Internal Assessment

Biology Higher Level

To what extent do the food preservatives Acetic Acid, Sodium Benzoate, and Citric Acid at the concentrations 0.5g/100mL and 1g/100mL affect the growth of *E.coli* and *L.acidophilus* as measured by the Radius of the Zone of Inhibition (mm)?

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1. Introduction

I have a habit of consuming large quantities of instant noodles as they are my comfort food. My interest was piqued by my Biology teacher's observation–instant noodles have a remarkably long shelf life. Instant noodles last 2-3 years mainly due to preservatives that inhibit microbial growth (Farboud). The most common are sodium benzoate and benzoic acid, which prevent mold and yeast growth (Burana-Osot). However, excess consumption of these preservatives has led to health consequences, explaining why government agencies have regulated preservative use depending on food products. The preservatives most relevant to instant noodles/processed foods are summarized in Table 1.

Table 1: Selected preservatives and their common sources, legal concentration limits, and reported health concerns.

Preservative	**Common sources**	**Legal limit in foods**	**Health concerns upon excess exposure**
Sodium benzoate	Instant noodles, soft drinks (EFSA)	≤0.1% (FDA); ≤0.25% (EFSA)	Oxidative stress; hormonal disruption; benzene formation upon heating (McCulloch)
Benzoic acid	Instant noodles, processed foods (EFSA)	≤0.1% (FDA); ≤0.25% (EFSA)	Allergic reactions; metabolic issues (Issa and Mohammed)
Citric acid	Soft drinks, jams, processed meats (Książek)	GMP[1]	Tooth erosion; gut irritation (FDA)
Acetic acid	Vinegar, condiments (EFSA)	GMP	Gastrointestinal discomfort (FDA)

I wanted to investigate the impact of these preservatives on gut health, as these effects are less understood. The gut microbiome is the community of bacteria, fungi and viruses essential for digestion, vitamin synthesis, and immune regulation (Hul et al.). The zone of inhibition (ZI)

[1] GMP stands for"Good Manufacturing Practice," meaning that government agencies impose no strict percentage limit but regulate the preservative through safe use.

refers to the area around an antimicrobial agent applied on an agar plate where bacteria fail to grow due to the inhibitory effect of the agent (Bhattacharjee). I will test the ZI of preservatives on two representative gut bacteria: *Escherichia coli*, a gram-negative, rod-shaped bacterium found especially in the lower intestine, providing vitamins K and B12, and *Lactobacillus acidophilus*, a gram-positive bacterium that produces lactic acid to lower gut pH and inhibit pathogens (Celebi et al.).

Previous literature on this topic is limited and centers around sodium benzoate inhibiting *E.coli*. A study regarding microbial growth on sweet potato puree showed that combinations of sodium benzoate and citric acid led to significant reductions of *E.coli* counts by 4 log units (Chen and Zhong). Another study regarding the antibacterial activity of sodium benzoate against *E.coli* found it effective in inhibiting growth under acidic conditions (Zaghloul et al.). This investigation widens the scope of both the preservatives and bacteria tested.

2. Preliminary Data

Pretrials at 0.1% for all four preservatives produced no inhibition of *E.coli* or *L.acidophilus*, so concentrations were raised to 0.5% and 1.0%; this simulates overuse or accidental excesses encountered during food processing, effectively evaluating food safety margins. It also explores possible adverse effects on beneficial microbes like *L.acidophilus*–and the health consequences of processed foods overconsumption. Due to the poor solubility and saturation limit of benzoic acid at 0.3%, it could not be directly compared to the other preservatives and was excluded from the investigation (FAO).

The methodology utilized in this experiment is the Disk Diffusion Assay, where small, sterile paper discs that are dipped into the food preservative solution are placed onto the surface of the agar plate that has been uniformly inoculated with either bacterium (Mounyr Balouiri et al.).

Table 2: Comparison of the benefits and limitations of the Disk Diffusion Assay, including potential mitigation methods.

Benefits	Limitations
High throughput–many agents tested simultaneously per plate.	Diffusion differences between preservatives; ZI influenced by molecular size and solubility.

Versatile–can test wide range of bacteria	Preparation variability: agar thickness, inoculum density, disc loading volume.
Standardized and reproducible–accepted in susceptibility testing	Species difference: *E.coli* grows fast and is pH sensitive, *L.acidophilus* grows slower and is acid-tolerant.
Rapid result production within 48 hours	Contamination risk from handling/air exposure. Can be mitigated through following proper aseptic technique.

3. Experimental Design

3.1 Hypothesis

H_1: Increasing concentration of sodium benzoate, benzoic acid, citric acid and acetic acid will produce a larger ZI in *E.coli.*

H_2: Increasing concentration of the preservatives will also produce a larger ZI in *L.acidophilus*.

3.2 Independent Variables (Mass of solution/volume of water)

0.5g/100mL and 1.0g/100mL solutions for Acetic acid, Citric acid, and Sodium benzoate.

3.3 Dependent Variable

Mean ZI radius (mm) of *E.coli* and *L.acidophilus* inhibition after 48 hours in a 25℃ incubator.

3.4 Control Variables

Table 3: Summary of controlled variables and the method through which they were controlled.

Variable	Method of Control
Incubation time	48 hours of incubation for comparable bacterial growth/preservative inhibition.
Incubation temperature	25℃ in the same incubator; uniform bacterial metabolism/preservative activity.
Preservative volume applied	Standardized by wicking excess on sterile filter paper.
Bacterial strain and concentration	Same strains of *E.coli* and *L.acidophilus* were used; spread uniformly on agar plates.
Type of growth media	Same agar medium and formula for all plates; ensures consistent nutrient availability for bacteria.
Preservative solution	Distilled water was used; same dissolution process and

preparation	equilibration temperature (20℃).
Incubation conditions	Same incubator was used for all plates; lack of light and humidity prevents external influences on bacterial growth.
Negative control	Plates with only *E.coli* and *L.acidophilus* grown to monitor normal growth.
Repeat trials	15 data points per condition for statistical validity–3 agar plates each with 5 paper discs.

