Antibacterial Activity of Lysozyme and Kanamycin in Inhibiting the Growth of *Escherichia coli*

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Content

Introduction

Background

The increasing use of antibiotics in agricultural industries like poultry farms has accelerated the emergence of antibiotic-resistant bacterial strains (N. Roth, 2018). Many poultry farmers spray and add antibiotics on eggshells and chicken feeds as preventative measures against infectious bacterial diseases (R. Abreu, 2023). Despite their bactericidal ("kill bacteria") and bacteriostatic ("suppress the growth of bacteria") properties, antibiotic overuse has profound disadvantages (Bernatová, 2013). With a limited number of available antibiotics, the available number of treatments for bacterial infection diminishes as multidrug-resistant bacteria become more prevalent (N. Roth, 2018). Consequently, the increasing pathogenicity and virulence of antibiotic-resistant bacteria pose a threat to public health (N. Roth, 2018).

Though banned from being added to poultry feeds, continuous administration of kanamycin in poultry has led to a proliferation of resistant strains of *Escherichia coli* and *Salmonella* in poultry farms (Richard, 1987). Kanamycin is an aminoglycoside antibiotic and its ability to bind to the A-site on the 16S RNA of the 30s ribosomal subunit causes misreading of t-RNA (Gallego, n.d.). Consequently, kanamycin's activity interferes with the translational process by introducing wrong amino acids in the polypeptide chain, depriving bacteria's ability to synthesize functional proteins vital for their survival (Gallego, n.d.). For instance, t-RNA misreading causes ribosomes to produce nonfunctional proteins integral to bacterial membrane structures (Gallego, n.d.). As their membranes are damaged by mistranslated proteins, more kanamycin enters and accelerates cell death, rendering kanamycin bactericidal (Gallego, n.d.).

Despite its prominent antibacterial activity, the overuse of antibiotics like kanamycin in poultry farms has accelerated the selection of drug-resistant bacteria (Richard, 1987). In the search for an alternative antimicrobial agent, many research focus on the bactericidal property of lysozyme (P. Ferraboschi, 2021). Lysozyme is a digestive enzyme found in the lysosome of all eukaryotic cells with antibacterial properties (P. Ferraboschi, 2021). By hydrolyzing the β-1,4-glycosidic linkage present between N-acetyl glucosamine and N-acetyl muramic acid in polysaccharides, peptidoglycan digestion creates pores in bacterial cell wall, and the hypertonic cytosol concentration induces cell lysis through osmosis (S. Min, 2005) (P. Ferraboschi, 2021).

Though many farms use antimicrobial agents to prevent spoilage of poultry products, the egg-white of poultry embryos is rich of innate immune defense factors like lysozyme to combat bacterial infection (Shahmohammadi, 2018). In fact, about 3.5% of total egg-white protein is lysozyme, making egg white one of the most abundant sources of lysozyme (Shahmohammadi, 2018). But unlike kanamycin, which is bactericidal against both gram-positive and gram-negative bacteria, lysozyme has a limited bactericidal effect on gram-negative bacterial strains (Drugbank, 2023). While lysozyme effectively neutralizes gram-positive bacteria that only have peptidoglycan cell wall, the presence of lipopolysaccharides outer membrane encapsulating gram-negative bacteria decreases its bactericidal properties by making it harder for lysozyme to approach the inner peptidoglycan layer (P. Ferraboschi, 2021). This is due to the hydrophilic surface of lysozyme protein (M. Jafari, 2016). As lipopolysaccharide is amphipathic owing to its polar outer polysaccharide (sugar) region and non-polar inner lipid (fatty acids) region, only a few lysozyme molecules can physically reach the peptidoglycan layer through the hydrophobic region of the outer membrane (M. Jafari, 2016) (Macrophi Inc, n.d.).

The antibacterial activity of lysozyme and kanamycin can be compared by measuring the diameter of the zone of inhibition formed after administering each antimicrobial agent on a bacterial colony cultured on an agar plate (Microchem, 2022). As antimicrobial agents diffuse through the agar medium, they will exert a growth-inhibiting effect on bacteria (Microchem, 2022). As the size of the inhibition zone correlates to how far an antimicrobial agent could inhibit bacterial growth with minimal concentration, the diameter of the zone of inhibition produced in a diffusion test is an accurate method to measure the antibacterial activity of the antimicrobial agent tested (Microchem, 2022).

