old chicks by oral gavage. However, no culturable *C. jejuni* 11168 cells were detected in the cloacal swabs of chickens during the whole experiment period. In summary, aerobic exposure promotes *C. jejuni* to enter VBNC state. Understanding the persistence and resuscitation of VBNC *C. jejuni* is critical for improving detection methods, ensuring food safety, and mitigating public health risks.

Introduction

Campylobacter jejuni is a leading foodborne bacterial pathogen in the United States and developed countries. *C. jejuni* is considered a microaerophilic bacterium. However, under aerobic stresses, it can enter into a viable but non culturable state (VBNC). It was determined that they could enter the VBNC when put under oxygenic stress (Zhang and Lu, 2017). The importance of this is exemplified when it is recognized that once the *Campylobacter* enters this state it may pass detection in food and confuse epidemiological studies (Yagi et al, 2022) as *C. jejuni* becomes persistent in environments and evade detection and control methods. This makes the study of this viable but nonculturable state important to help strengthen our understanding of food borne pathogens and possibly prevent future outbreaks from occurring.

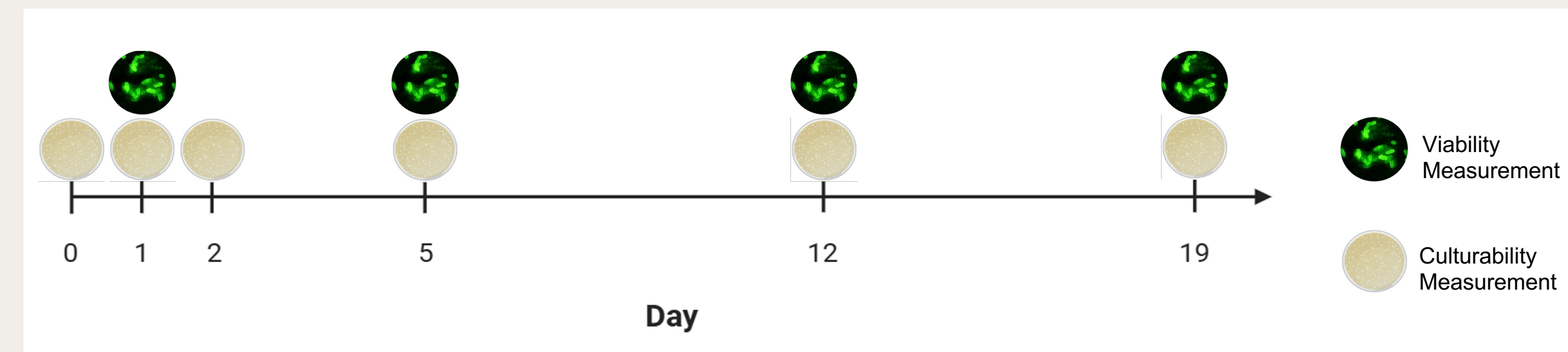
Since bacteria in VBNC state still maintain their membrane integrity, it's possible to differentiate the viable cells from dead cells by using staining dyes with different membrane permeability. The LIVE/DEAD BacLight Bacterial Viability Kits contains 2 two-color fluorescent dyes, propidium iodide (PI) and SYTO™ 9. Cells with a compromised membrane that are dead or dying will stain red with propidium iodide, whereas cells with an intact membrane will stain green with SYTO™ 9. Using such kit, we can study the dynamics of live/dead cells ratio during the bacteria enter VBNC state.

Previous studies had found bacteria can resuscitate from VBNC state, which may pose a hidden risk for food safety and public health. The favorable conditions for resuscitation include nutrient supplementation, external stress elimination, or supplementation with resuscitation-promoting substances. In this study, we also tested possible resuscitation out of VBNC state in an *in vivo* (chicken) model.

Stress *C. jejuni* Aerobically

C. jejuni was originally grown by spreading 100 µL of an isolated culture on 3 Mueller Hinton (MH) agar plates which were placed in a trigas incubator at 42°C for 48 hours. These cells were harvested using 3 mL of MH broth. The collected broth was then put through a 10 fold dilution. The OD₆₀₀ of this solution was measured and the solution was adjusted to have an OD₆₀₀ of 0.1. This was then split into 2 flasks (labeled A and B) each containing 30 mL of solution. These flasks were placed in a shaking incubator set to 25°C and 100 rpm. This provided an unfavorable oxygen rich environment. The flasks remained in the shaking incubator for the remainder of the experiment.