3.5 Safety and Ethics

Table 4: Common laboratory risks and methods of risk management.

Risk	Method of Risk Management
Handling teaching microorganisms	Use Aseptic Technique; keep lids ajar only briefly; label and tape plates with relevant information; incubate at ≤25-30℃; disinfect benches before and after; wash hands; no food/drink (CLEAPSS).
Aerosols and contamination when opening plates	Work next to a Bunsen burner to use the up-draught; open plates at an angle away from the face; keep doors and windows closed to avoid draughts (CLEAPSS).
Flame-sterilizing forceps and Bunsen burner burns	Tie hair back; clear bench of any flammable materials; set safety flame when not conducting the procedure; let forceps cool before use; wear goggles and a lab coat (CLEAPSS).
Hotplate usage for warm water dissolution of Benzoic acid	Use the lowest effective temperature; handle hot vessels with tongs; keep cords and liquids away from edges; cool solutions before pouring (CLEAPSS).
Mis-labelling and cross-contamination between bacterial strains	Label each plate with organism, date, plate #, student initials; segregate plates by organism; only open one plate at a time (CLEAPSS).

3.6 Method

1. Prepare 800mL nutrient agar; pour aseptically to ~5mm in plate; allow to set.
2. Prepare 100mL of 0.5g/100mL Acetic acid solution.
    a. Measure 0.500g of Acetic acid powder with a balance (±0.005g); pour into 100mL beaker filled with 100mL distilled water at 20℃.
    b. Stir with a glass stirring rod until powder is completely dissolved.
    c. Seal beaker with laboratory film; label solution and concentration on film with marker.
    d. Repeat step a-d with varying preservatives and concentrations; 6 solutions prepared in total.
3. Disk Diffusion Assay loading:
    a. Lay sterile filter papers in the aseptic zone.
    b. With sterile forceps, pick up a 6mm paper disc; dip 3s in preservative solution, drain 1s above solution, blot 5s/face on filter paper.
    c. Place on *E.coli* plate. Open the lid against the face, place the disc on the top position, and immediately close the lid. Place 5 discs evenly on each plate.
    d. Repeat for the remaining discs; 3 plates per solution yields 15 points total. Change filter paper every ~10 discs. Switch to *L.acidophilus* plates after completing all *E.coli* plates.
    e. Control: one plate per bacterium without discs; all other conditions identical.
    f. Tape and label dishes with markers.
4. Incubate inverted at 25℃ for 48h; dry interior and unlit, plates spaced so lids do not touch.
5. Photograph each plate against a lit surface after 48h; measure each disc's ZI radius (mm, ±0.05mm) using a caliper; take 3 radii ~120° apart; follow layout in §4.1.

4. Results

4.1 Qualitative Data

Images of petri dishes that have *E.coli* or *L.acidophilus* spread and paper discs dipped in certain IV solutions after 48 hours of incubation in 25℃:



Fig 1. 0.5g/100mL Acetic acid + *L.acidophilus* Dish A

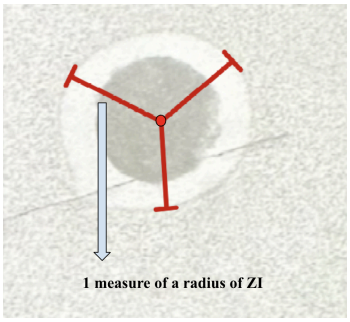


Fig 2. the ZI of *L.acidophilus* can be clearly observed and measured. Each combination of concentration and bacterium has 3 dishes (A, B, C) and 5 paper plates (1-5 determined clockwise when the label is on the bottom; 5 is always the plate in the center).

3 measurements of radii of the ZI are taken for each point, then averaged to produce the final ZI value of the plate.

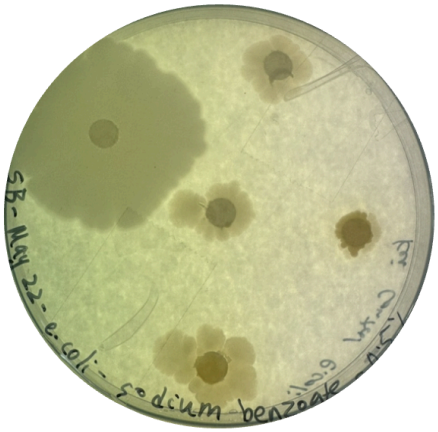


Fig 3. 0.5g/100mL Sodium benzoate + *E.coli* Dish B

Fig 4. Several petri dishes were contaminated, all surrounding the paper plates.

All 3 measurements surrounding a paper plate will be excluded if that paper plate has been contaminated.

4.2 Raw Data

Table 5: Raw data of *E.coli* inhibition with various food preservatives[2].

Independent variable	**ZI in mm (±0.05mm) after 48 hours in an incubator at 25℃**														
	Dish A					**Dish B**					**Dish C**				
	1	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
0.5g/100mL Acetic acid	7.2	6.3	7.4	6.5	0	7.3	6.5	0	0	0	6.7	0	0	0	0

[2] Contaminated points are not specifically highlighted as none of those points produced inhibition, hence not impacting the results.

