

Effects of combined pH and temperature stress on *Escherichia coli* K-12 viability

Ali Hammoud

ali2009hammoud@gmail.com

Dearborn Center of Math Science and Technology <https://orcid.org/0009-0004-9149-7186>

Research Article

Keywords: Escherichia coli K-12, pH stress, temperature stress, bacterial viability, combined stressors, acid stress, two-way ANOVA

Posted Date: October 28th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-11514588/v1>

License:   This work is licensed under a Creative Commons Attribution 4.0 International License.

[Read Full License](#)

Additional Declarations: The authors declare no competing interests.

Abstract

Bacteria are frequently exposed to environmental stress, including changes in both temperature and acidity. Understanding how these factors work in unison to affect bacterial survivability is beneficial to food safety, medicine, and environmental science. This project investigates how short-term exposure to acidic and basic conditions at different temperatures affects the viability of *E. coli* K-12. It was hypothesized that cold temperatures would cause greater reduction in viability under basic pH, while warm temperatures would cause greater reduction under acidic pH. This was done by creating 5 independent trials of overnight bacterial cultures. Samples of these bacteria were inoculated and mixed with a pH 4, pH 9, or PBS buffer solution before being exposed to either a cold (4 degree celsius) or warm (37 degree celsius) environment for approximately 90 minutes. After the exposure, each treatment was serially diluted before being plated onto an LB agar petri dish and incubated at 37 degrees celsius for approximately 20 hours to determine colony forming units per milliliter (CFU/mL). A baseline control using phosphate-buffered saline without exposure to temperature stress was measured. Results showed that the acidic pH conditions were associated with lower viability to the neutral control conditions. Basic conditions produced variable effects but generally less reduction compared to the acidic stress. Temperature did not have a statistically significant effect on survival. These findings indicate that short-term acidic stress has a stronger negative effect on *E. coli* viability than temperature changes, contributing to our understanding of how environmental stressors influence bacterial survival.

Introduction

Bacterial survival may be influenced by many environmental factors, including pH and temperature, two factors that are often encountered simultaneously, such as during food processing, in wastewater and in the gut, both of which can impact bacterial survival (1). Much research has been conducted on the effect of pH and temperature on bacteria, but few studies of the influence of pH and temperature on the same strain of bacteria over a short time period in the lab. Therefore, understanding the combined effect of these stressors is essential for accurately predicting bacterial survival under complex environmental conditions.

Escherichia coli K-12 is an ideal strain to use in microbiological experimentation studies due to its known physiology and genetics, and because it is considered Biosafety Level 1 and safe for laboratory experimentation (2). This has been a model strain since the 1940s (isolated in 1922) and no longer colonizes the gut, making it safe and accessible for standard laboratory experimentation.

The impact of pH on bacterial viability has been extensively studied in *E. coli*. Having evolved to relatively neutral conditions, *E. coli* can maintain an internal pH of 7.4–7.9 in an external pH of 5.0–9.0 (3, 4). However, when the pH is lower, internal pH can drop as well, causing denaturation of proteins, membranes, DNA, and enzymes. Because of this, very acidic environments tend to be particularly harmful (5).

E. coli survives acidic conditions by employing acid resistance mechanisms which help maintain its internal pH. It is important to recognize that these are generally only effective under certain conditions and can become overwhelmed under extreme acid stress (6). *E. coli* also employs membrane related strategies to enhance acid resistance, but without adaptation these responses may not be adequate. This can lead to cell damage, and in some cases death.

The response of *E. coli* to high pH environments have not been as thoroughly explored as with low pH, but with the available studies they indicate that increased pH also causes sufficient stress. Under the stress of alkaline conditions, *E. coli* responds to it by regulating intracellular pH (4). In the long term, *E. coli* K-12 can adapt to tolerate up to pH values of 9.0-9.3, implying pH 9.18 to be towards the upper limit of the normal pH range for *E. coli* growth (4).

E. coli growth and survival is heavily influenced by temperature. It has an optimum growth temperature of 37°C, and it has been reported to grow at temperatures as high as 46.5°C (7). As the temperature changes, the stress response is triggered.

When exposed to cold conditions, responses are activated that allow *E. coli* to maintain its core functions, which can keep it alive while inhibiting growth. These are fast and, under the right conditions, can be reversible responses (8). Importantly to keep in mind, 4°C is not normally fatal to *E. coli*, but will reduce its metabolic activity and growth.