Decreased culturability of *C. jejuni* 11168 when incubating aerobically



Culturability Measurement

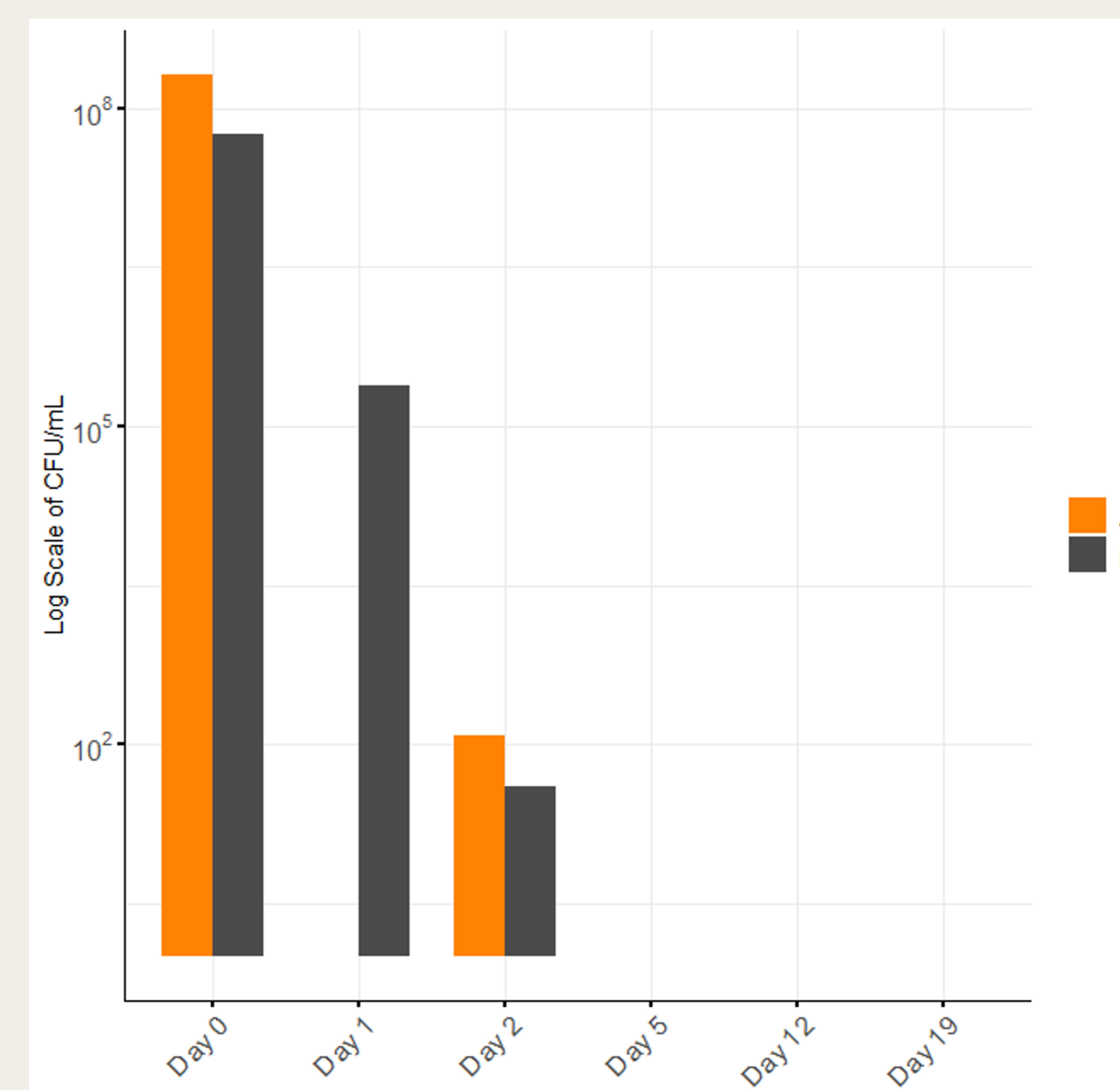


Figure 1. Culturability change of *C. jejuni* 11168 over the course of the aerobic stress. A and B represent each of the two replicate *C. jejuni* cultures. A sample of each flask was serially diluted to 10^{-6} . 100 µL of these dilutions were spread onto MH agar plates and allowed to grow in a 42°C trigas incubator for 48 hours. These plates were then counted and used to determine the CFU. The CFU/mL decreased daily and by day 5 there were no visible colonies on any plates.