1.0g/100mL Acetic acid	6.5	6.20	0	0	0	0	0	0	0	0	6.6	0	0	0	0
0.5g/100mL Citric acid	6.1	0	0	0	0	6.3	0	0	0	0	0	0	0	0	0
1.0g/100mL Citric acid	0	0	6.6	0	0	0	0	0	0	0	0	0	0	6.2	0
0.5g/100ml Sodium benzoate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.0g/100mL Sodium benzoate	0	0	0	0	0	0	0	5.8	0	5.7	5.7	0	5.9	0	0

* Radius of each ZI is the mean calculated from the three measurements as presented in §4.1

Table 6: Raw data of *L.acidophilus* inhibition with various food preservatives.

Independent variable	**ZI in mm (±0.05mm) after 48 hours in an incubator at 25℃**														
	Dish A					**Dish B**					**Dish C**				
	1	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
0.5g/100mL Acetic acid	6.8	6.1	8.1	7.5	5.8	9.3	7.5	9.1	6.6	5.8	7.7	8.1	10.0	9.5	7.8
1.0g/100mL Acetic acid	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.5g/100mL Citric acid	0	0	0	0	0	7.2	7.2	0	0	0	6.3	7.3	0	0	0
1.0g/100mL Citric acid	0	0	0	0	0	5.4	5.3	5.3	0	0	0	0	0	0	0
0.5g/100ml Sodium benzoate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.0g/100mL Sodium benzoate	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

4.3 Processed Data

Average radius of each independent variable is calculated from the 15 data points presented, excluding contaminated plates. Standard deviation (SD) is calculated based on all valid data for each independent variable.

Table 7: Processed data of *E.coli* inhibition, including no-effect data points[3].

[3] No-effect data points refer to plates that were not contaminated but did not demonstrate inhibition

Independent variable	Radius of ZI in mm (±0.05mm) after 48 hours in an incubator at 25℃	
	Average	SD
0.5g/100mL Acetic acid	3.2	3.5
1.0g/100mL Acetic acid	1.3	2.7
0.5g/100mL Citric acid	0.8	2.2
1.0/100mL Citric acid	0.8	2.3
0.5g/100mL Sodium benzoate	0	0
1.0g/100mL Sodium benzoate	1.5	2.6

Table 8: Processed data of *E.coli* inhibition only considering points demonstrating inhibition[4].

Independent variable	Radius of ZI in mm (±0.05mm) after 48 hours in an incubator at 25℃	
	Average	SD
0.5g/100mL Acetic acid	6.9	0.5
1.0g/100mL Acetic acid	6.4	0.2
0.5g/100mL Citric acid	6.2	0.1
1.0/100mL Citric acid	6.4	0.3
0.5g/100mL Sodium benzoate	0	0
1.0g/100mL Sodium benzoate	5.8	0.1

Table 9: Processed data of *L.acidophilus* inhibition, including no-effect data points.

Independent variable	Radius of ZI in mm (±0.05mm) after 48 hours in an incubator at 25℃	
	Average	SD
0.5g/100mL Acetic acid	7.7	1.3
1.0g/100mL Acetic acid	0	0
0.5g/100mL Citric acid	1.9	3.2
1.0/100mL Citric acid	0.9	2.2

[4] I report both the “all-discs” mean to analyze the frequency of inhibition and the “effect-only” mean to estimate the magnitude when inhibition occurs.

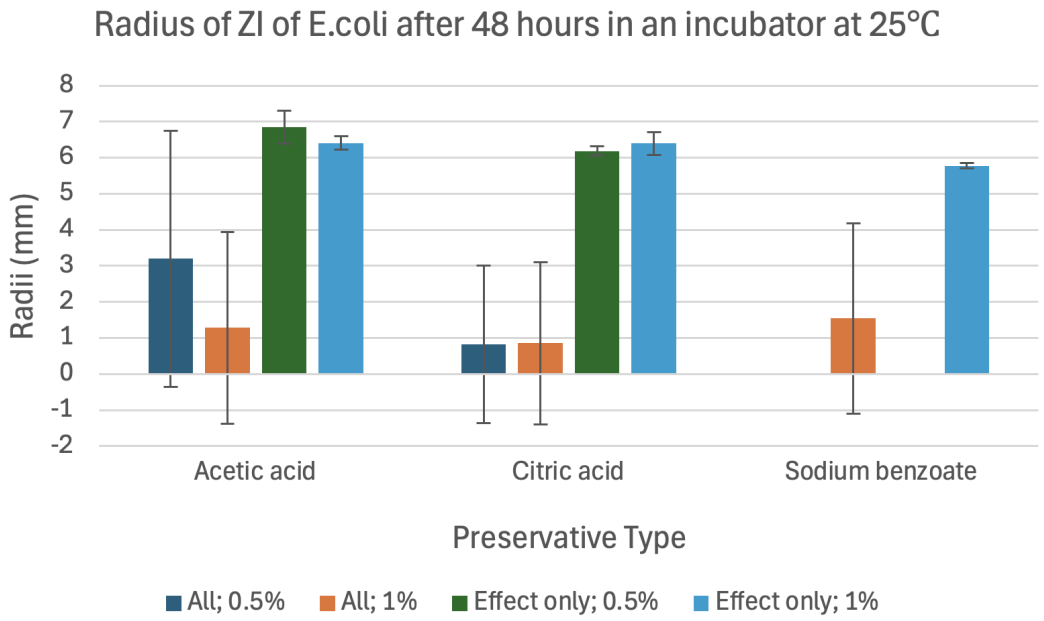
0.5g/100mL Sodium benzoate	0	0
1.0g/100mL Sodium benzoate	0	0

Table 10: Processed data of *L.acidophilus* inhibition only considering points demonstrating inhibition.