E. coli will undergo heat shock responses at higher temperatures. However, 37°C is still within its optimal range, so it would not be expected to reduce viability through thermal damage (7).

While the influence of pH and temperature individually are well-known, the combined influence of these factors are not. Hurdle technology (the combination of multiple mild preservation factors to inhibit microbial growth) helps explain this by showing that multiple stressors can control microbial growth more effectively than any single factor alone (9). This raises the question of whether combined stressors produced additive or interactive effects.

Kim et al. examined the effect of prior exposure to different pH levels and temperatures affected the ability of foodborne pathogens including *Salmonella* and *Staphylococcus*, to survive later heat treatment (1). They subjected the bacteria to a pH of 6.0, 7.4, and 9.0, and temperatures 15, 25, 35, and 45°C for 24 hours before treating them with lethal heat. They were primarily interested in the rate of death during heat treatment, rather than the survival of combined stress treatments. They found that the initial pH affected the bacteria's heat tolerance, which means that pH and temperature can have complex interactions. The influence of temperature was not as consistent, highlighting how challenging it is to predict the effects of multiple stressors from single-factor experiments.

Other studies of bacterial stress tolerance have also noted cross-protection, where tolerance to one stress can increase tolerance to another. Research has shown that in several bacterial species, including *E. coli*, the general stress response that is triggered by acid exposure can also increase the tolerance to

other stressors such as heat, oxidative damage, and salt stress (4, 6). This indicates the effects of pH and temperature stress may not always be straightforward and can vary depending on exposure conditions.

Despite extensive research on how pH and temperature individually affect *E. coli* and other bacteria, and despite the clear importance of combined stressors to real-world contexts where bacteria constantly face simultaneous changes in both pH and temperature, relatively few studies have directly measured the short-term viability of *E. coli* K-12 under simultaneous pH and temperature stress. Even fewer use an experimental design that can separate the individual effects of each variable from their combined impact. Most existing research on combined stressors focuses on long-term growth patterns, predictive modeling, or sequential exposure (exposing condition to one stressor, then after adaptation sees the effect of the second), rather than measuring viability when both stressors are applied at the same time. For example, Kim et al. exposed bacteria to pH and temperature stress sequentially rather than simultaneously (1). Similarly, cross-protection studies like those by Hamdallah et al. (4) and Li et al. (6), look at how prior exposure to one stressor affects how bacteria respond to another, meaning the stressors are still applied at different times, yet again not simultaneously. These studies help to understand how bacteria respond to changing environments, but do not illustrate the simultaneous effect of both stressors.

Additionally, predictive models such as those created by Koutsoumanis et al. for long-term growth prediction of spoilage bacteria in ground meat, primarily look at growth during storage rather than immediate stress (10). As a result, it remains unclear how pH and temperature interact under simultaneous short-term exposure conditions.

This study looks at this gap by testing how *E. coli* K-12 responds to different pH and temperature levels at the same time. The bacteria were exposed to pH 4, 7, and 9 and were kept at about 4°C or 37°C for 90 minutes. After that, viability was measured using colony-forming unit (CFU) counts. A two-way ANOVA was used to look at the effects of pH and temperature separately, as well as whether they interact. The main question was how short-term exposure to acidic and basic conditions under cold and warm temperatures affect *E. coli* K-12 viability. I hypothesized that cold temperatures would cause greater reduction in viability under basic pH, while warm temperatures would cause greater reduction under acidic pH.

Results

The viability of bacteria under all treatment conditions was expressed as log₁₀(CFU/mL) based on counts of colony-forming units (CFU) following 90 minutes exposure to each pH and temperature treatment. Because logarithmic transformation is not defined for zero, the zero-colony counts in the pH 4-cold condition (trials 1, 2, and 3) were assigned the detection limit of 3.70 log₁₀(CFU/mL). Measurements taken from unstressed overnight bacterial cultures in phosphate buffered saline (PBS) immediately before treatments demonstrated a mean log₁₀(CFU/mL) of 6.56 (range 4.65–9.06 over five

trials) confirming that all cultures had viable bacteria at the beginning of each experiment, and providing a reference point for interpreting the level of treatment-induced reductions in viability.