Aim of Research

Among the three main bacterial strains found on the surface of poultry eggs and in egg yolk, *Staphylococcus spp*. is gram-positive while *Salmonella* and *Escherichia coli* are gram-negative (Chaemsanit, 2015). Assuming that lysozyme is effective in inhibiting the growth of gram-positive *Staphylococcus* strains, its challenge of passing through the outer membrane led me to question whether lysozyme present in egg-white is enough to neutralize gram-negative bacteria like *E. coli* that penetrate the eggshell (*Salmonella* was not experimented with owing

to its virulence and pathogenicity). Since egg yolk itself is a rich source of antimicrobial agents, I wanted to investigate whether industries are administering antibiotics unnecessarily when lysozyme in poultry products can sufficiently combat against *E. coli* or whether there is a pertinent need for supplementary antibiotics.

Hence, this research will first explore how increasing lysozyme concentration affects its antibacterial activity and how the antibacterial activity of lysozyme compares to that of kanamycin. Furthermore, the presence of a synergic effect between lysozyme and kanamycin in inhibiting *E. coli's* growth will also be investigated by administering both antimicrobial agents together, examining the possibility of utilizing lysozyme alongside kanamycin to produce a higher antibacterial activity while reducing the amount of kanamycin used. Using the data collected, this research will determine the relative antibacterial activity of each antimicrobial agent tested to conclude whether there is sufficient concentration of lysozyme in poultry egg white to inhibit the growth of *E. coli* or if the addition of supplementary antibiotics appears necessary to fully inhibit bacterial growth.

Research Question

To find the antibacterial activity of lysozyme and kanamycin against *Escherichia coli*, this research has made the following research question: how does the administration of increasing concentrations of lysozyme (100µg, 1mg, 3,85mg, 10mg, and 100mg per mL) and kanamycin (100µg per mL) on pure *Escherichia coli* culture grown in agar plate affect the diameter of zone of inhibition formed after 24 hours of incubation period at 37°C, measured through diffusion test?

Hypothesis

This research hypothesizes that increasing the concentration of lysozyme will produce a larger diameter of the zone of inhibition by increasing lysozyme's antibacterial activity. Though the presence of amphipathic lipopolysaccharide membrane renders it difficult for hydrophilic lysozyme to diffuse through the hydrophobic lipid region, the rise in concentration will increase the sheer chance that some lysozyme molecules diffuse through the outer membrane and reach *E. coli's* peptidoglycan cell wall (Macrophi Inc, n.d.). Furthermore, this

research expects kanamycin to be more bactericidal than lysozyme of the same concentration as the outer membrane does not interfere with its antibacterial activity. In fact, kanamycin enters *E. coli* through a self-promoted uptake mechanism by displacing cations like Ca^{2+} and Mg^{2+} from both lipopolysaccharide outer membrane and inner membrane, rendering the two membranes more permeable to kanamycin (Gallego, 2023). Hence, kanamycin would exert its full bactericidal effect on *E. coli* by disrupting their protein synthesis unlike lysozyme whose antibacterial activity is limited owing to the presence of the outer membrane (Gallego, n.d.).

Variables

Table 1 Discussion of the independent variable

Independent variable	How it was changed
Lysozyme concentration (100µg/mL, 1mg/mL, 3.85mg/mL, 10mg/mL, and 100mg/mL)	The initial stock solution (100mg/mL) was diluted into concentrations orders of magnitude lower using deionized water. 3.85mg/mL of lysozyme was also chosen as it is the lysozyme concentration found in poultry egg white.

Table 2 Discussion of the dependent variable

Dependent variable	How it will be measured
The diameter of the zone of inhibition (mm)	A ruler will be used to measure the zone of inhibition formed after applying the antimicrobial agent and incubating the *E. coli* culture on agar plates in 37 degrees for 24 hours.

Table 3 Discussion of the Controlled variables

Controlled variable	Why it was controlled	How it was controlled
The volume of lysozyme and kanamycin added	The volume of antimicrobial agents added must be constant to examine the effect concentration has on lysozyme's antibacterial activity.	Micropipettes were used to add 50µL of antimicrobial agent to each cylindrical hole bored on the agar plate.
The amount of *E. coli* inoculated on each plate	Each agar plate must be covered by an equally thick *E. coli* colony to ensure that the diameter of the zone of inhibition measured reflects antimicrobial agent's antibacterial activity accurately.	1mL of the pure *E. coli* culture grown in the LB medium was spread out evenly on each agar plate using a brush.
The temperature of *E. coli* culture	Since bacterial growth through binary fission is influenced by temperature, the temperature during the incubation period must stay constant.	The laboratory incubator maintained the constant temperature at 37 degrees

Methodology

Apparatus

Table 4 Lists of materials and equipment used in the experiment.