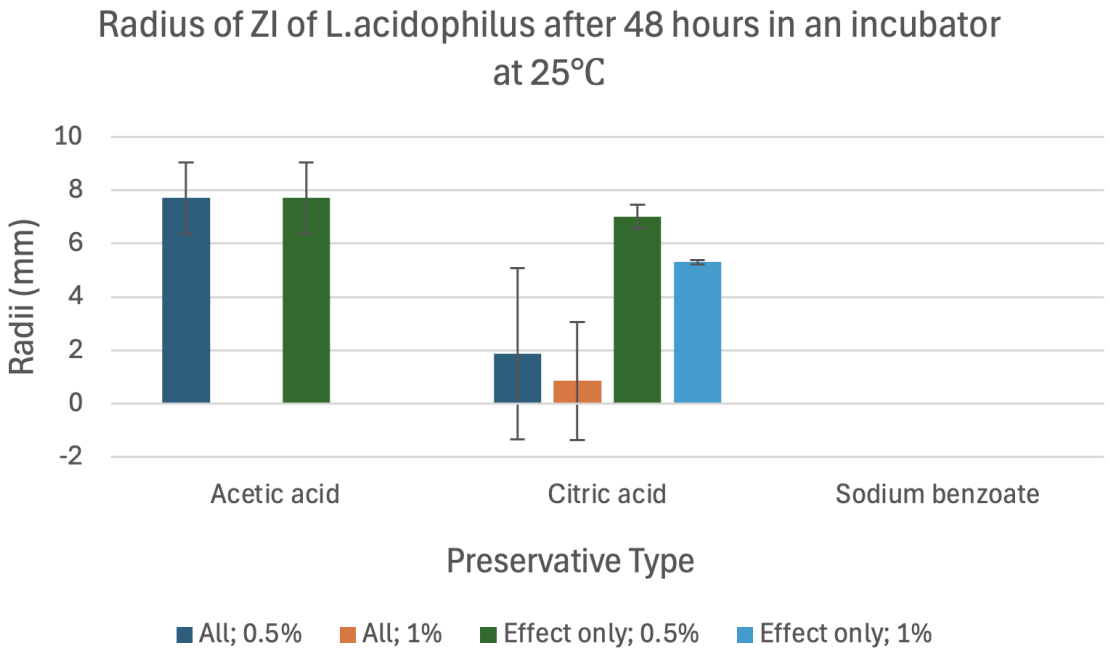
Independent variable	**Radius of ZI in mm (±0.05mm) after 48 hours in an incubator at 25℃**	
	Average	**SD**
0.5g/100mL Acetic acid	7.7	1.3
1.0g/100mL Acetic acid	0	0
0.5g/100mL Citric acid	7.0	0.5
1.0/100mL Citric acid	5.3	0.1
0.5g/100mL Sodium benzoate	0	0
1.0g/100mL Sodium benzoate	0	0

4.4 Graphs

Graph 1: Mean radius of inhibition zone (mm) of *E.coli* after 48h of incubation at 25℃ with 0.5% and 1% preservatives (All data points vs effect-only points, ±SD)



Graph 2: Mean radius of inhibition zone (mm) of *L.acidophilus* after 48h of incubation at 25℃ with 0.5% and 1% preservatives (All data points vs effect-only points, ±SD)



5. Analysis

5.1 Data Analysis

Graph 1 shows how acetic acid inhibits *E.coli* at both concentrations with an effect-only mean of ~6-7mm; the all-discs mean falls to ~1-3mm at 1.0% concentration because of lower frequency. Referring to Graph 2, 0.5% acetic acid effectively inhibits *L.acidophilus*, with an effect-only mean of ~7.70mm and all discs inhibited, whereas 1.0% shows no zones. Thus, for *E.coli* a higher concentration of acetic acid reduces frequency, while for *L.acidophilus* a higher concentration reduces overall activity.

Citric acid is dose-indifferent for inhibiting *E.coli*–effect-only means are ~6.2-6.4 mm at both concentrations and all-discs means are both ~0.8mm due to low frequency. Rather, for *L.acidophilus*, a higher concentration reduces both the frequency and the radius, with the all-discs mean dropping from ~1.9mm to ~0.9mm. Hence, the effect of citric acid concentration depends on the bacterium.

Based on Graph 1, 0.5% sodium benzoate does not inhibit *E.coli*; at 1.0%, the effect-only radii averages ~5.8mm, but the all-discs mean remains at ~1.54mm due to low frequency. Sodium benzoate is not effective on *L.acidophilus* within this concentration range, as no zones were observed at either dose.

5.2 Analysis of Variance

5.2.1 Methodology of ANOVA and effect size

A two-way ANOVA with replication determines two factors that influence ZI radii–type of preservative (acetic acid, citric acid, sodium benzoate) and level of concentration (0.5%, 1.0%), and the interaction between them (Will). Down-sampling using Excel RAND()+sort ensured equal replicates excluding contamination; the smallest valid count was 8 for *E.coli* and 13 for *L.acidophilus*.

Three hypotheses are made when conducting the ANOVA:

1. Preservative effect:

H_0: ignoring concentration, the average radius of ZI is the same for Acetic acid, Citric acid and Sodium benzoate.

H_1: At least one preservative produces a different average ZI radius.

2. Concentration effect:

H_0: ignoring the type of preservative, the concentrations 0.5% and 1.0% give the same average ZI radius.