Table 1 presents the mean log₁₀(CFU/mL) values for each of the six treatment conditions. Across both temperatures, the PBS (pH 7, neutral control) condition often yielded the highest viability, with a mean of 7.31 at cold temperatures and 7.67 at warm temperatures. Acidic conditions produced the lowest recorded viability in both temperature groups, having means of 4.61 at cold and 5.33 at warm temperatures. The basic condition falls between these two extremes with means of 6.50 at cold and 8.03 at warm temperatures. Notably, the pH 9-warm condition produced a mean that exceeded that of the PBS-warm condition, although a driving factor was the high variability in trials 4 and 5, with log₁₀ values of 9.69 and 9.88.

Table 1
Mean log₁₀(CFU/mL) across
pH and temperature
conditions.

Condition	Cold	Warm
pH 4	4.61	5.33
PBS	7.31	7.67
pH 9	6.50	8.03

Figure 1 shows the mean values across all 6 conditions as a grouped bar graph. Statistical significance is presented in Table 2.

Table 2
Two-way ANOVA results for effect of pH and temperature on log₁₀(CFU/mL).

Source of Variation	SS	df	MS	F	P-value	Statistically significant?	Effect size (η ²)
pH	38.76	2	19.38	7.09	0.004	Yes	0.35
Temperature	5.69	1	5.69	2.08	0.16	No	0.05
pH x Temperature	1.8	2	0.9	0.33	0.72	No	0.02
Residual	65.61	24	2.73				

Table 2 lists the two-way ANOVA test that was applied to the log₁₀(CFU/mL) data to assess the effect of the interaction between pH (4.01, 7.0, and 9.18) and temperature (cold and warm) on bacterial viability. The statistical test was chosen to determine the main and interaction effects of two independent variables upon the bacteria's viability. Table 2 shows the ANOVA results, and its effect sizes.

The main effect of pH on bacterial viability was statistically significant ($p = 0.004$), with an eta-squared of 0.35. Thus, pH accounted for 35% of the total variance in $\log_{10}(\text{CFU/mL})$, demonstrating that it had a large effect size. The means in Table 1 reveal that pH 4 was the least viable in both groups. Under the cold conditions, pH 9 was lower than the PBS-cold mean but higher under the warm conditions due to the high variability in trials 4 and 5.

Temperature did not have a significant effect on viability ($p = 0.16$, $\eta^2 = 0.05$), explaining only 5% of the variance. Under PBS, warm showed slight increase in viability compared to cold which is consistent with the assumption that the optimum temperature for *E. coli* K-12 growth is around 37°C and 4°C is suppressive. For pH 4, viability was slightly higher in warm incubation than in cold, suggesting that warm incubation did not amplify the suppressive effect of acid stress as the hypothesis predicted. However, this difference was not significant.

The interaction between pH and temperature was not significant ($p = 0.72$), and had a small effect size ($\eta^2 = 0.02$). This implies that the effect of pH on bacterial viability was not significantly different at different temperature levels and the effect of temperature was not significantly different between pH levels. Therefore, the original hypothesis that cold incubation would interact with basic pH to produce greater reduction in bacterial viability, and warm incubation would interact with acidic pH to produce greater reduction in bacterial viability was not supported.

There was considerable variability between trials. For instance, in the PBS cold group, the $\log_{10}(\text{CFU/mL})$ values for each trial ranged from 5.23 to 9.06, a difference of nearly four $\log_{10}$ units. A similar variation was observed in other conditions and represents the inherent biological variability of overnight bacterial cultures, combined with procedural variability (pipetting, diluting, and agar plate differences). Despite this variability, pH 4 produced the lowest viability in 9 of the 10 trial and temperature combinations, while the order of PBS and pH 9 varied between trials.

Discussion

The experiment has addressed the question, “How do short-term acidic and basic conditions across cold and warm temperature regimes affect *E. coli* K-12 viability?” The results from the two-way ANOVA show that the main effect of pH accounted for 35% of the variance in $\log_{10}(\text{CFU/mL})$ ($p = 0.004$, $\eta^2 = 0.35$), whereas temperature accounted for only 5% and was not significant ($p = 0.16$, $\eta^2 = 0.05$). The interaction between pH and temperature ($p = 0.72$, $\eta^2 = 0.02$) was also not significant, which means that the effect of one did not depend on the other. Only pH significantly affected the viability of *E. coli* K-12, and the two stressors did not interact.