A list of material used	A list of equipment used
• Crystallized Sodium Chloride (NaCl) • Peptone powder • Yeast extract • Agar powder • Deionized water • Crystalized lysozyme • Kanamycin solution (50mg/mL) • *Escherichia coli* BL21 (DE3) • 60% ethanol	• Micropipettes (2µL-20µL, 10µL-100µL, and 100µL-1000µL) • 500ml volumetric flask • Glass jars • TOMY AUTOCLAVE sterilizer • Ventilation chamber • Digital scale (±0.1mg) • Petri dish • Incubator • Brush • Ruler (±0.5mm)

Experimental procedure

- Part one: Creating lysogeny broth (LB) for *E. coli* culture.

According to the LB broth recipe, 5g of NaCl, 5g of peptone, 2.5g of yeast extract, and 10g of agar were measured using a digital scale and mixed with 500ml of deionized water measured using a volumetric flask (Burgess, 2016). The solution was transferred into a glass jar labeled "LB medium with agar". In another glass jar, the identical LB medium was prepared without agar. TOMY AUTOCLAVE was used to sterilize potential microorganisms inside both LB medium by exposing the jars in $120^{\circ}C$ for 20 minutes. Once the jars cooled, the LB medium with agar was poured into Petri dishes to make a 1cm thick agar medium in each Petri dish. A sample of *Escherichia coli* BL21 strain resistant to ampicillin was cultured in pure LB medium without agar for 3 days and treated with ampicillin to prevent the growth of bacterial strains other than *E. coli*.

- Part two: Preparing the lysozyme and antibiotic solutions.

100mg of lysozyme crystal was measured using a digital weight and dissolved in 1mL of deionized water measured by micropipette to prepare the lysozyme stock solution. Less concentrated lysozyme solutions were prepared by diluting solutions of higher concentration:

Solution A (100mg/mL stock solution)

Solution B (10mg/mL): Mixing 100µL of solution A and 900µL of deionized water

Solution C (1mg/mL): Mixing 100µL of solution B and 900µL of deionized water

Solution D (100µg/mL): Mixing 100µL of solution C and 900µL of deionized water

In addition, 3.85mg/mL lysozyme solution was prepared by mixing 38.5µL of solution A and 961.5µL of deionized water to measure the antibacterial activity of the lysozyme concentration found in poultry egg white (Egg white of one egg weight 30g and measures 30mL. 11% of egg white is made of protein, and 3.5% of egg white protein is lysozyme (Silvetti, 2017)).

- Egg white in one egg: 30g (30mL)
- Lysozyme in egg white of one egg: 0.1155g
- Lysozyme concentration in egg white: 115.5mg/30mL = 3.85mg/mL

100µg/mL kanamycin solution was prepared by adding 2µL of 50mg/mL stock solution into 998µL of water. As the final treatment, 25µL of 3.85mg/mL lysozyme solution and 25µL of 100µL/mL kanamycin solution were mixed to form an antimicrobial mixture to investigate the synergic effects of the two antimicrobial substances. Mixing two volumes diluted the concentration of each antimicrobial agent, resulting in 1.925mg/mL of lysozyme and 50µg/mL of kanamycin concentrations, respectively.

- Part three: Inoculating *E. coli* onto agar plate.

1mL of *E. coli* BL21 strain cultured in LB medium without agar was measured using a micropipette to ensure that roughly the same amount of *E. coli* was being inoculated to each agar plate. Using a sterile swab, the suspension of the pure culture was spread evenly over the surface of the sterile agar plate, and three small cylindrical well was bored on the agar plate with sufficient distance between each other to prevent overlap of different inhibition zones. Every treatment (100µg/mL, 1mg/mL, 3.85mg/mL, 10mg/mL, 100mg/mL of lysozyme, 100µg/mL of kanamycin, and the antimicrobial mixture) was repeated 5 times, using micropipette to fill each well with 50µL of each antimicrobial agents tested. Agar plates were then incubated in laboratory incubator for 24 hours at $37^{\circ}C$.

- Part four: Measuring the zone of inhibition.