H_1: 0.5% and 1.0% produce different average ZI radii

3. Interaction between preservative type and concentration:

Let $\mu_{i,j}$ be the true mean ZI radius for preservative *i* and concentration *j:*

$$\Delta_{Acetic} = \mu_{Acetic,\ 1.0} - \mu_{Acetic,\ 0.5}$$

$$\Delta_{Citric} = \mu_{Citric,\ 1.0} - \mu_{Citric,\ 0.5}$$

$$\Delta_{NaB} = \mu_{NaB,\ 1.0} - \mu_{NaB,\ 0.5}$$

H_0: $\Delta_{Acetic}=\Delta_{Citric}=\Delta_{NaB}$

H_1: At least one of the interactions (as represented by the Δs) is different

Effect size is reported as partial η^2:

$$\text{partial } \eta^2 = \frac{SS_{\text{effect}}}{SS_{\text{effect}} + SS_{\text{Within}}}$$

5.2.2 ANOVA of *E.coli*

Table 11-14: Two-way ANOVA of preservative type and concentration effect on *E.coli*

0.5%	**Acetic**	**Citric**	**NaB**	**Total**
Count	8	8	8	24
Sum	41.24	12.37	0	53.61
Average	5.155	1.54625	0	2.23375
Variance	10.3000857	8.1999125	0	10.4986071

1.0%	**Acetic**	**Citric**	**NaB**	**Total**
Count	8	8	8	24
Sum	19.23	12.79	23.11	55.13
Average	2.40375	1.59875	2.88875	2.29708333
Variance	11.0160554	8.77789821	9.5398125	9.22301286

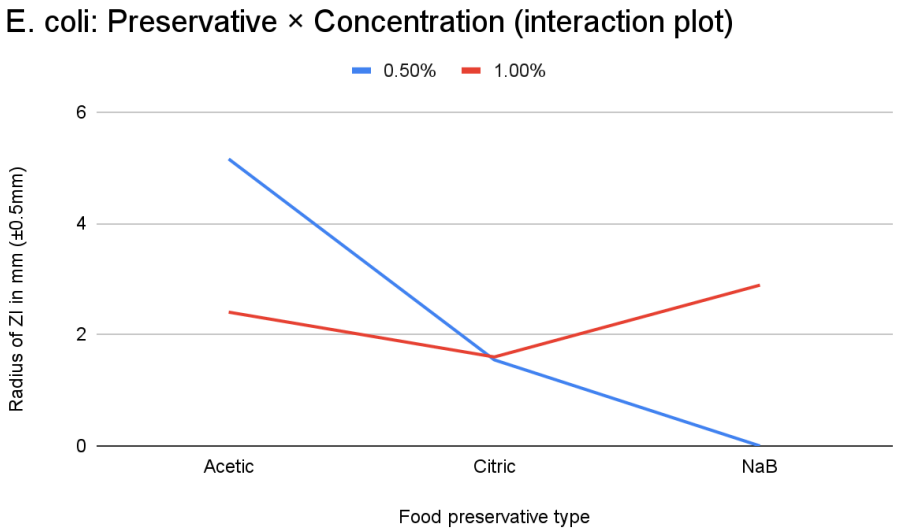
Total	**Acetic**	**Citric**	**NaB**
Count	16	16	16
Sum	60.47	25.16	23.11

Average	3.779375	1.5725	1.444375
Variance	11.9660329	7.922371333	6.67721292

Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Sample	0.04813333	1	0.04813333	0.00603758	0.93843423	4.07265376
Columns	55.1410042	2	27.5705021	3.45828966	0.04070591	3.21994229
Interaction	63.6199042	2	31.8099521	3.99006257	0.02591141	3.21994229
Within	334.83635	42	7.97229405			
Total	453.645392	47				

1. Concentration: $F \leq F$ crit, $p \geq 0.05$, null hypothesis is accepted; concentration change did not affect the average ZI.
    a. Partial $\eta^2 = 0.00014373$; no overall concentration effect.
2. Preservative: $F \geq F$ crit, $p \leq 0.05$, null hypothesis is rejected; the mean ZI are significantly different between the preservatives.
    a. Partial η^2 value is 0.1413954; 14% of variance is explained by the type of preservative.
3. Interaction: $F \geq F$ crit, $p \leq 0.05$, null hypothesis is rejected; the dose effect depends on the preservative.
    a. Partial η^2 value is 0.15866597; 16% of variance is explained by the interaction between concentration and preservative type.

Graph 3: Interaction plot for *E.coli*: mean ZI radius by preservative and concentration



The 0.5% and 1.0% lines are not parallel, so dose effect depends on preservative. On average, between 0.5% and 1.0%, acetic acid decreases by 2.8mm, sodium benzoate increases by 2.9mm and citric acid remains largely unchanged.

5.2.3 ANOVA of *L.acidophilus*

Table 15-18: Two-way ANOVA of preservative type and concentration effect on *L.acidophilus*

0.5%	**Acetic**	**Citric**	**NaB**	**Total**
Count	13	13	13	39
Sum	98.2	28.02	0	126.22
Average	7.5538462	2.1553846	0	3.2364103
Variance	1.7946256	11.374994	0	14.518876

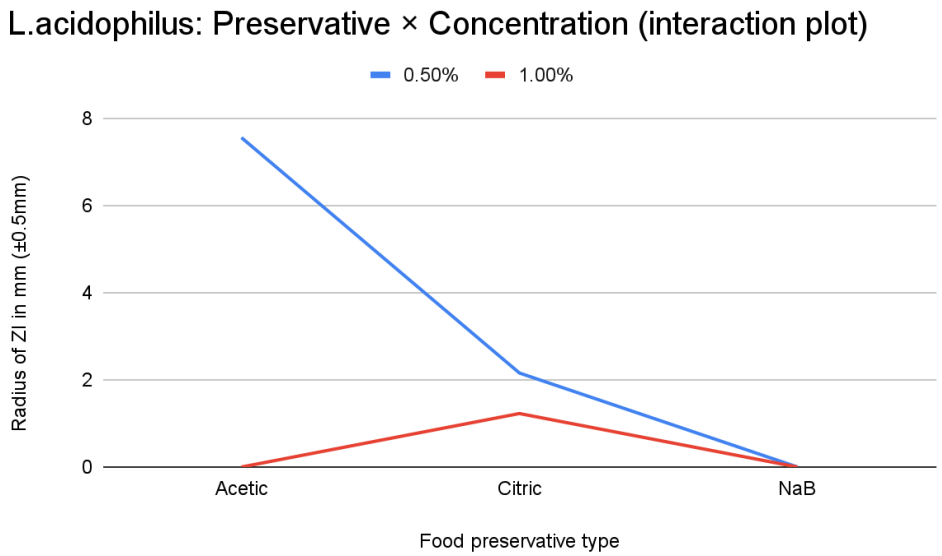
1.0%	**Acetic**	**Citric**	**NaB**	**Total**
Count	13	13	13	39
Sum	0	15.94	0	15.94
Average	0	1.2261538	0	0.4087179
Variance	0	5.430309	0	2.0577273