The effect of pH on viability is supported by the literature review. Brameyer et al. found that at low external pH (pH 4.4), the internal pH will be reduced to around pH 6.0, resulting in damage to proteins, membranes, and DNA (5). Since the pH 4 used in the current study is more extreme than pH 4.4, it would be expected that the damage to the cells would be equivalent or greater, as indicated by the fact that the pH 4 condition had the lowest mean viability (4.61 at cold, 5.33 at warm) of the conditions studied. When

viable colonies were found, viability at pH 4 was lower than the PBS control and pH 9 at both cold and warm temperatures. Li et al. outlined some of the mechanisms of acid resistance in *E. coli* but extreme acid can overwhelm these (6). In this case, pH 4 was severe enough to cause a loss of viability in spite of intrinsic resistance.

However, the pH 9 conditions were less extreme and more unpredictable. Hamdallah et al. showed that *E. coli* K-12 can grow at pH 9.0-9.3, if there is sustained selective pressure (meaning repeated or prolonged exposure to the new environment selects for more tolerant cells) (4). This would suggest that pH 9 is the upper limit of the growth range for *E. coli*, and therefore has limited growth. pH 9 was therefore not as inhibitory to viability as pH 4, as it falls within the upper end of the normal growth range where *E. coli* can still carry out many of its natural functions. The average viability of pH 9 in the warm group (8.03) was greater than the control (7.67). This was likely due to the very high values (9.69 and 9.88 log₁₀) in trials 4 and 5. Given that Hamdallah et al. showed pH 9 is in the growth range of *E. coli* K-12, the slightly higher viability observed at 37°C suggests pH 9 was not enough to cause a reduction in viability below neutral pH (4). However because of the high variability in these trials, this is a rather tentative conclusion.

The lack of a significant temperature effect is also in line with previous research. *E. coli* is typically grown at 37°C but at 4°C the cells' metabolic activity is slowed without death (7, 8). A 90-minute cold exposure would therefore not be expected to reduce viability, which is reflected in the small effect size ($\eta^2 = 0.05$).

The lack of significant interaction effect supports the notion that under short-term simultaneous exposure to pH and temperature, the effects of pH and temperature were not interactive. This is unlike cross-protective studies that reported an effect of one stressor on tolerance to another and usually involved sequential exposure of stressors (4, 6). This might be because pH and temperature affect different aspects of cellular function (pH affecting structural integrity and temperature affecting metabolic reaction rates) and the effect was additive.

The baseline mean (6.56 log₁₀(CFU/mL)) confirms viable bacteria were present at the start of each trial. Because each treatment tube contained a 1:10 dilution of the culture, treated values are about 1 log₁₀ unit lower than they would be on the same basis as the baseline. After accounting for this dilution, mean viability at pH 4 (5.61 cold, 6.33 warm) remained below baseline, while PBS and pH 9 means were at or above baseline, likely reflecting the high variability between trials.

There was variation between trials. For instance, the PBS-cold group varied from 5.23–9.06 log₁₀(CFU/mL) (a difference of almost four log₁₀ units). This is likely due to pipetting inaccuracies, different dilutions, and plating issues (17 of 27 colony counts fell outside the optimal 30–300 range, most of them below 30). While this obscured the effects of pH and temperature, the ANOVA was able to detect the pH effect, and gives confidence that the trends observed reflect real biological effects.

Zero colony counts in the cold pH 4 treatments in trials 1–3 should be viewed with caution. Because log₁₀(0) is undefined, these values were assigned the detection limit (3.70 log₁₀ CFU/mL). Since the

true values could be lower, this approach is conservative. The three zero-colony samples were plated on the same plate so it is not clear if the zero counts were due to treatment or plate effects. However, the other data still point to the lowest viability being at pH 4 and because these values were set to the detection limit, the observed pH effect is likely conservative, underestimating the true impact of acid stress.

Despite the plate issue and considerable variability, the conclusions remain valid. pH 4 had the lowest viability in 9 of the 10 trial and temperature combinations, although the ranking of PBS and pH 9 was inconsistent. The ANOVA was significant for pH despite the variability, so the biological effect was strong enough to be detected, despite the small sample size and variability.

These results indicated that the main factor that affected survival of bacteria under the tested conditions was pH, with the greatest loss of survival at an acidic pH (pH 4), regardless of temperature. Temperature did not have a significant effect. The effect of pH and temperature were not dependent on one another and no interactions were found. Therefore, the original hypothesis that certain combinations of pH and temperature would have a larger effect was not supported.