Aerobically stressed *C. jejuni* 11168 displayed decreased viability

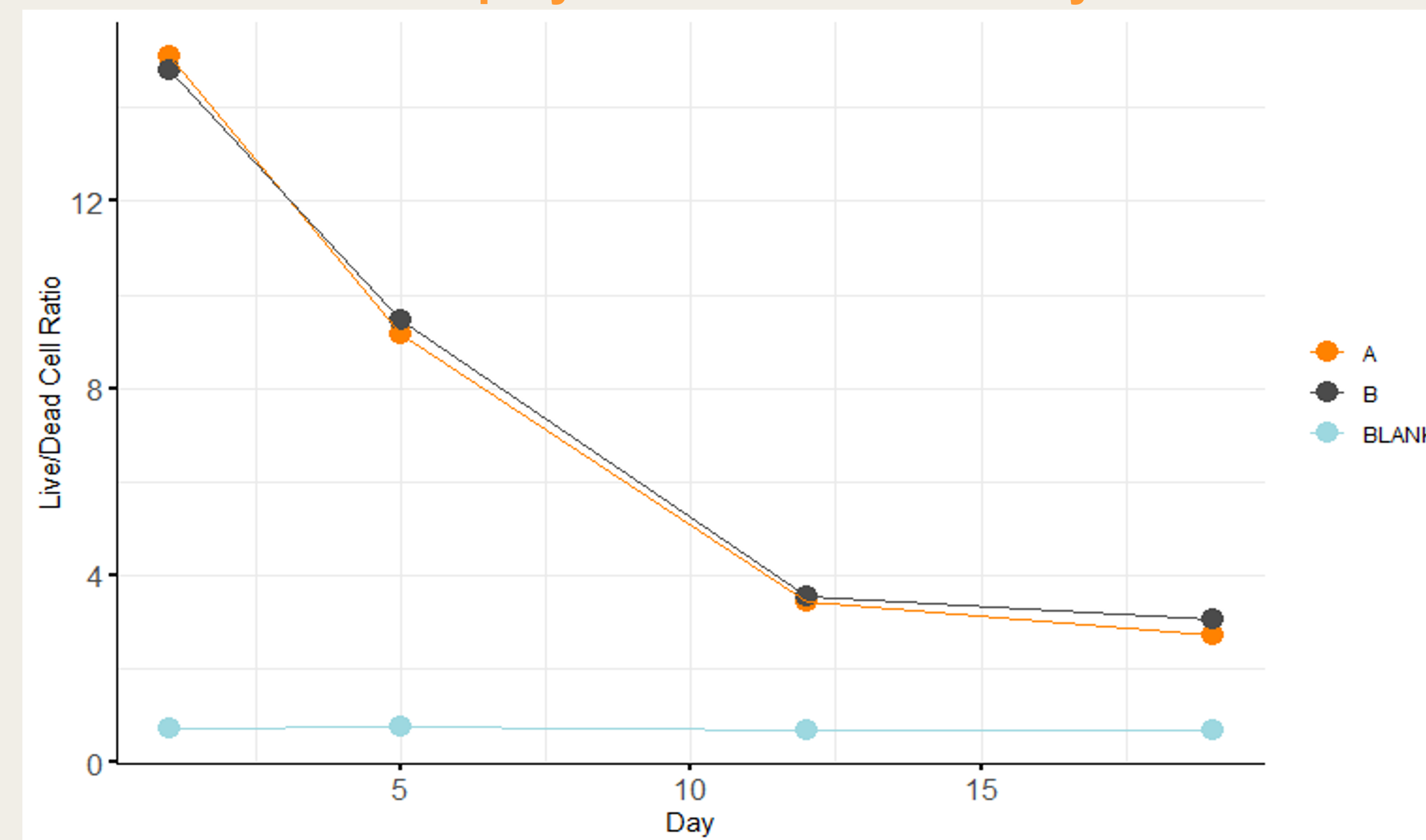


Figure 2. Cell viability assessment (live/dead cell ratio) through Experiment. Weekly, a sample of each of the flasks was also collected. The cultures were diluted so the OD₆₀₀ was equal to 0.1. Using a fluorescent microplate reader centered at 485 nm, intensity at 530 nm and 630 nm was measured. After measuring the fluorescence intensity using the fluorescence microplate reader each value was ascribed to represent live or dead cells. The intensity at 530 nm (from SYTO 9) represents to the number of total cells (both live and dead cells) in the well, and the intensity at 630 nm (from PI) represents to the number of dead cells in the well. Similarly to the last figure, A and B represent the two *C. jejuni* 11168 cultures.