Total	Acetic	Citric	NaB
Count	26	26	26
Sum	98.2	43.96	0
Average	3.7768231	1.6907692	0
Variance	15.697174	8.2910474	0

Source of Variation	SS	df	MS	F	P-value	F crit
Sample	155.91895	1	155.91895	50.296631	$7.48 \cdot 10^{-10}$	3.973897
Columns	186.12435	2	93.062174	30.020172	$3.30 \cdot 10^{-10}$	3.1239074
Interaction	220.58745	2	110.29372	35.578758	$1.80 \cdot 10^{-11}$	3.1239074
Within	223.19914	72	3.099988			
Total	785.82989	77				

1. Concentration: $F \geq F$ crit, $p \leq 0.05$, null hypothesis is rejected; changing the concentration impacts the mean ZI.
    a. Partial η^2 value = 0.41126751; 41% of variance is explained by the concentration.
2. Preservatives: $F \geq F$ crit, $p \leq 0.05$, null hypothesis is rejected; mean ZI are significantly different between preservatives.
    a. Partial η^2 value = 0.45471212; 45% of variance is explained by the type of preservative.
3. Interaction: $F \geq F$ crit, $p \leq 0.05$, null hypothesis is rejected; the dose effect depends on the preservative
    a. Partial η^2 value = 0.49705749; 50% of variance is explained by the interaction between concentration and preservative type.

Graph 4: Interaction plot for *L.acidophilus*: mean ZI radius by preservative and concentration



The 0.5% and 1.0% lines are not parallel, so dose effect depends on preservative. Acetic acid demonstrates an average ZI of 7.6mm at 0.5% but no inhibition at 1.0%, citric acid falls from 0.5% to 1.0% by 0.9mm, and sodium benzoate demonstrates no inhibition for either concentration.

5.3 Uncertainty

Each ZI radius measurement was taken with a caliper of ±0.05mm uncertainty. As I took three measurements of radii per disc and averaged them, the uncertainty per disc is ± 0.05mm / $\sqrt{3} \approx \pm 0.029$mm. A condition mean over n number of discs has a reading uncertainty of $\pm 0.05 / \sqrt{(3n)}$. With 15 discs per preservative and concentration, $\pm 0.05 / \sqrt{(45)} = \approx \pm 0.0075$mm. Rather, the observed SDs were several mm large, meaning instrument reading error is negligible.

Other uncertainties were related to the solution preparation. Preservative powders were weighed on a ±0.005g balance, giving ±1.0% relative uncertainty at 0.5g and ±0.5% at 1.0g. Volumes were measured in a 100mL graduated cylinder with ±0.5mL uncertainty (0.5% of the 100mL water volume). Combining balance and volume yields uncertainties of ±1.12% for 0.5% solutions and ±0.71% for 1.0% solutions. Any concentration errors would affect all discs within a solution equally rather than biasing comparisons.

6. Discussion and conclusion

H_1 on *E.coli* is partially supported: sodium benzoate demonstrates a threshold–no zones at 0.5% but inhibition at 1.0%–whereas acetic and citric acids do not show a consistent

concentration-increase in radius. Acetic acid maintains ~6–7 mm radii when zones occur but has lower frequency at 1.0%, while citric acid is dose-indifferent. Consistent with these patterns, the ANOVA for *E.coli* shows insignificant effects of concentration ($p=0.938$), but significant effects of preservative ($p=0.0407$) and preservative-concentration interaction ($p=0.0259$).

The threshold-like inhibition by sodium benzoate can be explained by the inhibition of metabolic enzymes like phosphofructokinase and succinate dehydrogenase in *E.coli*, which disrupts energy production and growth; this only becomes pronounced at higher concentrations (Santiesteban-López et al.). Rather, acetic and citric acids lower the internal pH and disrupt membrane permeability of *E.coli*, to which the bacterium has no response (Musfirah et al.). This antibacterial effect saturates at 0.5% concentration, explaining the consistent ZI radii between doses (Musfirah et al.). However, at higher concentrations, weak acids like acetic acid diffuse into *E.coli* cells and dissociate, causing internal acidification and anion accumulation that can kill cells near the disc but not expand the inhibition radius, so fewer visible zones form; this explains the lower frequency at 1.0% (Etsuzo Entani et al.)

H_2 on *L.acidophilus* is mainly unsupported. Acetic acid inhibits all points at 0.5% but none at 1.0%; citric acid demonstrates reduced frequency and smaller radii at 1.0%; sodium benzoate has no effect at either dose. The ANOVA confirms dependence on both concentration ($p=7.48\times10^{-10}$) and preservative type ($p=3.30\times10^{-10}$), and a significant interaction ($p=1.80\times10^{-11}$).