The findings need to be interpreted with caution. The small sample size ($n = 5$) may have decreased the statistical power and the variability of the samples' means were affected by the variability of the samples themselves. However, the ANOVA was significant for pH, confirming the main result.

The other limitation is that zero colony counts were assigned the detection limit ($3.70 \log_{10}$ CFU/mL) rather than their true value, which could be lower if the bacteria were completely eliminated. This may mean that the impact of pH 4, the most influential factor, may be an underestimate of the impact of acid stress on *E. coli* survival. Future research could use a statistical test designed for count data to handle zero values directly. The consistently low viability at pH 4 across trials supports the conclusion that the acid effect is real and not due to random error.

In addition to the above study restriction, the lack of agitation of the tubes during exposure to stress for 90 minutes might have resulted in a non-uniform distribution of bacteria. The tubes were inverted prior to exposure to the temperature condition, but the absence of mixing during the exposure period is acknowledged as a potential source of variability.

In addition to the restrictions mentioned above, the pH of the buffer solutions was taken from the product labels rather than measured with a pH meter, so the actual pH may have differed slightly. There are also considerations for material and environment. The strain of *E. coli* used, K-12, is a lab strain and may not give the same information about stress response as wild-type *E. coli*. Also, the independence observed here is likely to hold for the pH and temperature ranges examined (pH 4.01–9.18 and 4–37°C) where the pH and temperature stresses affect different parts of the cell. At a different scale, where the effects of pH on cell structure and temperature on proteins converge, this independence may not hold, limiting projected interpretations. This constrains the interpretation of the results to short-term impacts and may have led to less precise measurements.

These findings contribute to the understanding of the interaction of biological stressors under short-term co-exposure. The non-interaction between stressors observed here implies that stressors can have different effects on different parts of the cell (pH having an effect on cell structure and temperature affecting the rate of metabolic activities), and no interaction between the stressors was detected. This result is contrary to what would be expected based on the outcome of cross-protection experiments where the effect of one stressor on the response to a second has been reported (4, 6). The absence of this effect with simultaneous stressors implies that the findings of sequential stressors should not be applied to simultaneous stressors.

For those performing multi-factor stress experiments, or modelling bacterial survival to multiple stressors, this study found no evidence that pH and temperature interact in the ranges investigated, although a larger sample size would be needed to rule out a small interaction. This independence is likely to be conditional on the mechanisms of the stressors being independent. This may not be the case under more extreme conditions, such as 45°C and higher, where the effects of acid damage to proteins and heat shock overlap, and so this is an important area for future investigation.

In addition to increasing the range of pH and temperature to test whether independence holds under more extreme conditions, future research could also vary the exposure time to determine how rapidly the bacteria respond and adapt to these stressors, increase the number of trials to increase statistical power, and measure not only colony formation, but other measures such as metabolic activity or expression of stress response genes to investigate the full cell-level response of bacteria exposed to multiple stressors; providing insight into not only how bacteria respond to combined stressors, but also why they respond the way they do at the cellular level.

Overall, this study highlights that pH is the most important factor affecting the survival of *E. coli* K-12 under short-term combined stress, while temperature did not have a significant effect. Although the findings are limited due to the sample size and variability, and the duration of exposure, the trends observed and statistical significance offer a strong foundation for future studies.

Materials and Methods

A quantitative experimental design was employed to study the effect of short-term exposure to combined pH and temperature stress on the survival of *E. coli* K-12. A two-factor design was selected since it allows for the independent assessment of the effect of each variable, as well as the interaction of variables to understand the response of bacteria under stress conditions, which cannot be achieved with a single-factor design (1). The experimental factors were pH (3 levels: 4.01, 7.0, and 9.18) and temperature (2 levels: ~4°C and ~37°C), resulting in 6 conditions. The response variable, bacterial viability, was measured in colony forming units per milliliter (CFU/mL) and log₁₀ transformed for statistical analysis. This was replicated 5 times for each condition.

Bacterial Strain and Culture Preparation

The bacterial strain used for this study was *Escherichia coli* K-12, which is commonly used in microbiology research, has well-defined genetics and is a biosafety level 1 as a result of its non-pathogenic properties (2). For each of the five independent trials, an overnight culture was prepared by inoculating 100 microliters of *E. coli* K-12 stock (which had been stored at 4°C according to the manufacturer's storage instructions) into 2 mL of Luria-Bertani (LB) broth. The overnight cultures were then grown for approximately 24 hours at 37°C, the optimal growth temperature for *E. coli* K-12 (7). Each trial was conducted with a new culture, rather than dividing a single culture, to ensure that each trial was a biological replicate. Overnight culturing was used to ensure uniform growth.