No resuscitated VBNC *C. jejuni* 11168 in a chicken model

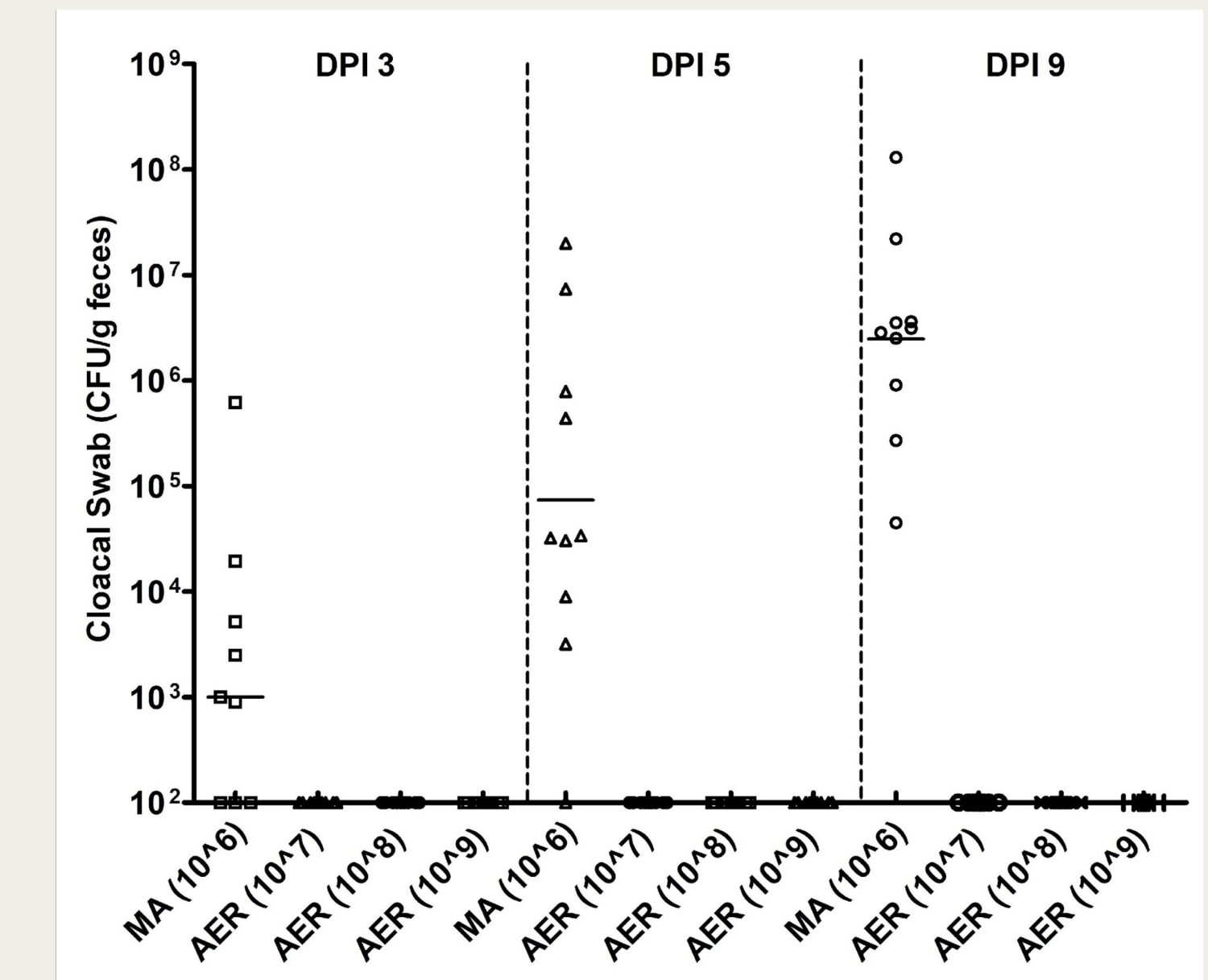


Figure 3. *In vivo* experiment of aerobically stressed *Campylobacter jejuni*. 3-day old chickens were split into four group (10 birds each). Each group received different treated *C. jejuni* 11168: group 1 chickens were inoculated with 100 µL of *C. jejuni* NCTC 11168 cultivated in microaerobic (MA) condition (85% N₂, 10% CO₂, and 5% O₂), group 2-4 chickens were inoculated with 100 µL of aerobically stressed (AER) VBNC *C. jejuni* 11168 with 10^7 , 10^8 and 10^9 cells/chicken, respectively. This measurement was calculated on Day post infection (DPI) 3, 5, and 9. The detection limit is 10² CFU/gram feces.

Conclusion

1. At Day 5, there are no colonies detected on the plates indicating that the bacteria is no longer culturable (**Fig. 1**).
2. Despite the Live/Dead Cell ratio continuing to decrease from Day 5 to Day 12, the curve not being flat indicates that there are still a decent amount of viable cells at Day 5 (**Fig. 2**).
3. When these VBNC *C. jejuni* were fed to 3-day old chicken, no resuscitated *C. jejuni* were detected in fecal samples (**Fig. 3**).

Discussion

There are a lot of studies reporting the resuscitation of VBNC bacteria under *in vitro* conditions. When these VBNC *C. jejuni* 11168 were introduced into the 3-day old chicks, they remained undetected in fecal samples, which suggested that these bacteria were not able to be resuscitated when passing through the chicken GI tract. It would be beneficial for further studies on the resuscitation of *Campylobacter* out of the VBNC state in elder chicken or other animal models, or possible by using a GI tract tissue model.

Acknowledgements

This research was supported by the University of Tennessee AgResearch. Care of chickens upheld by Wei Wang and Xinge Hu.

References:

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