As frequency and ZI radii both decreased at 1.0% compared to 0.5% for acetic and citric acids on *L.acidophilus*, the underlying mechanism can be explored. As weak organic acids, higher doses of acetic and citric acids causes *L.acidophilus*, a Gram-positive probiotic, to alter its cell membrane composition; the bacterium increases cyclopropane fatty acids by upregulating the cyclopropane synthase gene (Bjornsdottir et al.). Through decreasing membrane permeability, the cells maintain internal pH homeostasis, surviving better under acidic conditions (Srisukchayakul et al.). Rather, sodium benzoate is a salt, meaning it interferes with microbial metabolism and enzyme function; *L.acidophilus* contains detoxification mechanisms for expelling benzoate ions, explaining the lack of inhibition (Petrova et al.).

Overall, preservative impacts on the gut microbiome depend strongly on its type and concentration. Their impact on beneficial probiotics like *L.acidophilus* is inconsistent, where the

bacterium shows resilience or adaptation to some preservatives. This indicates typical consumption levels of these preservatives may not severely disrupt gut balance, but the weak organic acids were more inhibitory at lower concentrations. Since pretrials showed no inhibition at 0.1% but clear inhibition at 0.5%, chronic exposure within this range could still suppress probiotics and unbalance the gut community, increasing susceptibility to opportunistic pathogens and gastrointestinal upset.

7. Evaluation

7.1 Weaknesses

Table 19: Impact of experimental errors and future mitigation strategies.

Source of error	Impact on results	Potential improvements
Dip-loading technique + learning curve	Dipping discs into solutions carried contamination risk; results may have improved for *L.acidophilus* because it was done after gaining practice with *E.coli*.	Randomize run order, practice on test plates, work closer to the flame.
Variable liquid uptake in dip-and-blot method	Dip-and-blot caused unequal disc saturation, increasing variability and affecting inhibition frequency.	Choose another method such as 10 µL pipetting on each disc; strictly standardize dip/blot timing.
Uncontrolled pH	Weak-acid inhibition of bacteria depends on agar pH, so preservative type and concentration effects may reflect acidity.	Measure and report solution pH; use buffered agar at fixed pH.

The factor with the largest impact was the uncontrolled pH, as this confounds the effect of the preservative. Weak acids both acidify the medium and act via their undissociated acid form, so the differences across preservative type and concentration may reflect acidity rather than antimicrobial potency (Mira and Teixeira). Solution and agar surface pH were not measured or fixed, limiting causal conclusions, making buffered agar and controlled solution pH essential for further trials.

7.2 Extension

1. I would add minimum inhibitory concentration (MIC) testing. None of the preservatives demonstrated effects at 0.1%, but different preservatives started inhibiting the bacteria at different concentrations–sodium benzoate only inhibited *E.coli* at 1.0%, while acetic acid only inhibited *L.acidophilus* at 0.5%. MIC testing through broth microdilution would allow me to pinpoint the exact concentration that fully prevents visible bacterial growth, rather than inferring preservative potency from ZI radius (Nikol Kadeřábková et al.). In a broth microdilution, I would find the lowest dilution that remains clear after incubation; this eliminates the confounding influence of diffusion rates in agar and provides precise thresholds to compare across preservatives.
2. Investigations could attempt realistic modelling by testing common preservative pairs in food, such as citric acid with sodium benzoate in soft drinks, at label-level doses, to observe how mixtures inhibit bacteria compared to single agents. Alternatively, co-culturing Gram negative and Gram positive bacteria together in a 1:1 ratio could model gut competition directly. Exposing them to label-level doses of preservatives and examining the change in their relative abundance shows whether each preservative selects for or against the types of bacteria. This provides a realistic assessment of preservative effects on the gut community structure, rather than isolated species responses.
3. I would repeat the assays under stomach-like pH (~1.5-3.5) and intestinal pH (~6-7.4) using buffered media and pH-controlled preservative solutions; this shows how much preservative activity survives gastric conditions such as stomach acid exposure, and reach the gut, which is where probiotics reside (Yamamura et al.). Comparing activity across the two environments distinguishes stomach-only effects from gut-relevant effects, indicating which outcomes matter in vivo.

Works Cited

Bhattacharjee, Mrinal K. "Better Visualization and Photodocumentation of Zone of Inhibition by Staining Cells and Background Agar Differently." *The Journal of Antibiotics*, vol. 68, no. 10, Springer Science and Business Media LLC, May 2015, pp. 657–59, https://doi.org/10.1038/ja.2015.49.

Bjornsdottir, K., et al. "Protective Effects of Organic Acids on Survival of *Escherichia Coli* O157:H7 in Acidic Environments." *Applied and Environmental Microbiology*, vol. 72, no. 1, American Society for Microbiology, Jan. 2006, pp. 660–64, https://doi.org/10.1128/aem.72.1.660-664.2006.

Burana-Osot, Jankana, et al. "Validation of a HPLC Method for the Determination of Benzoic Acid and Sorbic Acid in Noodles." *Chiang Mai J. Sci*, vol. 41, no. 2, 2014, pp. 370–82, thaiscience.info/Journals/Article/CMJS/10932726.pdf.

Celebi, Ozgur, et al. "Effect of the Combination of *Lactobacillus Acidophilus* (Probiotic) with Vitamin K3 and Vitamin E on *Escherichia Coli* and *Staphylococcus Aureus*: An *in Vitro* Pathogen Model." *Molecular Medicine Reports*, vol. 27, no. 6, Spandidos Publishing, May 2023, https://doi.org/10.3892/mmr.2023.13006.

Chen, Huaiqiong, and Qixin Zhong. "Antibacterial Activity of Acidified Sodium Benzoate against Escherichia Coli O157:H7, Salmonella Enterica, and Listeria Monocytogenes in Tryptic Soy Broth and on Cherry Tomatoes." *International Journal of Food Microbiology*, vol. 274, Elsevier BV, Mar. 2018, pp. 38–44, https://doi.org/10.1016/j.ijfoodmicro.2018.03.017.