Treatment Solutions

The treatment solutions were an acidic buffer at about pH 4.01, a neutral phosphate-buffered saline (PBS) solution (pH 7), and an alkaline buffer at about pH 9.18. The pH 4.01 and 9.18 solutions were made by dissolving pH buffer powder packets in distilled water as instructed by the manufacturer. These pH values were selected because they represent biologically relevant stressful conditions outside of the normal external growth pH of *E. coli* (around pH 5.0 to 9.0) (3, 4). A pH of 4.01 is below the lower growth limit, and so it provides extreme acid stress to the cells, whereas pH 9.18 is at the upper limit of the growth range, so this allows a comparison of the effects of these two extremes. The PBS solution acted as a control that introduced no pH stress to the bacteria, so that any loss of viability could be attributed to the treatment. The pH was determined from the labels rather than being measured with a pH meter, creating a potential source of error that is discussed in the Discussion.

Experimental Exposure Procedure

In each experiment, six treatment tubes were set up, corresponding to the six combinations of pH and temperature treatments. To each tube, 900 microliters of the treatment solution and 100 microliters of the overnight *E. coli* K-12 culture were added. A calibrated micropipette was used and sterile tips were changed between each transfer to ensure no contamination. This 9:1 ratio ensured the pH of the buffer remained the final pH in the solution, rather than being changed by the culture medium. The six tubes were prepared together to ensure the pH conditions were consistent for each trial. Prior to temperature exposure, each tube was mixed by gentle inversion 10 times to ensure uniform distribution of bacteria within the treatment solution.

Following preparation, three of the six tubes were placed in a refrigerator maintained at approximately 4°C. This was confirmed by a thermometer every 30 minutes and was classified as the cold environment. The other three tubes were placed in a water bath at 37 degrees before being placed in a conventional oven in order to maintain that temperature. Again, this was confirmed with a thermometer every 30 minutes or so. 37°C is the optimal growth temperature for *E. coli*, whereas 4°C is a temperature in which the metabolic rate is reduced (1, 8). This allowed for a direct comparison between active and metabolically suppressed cellular states. All tubes were exposed for 90 minutes, a duration selected to

capture acute short-term stress responses rather than long term adaptation, which is consistent with the acute challenge duration used in previous research on acid stress in *E. coli* O157:H7 (11).

Serial Dilution and Plating

After the 90-minute incubation period, a serial dilution of the sample in each tube was prepared using PBS immediately. For this, 100 microliters of the sample were added to 900 microliters of PBS to obtain a 1:10 dilution. To prevent cross-contamination, new sterile pipette tips were used for each step of the dilution process, just as for the exposure process. This was done to create serial dilutions of 10^{-2} , 10^{-4} , and 10^{-6} . The serial dilution was necessary to get a countable number of colonies. The recommended range for accurate CFU counting is 30–300 colonies per plate. Lower counts include more statistical variability and higher counts include the possibility of overlapping colonies and can result in reduced accuracy (12).

Twelve LB agar plates were used across all 5 trials. Each plate was sectioned into three parts, with a marker on the bottom of each of these sections corresponding to one of the treatments. For each section, 20 microliters from each dilution (10^{-2} , 10^{-4} , and 10^{-6}) was placed on the agar. Due to the low volume, the sample was not spread with a specific instrument, but simply left to be absorbed into the agar. This drop plating was done rather than spread plating; which despite being an accepted technique, could have led to uneven colony growth. The dilution that produced a count between the optimal 30–300 colony range was used for calculating CFU from the three dilutions plated for each condition. If none of the dilutions were in this range, the nearest dilution was chosen.

CFU/mL was calculated as: $\text{CFU/mL} = (\text{number of colonies} \times \text{dilution factor}) / \text{volume plated (mL)}$. CFU/mL was then log₁₀ transformed to reduce skewness and variance, and improve the validity/accuracy of the statistical analysis (12). Because log(0) is undefined, plates with zero colonies were assigned the detection limit of $3.70 \log_{10}(\text{CFU/mL})$, equal to one colony at the 10^{-2} dilution, so that all samples could be included in the analysis. CFU/mL values for treated samples represent the concentration in the treatment tube, which contained a 1:10 dilution of the overnight culture.