The diameter (mm) of the zone of inhibition surrounding each cylindrical well was measured using a ruler under light. Subsequently, the agar plates with *E. coli* culture were disposed in a toxic waste container. Ethanol (60%) was used to sanitize other experimental apparatus used.

Safety ethics and environment

Due to dangers of experimenting with pathogens in a school laboratory, the entirety of experiment was conducted in a laboratory at the University of Oslo (UiO). A biochemistry professor at UiO supervised my research throughout the experiment to prevent accidents when I was handling *E. coli* cultures, TOMY AUTOCLAVE sterilizer, and laboratory incubator. Though incubating bacteria at 37 degrees, the temperature of the human body, is discouraged according to IB guidelines, the BL21 (DE3) *E. coli* strain used in this research is non-pathogenic. Furthermore, all procedures involving bacteria were conducted within a ventilation chamber, and protections (lab coats, goggles, and gloves) were always worn to preclude exposure to

bacterial samples. After the experiment, *E. coli* cultures and agar plates were disposed into biohazard containers, and all equipment were sanitized with 60% ethanol. Remaining solutions were stored for future uses to prevent unnecessary disposal of chemicals.

Data Analysis

Raw data

Quantitative data

Table 5 Diameter of the zone of inhibition measured after 24 hours of incubation at 37°C, with average results and standard deviation calculated.

Types of antimicrobial agent applied (50µL)	Diameter of the zone of inhibition (mm±0.5mm)						
	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Average	Standard deviation
Lysozyme (100µg/mL)	No results	No results	No results	No results	No results	No results	No results
Lysozyme (1mg/mL)	No results	No results	No results	No results	No results	No results	No results
Lysozyme (3.85mg/mL)	4	4	5	4	4	4	0.4
Lysozyme (10mg/mL)	6	6	7	5	6	6	0.7
Lysozyme (100mg/mL)	14	11	14	15	13	13	1.5
Kanamycin (100µg/mL)	13	12	14	10	11	12	1.6
25µL of Lysozyme (3.85mg/mL) and 25µL of Kanamycin (100µg/mL)	15	15	16	15	17	16	0.89

Qualitative data:

As shown in figure 1, there is a clear zone of inhibition surrounding the cylindrical well in which antimicrobial agents were administered. The lighter area indicates the absence of *E. coli* whereas the rest of opaque area shows the *E. coli* colony grown on agar plate.

Figure 1 Antibacterial activity of 100mg/mL lysozyme on an agar plate coated with E. coli culture.

Processed data

The area of the zone of inhibition was calculated by assuming that a zone of inhibition is a perfect circle. Since the measured diameter includes the diameter of the well, the area of the well was subtracted from the area produced by the measured diameter when calculating the area of the zone of inhibition. The example below shows calculation of the area of zone of inhibition for trial 1 with 100mg/mL of lysozyme.

Diameter of the well = 3mm,

Diameter measured = 14mm

Area of zone of inhibition = $(7mm \times 7mm \times \pi) - (1.5mm \times 1.5mm \times \pi) = 146.8694 \ldots mm^2 \approx \mathbf{147mm^2}$

Table 6 Areas of zone of inhibition for each antimicrobial agent, including the average value and their standard deviation.

Types of antimicrobial agent applied (50µL)	Area of zone of inhibition (mm^2)						
	Trial 1	Trial 2	Trial 3	Trial 4	Trial 5	Average	Standard deviation
Lysozyme (100µg/mL)	No results	No results	No results	No results	No results	No results	No results
Lysozyme (1mg/mL)	No results	No results	No results	No results	No results	No results	No results
Lysozyme (3.85mg/mL)	5.50	5.50	12.6	5.50	5.50	6.91	3.16
Lysozyme (10mg/mL)	21.2	21.2	31.4	12.6	21.2	21.5	6.68
Lysozyme (100mg/mL)	147	88.0	147	170	126	135	30.7
Kanamycin (100µg/mL)	126	106	147	71.5	88.0	108	29.8
Mixture of 25µL of Lysozyme (3.85mg/mL) and 25µL of Kanamycin (100µg/mL)	170	170	194	170	220	185	22.4

T-test to confirm the statistical significance of synergic effect between lysozyme and kanamycin in the antimicrobial mixture:

H_0: The zone of inhibition produced by antimicrobial mixture is not significantly bigger than that of 100µg/mL kanamycin solution.

H_1: The zone of inhibition produced by antimicrobial mixture is significantly bigger than that of 100µg/mL kanamycin solution.