CLEAPSS Hazcards.pdf. “CLEAPSS Hazcards.pdf.” *Google Docs*, 2019, drive.google.com/file/d/1qhouZxV1UDoJwEEaXsp0j8ZDocQKf42C/view.

EFSA. *Opinion of the Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers*. 4 June 2002, ec.europa.eu/health/ph_risk/committees/sccp/documents/out166_en.pdf.

Etsuzo Entani, et al. “Antibacterial Action of Vinegar against Food-Borne Pathogenic Bacteria Including Escherichia ColiO157:H7.” *Journal of Food Protection*, vol. 61, no. 8, Elsevier BV, Aug. 1998, pp. 953–59, https://doi.org/10.4315/0362-028x-61.8.953.

Farboud Shahabinejad, et al. “Functional Instant Noodle Formulation for Emergency Conditions: Sensory and Stability Characteristics.” *Food Science & Nutrition*, vol. 12, no. 7, Wiley, Mar. 2024, pp. 4605–14, https://doi.org/10.1002/fsn3.4062.

FDA. “21 CFR 184.1733 -- Sodium Benzoate.” *Ecfr.gov*, 2025, www.ecfr.gov/current/title-21/chapter-I/subchapter-B/part-184/subpart-B/section-184.1733#page-top.

Food and Agriculture Organization of the United Nations. *Benzoic Acid and Its Salts 92nd JECFA -Chemical and Technical Assessment (CTA), 2021 Benzoic Acid and Its Salts Chemical and Technical Assessment (CTA)*. 2021, openknowledge.fao.org/server/api/core/bitstreams/b36d6383-63d6-4b88-afe3-85e013878861/content.

Hul, Matthias Van, et al. “What Defines a Healthy Gut Microbiome?” *Gut*, vol. 73, no. 11, BMJ, Sept. 2024, pp. 1893–908, https://doi.org/10.1136/gutjnl-2024-333378.

Issa, Hayder M., and Dania H. Mohammed. “A Critical Review on the Journey of Benzoic Acid in the Pharmaceutical Industry from Manufacturing Processes through Various Uses to Disposal: An Environmental Perspective.” *Environmental Analysis Health and Toxicology*, vol. 40, no. 1, Korean Society of Environmental Helath and Toxicology, Mar. 2025, pp. e2025007–7, https://doi.org/10.5620/eaht.2025007.

Książek, Ewelina. “Citric Acid: Properties, Microbial Production, and Applications in Industries.” *Molecules*, vol. 29, no. 1, MDPI AG, Dec. 2023, p. 22, https://doi.org/10.3390/molecules29010022.

McCulloch, Marsha. “What Is Sodium Benzoate? Everything You Need to Know.” *Healthline*, Healthline Media, 3 July 2023, www.healthline.com/nutrition/sodium-benzoate.

Mira, Nuno P., and Miguel C. Teixeira. “Microbial Mechanisms of Tolerance to Weak Acid Stress.” *Frontiers in Microbiology*, vol. 4, Frontiers Media, Jan. 2013, https://doi.org/10.3389/fmicb.2013.00416.

Mounyr Balouiri, et al. “Methods for in Vitro Evaluating Antimicrobial Activity: A Review.” *Journal of Pharmaceutical Analysis*, vol. 6, no. 2, Elsevier BV, Dec. 2015, pp. 71–79, https://doi.org/10.1016/j.jpha.2015.11.005.

Musfirah, Nur, et al. “Effect of Different Conditions of Citric Acid and Acetic Acid Decontamination against Escherichia Coli in Lettuce.” *Journal of Agrobiotechnology*, vol. 9, 2018, pp. 54–61, core.ac.uk/download/pdf/267943209.pdf.

Nikol Kadeřábková, et al. “Antibiotic Susceptibility Testing Using Minimum Inhibitory Concentration (MIC) Assays.” *Npj Antimicrobials and Resistance*, vol. 2, no. 1, Nov. 2024, https://doi.org/10.1038/s44259-024-00051-6.

Petrova, Penka, et al. “The Complex Role of Lactic Acid Bacteria in Food Detoxification.” *Nutrients*, vol. 14, no. 10, MDPI AG, May 2022, p. 2038, https://doi.org/10.3390/nu14102038.

Santiesteban-López, N. Angélica., et al. “Growth Response of Escherichia Coli ATCC 35218 Adapted to Several Concentrations of Sodium Benzoate and Potassium Sorbate.” *Journal of Food Protection*, vol. 72, no. 11, Elsevier BV, Nov. 2009, pp. 2301–7, https://doi.org/10.4315/0362-028x-72.11.2301.

Srisukchayakul, Pornpoj, et al. “Study on the Effect of Citric Acid Adaptation toward the Subsequent Survival of Lactobacillus Plantarum NCIMB 8826 in Low PH Fruit Juices during Refrigerated Storage.” *Food Research International*, vol. 111, Sept. 2018, pp. 198–204, https://doi.org/10.1016/j.foodres.2018.05.018.

Will. “Difference between ANOVA Two-Factor with Replication and without Replication.” *TidyStat*, 25 Jan. 2023, tidystat.com/difference-between-anova-two-factor-with-replication-and-without-replication-in-excel/.

Yamamura, Ryodai, et al. “Intestinal and Fecal PH in Human Health.” *Frontiers in Microbiomes*, vol. 2, Frontiers Media SA, July 2023, https://doi.org/10.3389/frmbi.2023.1192316.

Zaghloul, et al. “Comparison of Antibacterial Activity of Fungal Chitosan and Some Preservatives against Some Foodborne Pathogenic Bacteria.” *Egypt. J. Microbiol*,

vol. 50, 2015, pp. 31–42,

ejm.journals.ekb.eg/article_233_505e56443f69a8294352b2272bb1bad8.pdf.