Baseline Measurement

A baseline measurement was taken for each trial by performing the same serial dilution and plating procedure on a sample of the overnight culture prior to treatment exposure. These baseline CFU/mL values were also log₁₀ transformed and used to confirm that each overnight culture contained viable bacteria at the start of each trial and to provide a reference point for interpreting the magnitude of any treatment-related reductions in viability. The baseline samples were not included in the two-way ANOVA, as their role was to establish a reference rather than for actual comparison.

Incubation and Colony Counting

All plates were incubated at 37°C for approximately 20 hours following plating, after which visible colonies were counted manually. Plates were incubated at this temperature as this is the standard incubation temperature for growing *E. coli* colonies on LB agar plates (7). Colony counts from the selected dilution were used in the CFU/mL calculations as described above.

Statistical Analysis

A two-way analysis of variance (ANOVA) was used to evaluate the effects of pH and temperature on log₁₀(CFU/mL) across all trials. A two-way ANOVA was chosen because the experimental design involves two independent factors which allows for a test of both individual and combined effects, which is directly linked to the research question in terms of combined effect of the stressors. Effect sizes (eta-squared, η^2) were calculated for each factor, and the interaction. In this way it helps demonstrate the contribution of each variable to the bacterial viability.

Declarations

Conflicts of Interest

The author declares they have no conflicts of interest.

Ethics

This study did not involve human participants or vertebrate animals. All work used *E. coli* K-12, a Biosafety level 1 strain.

Acknowledgements

This research received no external funding.

References

1. Kim C et al (2019) Influence of Prior pH and Thermal Stresses on Thermal Tolerance of Foodborne Pathogens. *Food Sci Nutr* 7(6):2033–2042. <https://doi.org/10.1002/fsn3.1034>
2. Browning DF et al (2023) Laboratory Strains of *Escherichia coli* K-12: Things Are Seldom What They Seem. 9(2):mgen000922. *Microbial Genomics* <https://doi.org/10.1099/mgen.0.000922>
3. Harden MM et al (2015) Acid-Adapted Strains of *Escherichia coli* K-12 Obtained by Experimental Evolution. *Appl Environ Microbiol* 81(6):1932–1941. <https://doi.org/10.1128/AEM.03494-14>
4. Hamdallah I et al (2018) Experimental Evolution of *Escherichia coli* K-12 at High pH and with RpoS Induction. *Appl Environ Microbiol*, 84, 15, <https://pmc.ncbi.nlm.nih.gov/articles/PMC6052260/>

5. Brameyer S et al (2022) Division of Labor and Collective Functionality in *Escherichia coli* under Acid Stress. 5:327. *Communications Biology* <https://doi.org/10.1038/s42003-022-03281-4>
6. Li Z et al (2024) Response of *Escherichia coli* to Acid Stress: Mechanisms and Applications. *Narrative Rev Microorganisms* 12(9):1774. <https://doi.org/10.3390/microorganisms12091774>
7. Ezemaduka AN et al (2014) A Small Heat Shock Protein Enables *Escherichia coli* to Grow at a Lethal Temperature of 50°C Conceivably by Maintaining Cell Envelope Integrity. *J Bacteriol* 196(11):2004–2011. <https://doi.org/10.1128/jb.01473-14>
8. Ivancic MM et al (2013) Cold Shock CspA and CspB Protein Production during Periodic Temperature Cycling in *Escherichia coli*. *BMC Res Notes* 6:248. <https://doi.org/10.1186/1756-0500-6-248>
9. Leistner L (2000) Basic Aspects of Food Preservation by Hurdle Technology. *Int J Food Microbiol* 55:1–3. [https://doi.org/10.1016/S0168-1605\(00\)00161-6](https://doi.org/10.1016/S0168-1605(00)00161-6)
10. Koutsoumanis K et al (2006) Development of a Microbial Model for the Combined Effect of Temperature and pH on Spoilage of Ground Meat and Validation under Dynamic Temperature Conditions. *Appl Environ Microbiol* 72(1):124–134. <https://doi.org/10.1128/AEM.72.1.124-134.2006>
11. Jordan KN et al (1999) Survival of Low-pH Stress by *Escherichia coli* O157:H7: Correlation between Alterations in the Cell Envelope and Increased Acid Tolerance. *Appl Environ Microbiol* 65(7):3048–3055. <https://doi.org/10.1128/aem.65.7.3048-3055.1999>
12. Sutton S (2011) Accuracy of Plate Counts. *J Validation Technol* 17(3):42–46

Figures

Mean log₁₀(CFU/mL) Across pH and Temperature Conditions

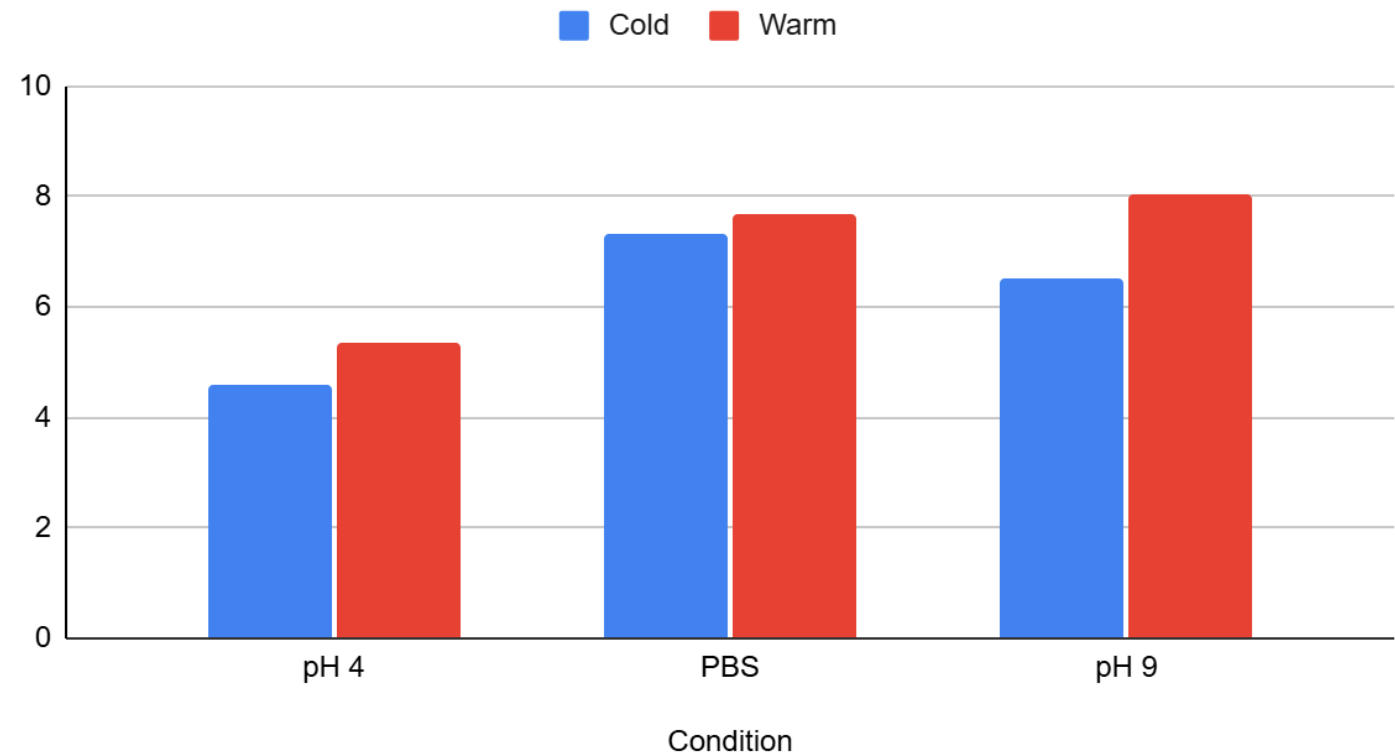


Figure 1

Effect of combined pH and temperature stress on *E. coli* K-12 viability.

Mean log₁₀(CFU/mL) of *E. coli* K-12 after 90-minute exposure to pH 4.01, PBS (pH 7), or pH 9.18 at ~4 °C (cold, blue bars) or ~37 °C (warm, red bars). Viability was measured by drop plating serial dilutions on LB agar. Zero-colony counts were set to the detection limit (3.70 log₁₀ CFU/mL). Bars show means of n = 5 independent trials. Two-way ANOVA: pH, p = 0.004; temperature, p = 0.16; pH × temperature, p = 0.72.