Let's set p=0.01 as the critical value for the statistical level of significance of this T-test with degrees of freedom (DF) of 8. A p value lower than 0.01 would indicate that the area of zone of inhibition produced by the antimicrobial mixture is significantly higher than that of kanamycin. Conducting a T-test using the five areas of the zone of inhibition for the antimicrobial mixture and kanamycin results in $p = 8.51 \times 10^{-4}$. Since the calculated p value

is lower than p=0.01, there is less than 1% chance that the observed differences in zone of inhibition are due to randomness. Hence, the area of the zone of inhibition produced by the antimicrobial mixture is significantly bigger than that of 100µg/mL kanamycin with more than 99% certainty, allowing this research to reject H_0 and accept H_1.

H_0: The zone of inhibition produced by antimicrobial mixture is not significantly bigger than that of 100mg/mL lysozyme solution.

H_1: The zone of inhibition produced by antimicrobial mixture is significantly bigger than that of 100mg/mL lysozyme solution.

Conducting a T-test using areas of zone of inhibition calculated for the antimicrobial mixture and 100mg/mL gives p =0.01004. Setting p=0.05 as the critical value of this T-test with DF of 8, there is more than 95% statistical significance that the zone of inhibition produced by the antimicrobial mixture is significantly bigger than that of 100mg/mL lysozyme solution (H_1 accepted).

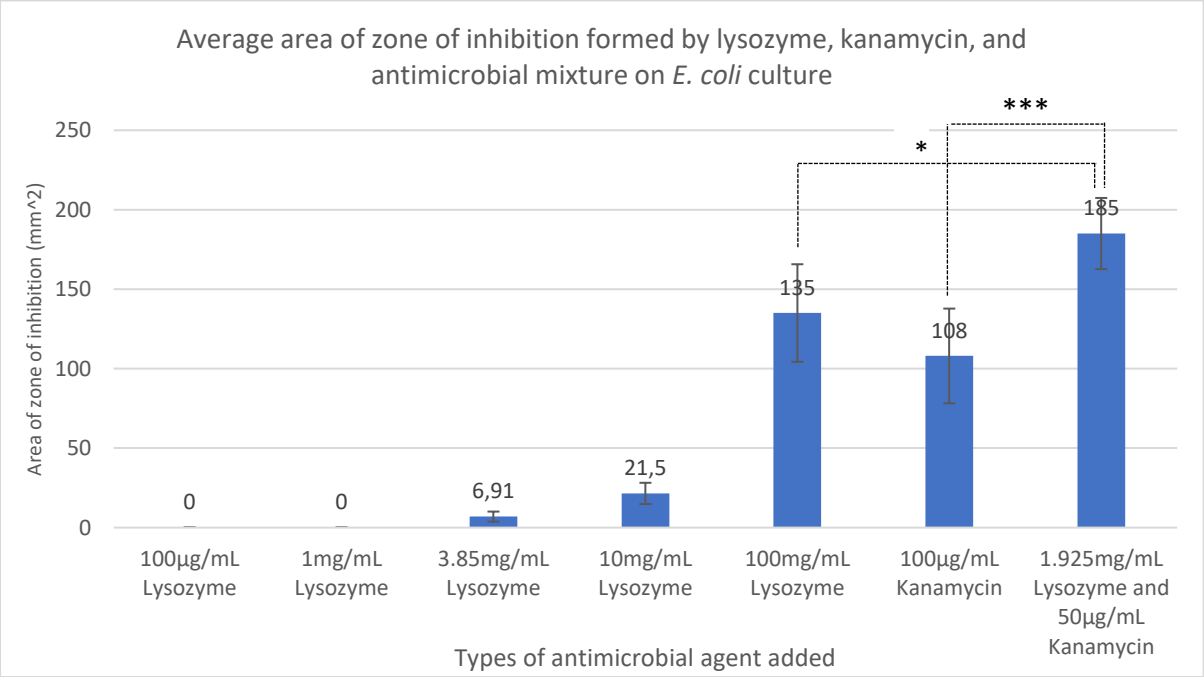

*Figure 2 Effect of increasing lysozyme concentration as well as the effect of kanamycin and the antimicrobial mixture on the area of the zone of inhibition formed. The vertical error bars represent the standard deviation for the average area of the zone of inhibition calculated for each type of antimicrobial agent. * indicates the statistical significance that exceeds the critical value of p=0.05. *** indicates the statistical significance that exceeds the critical value of p=0.01.*

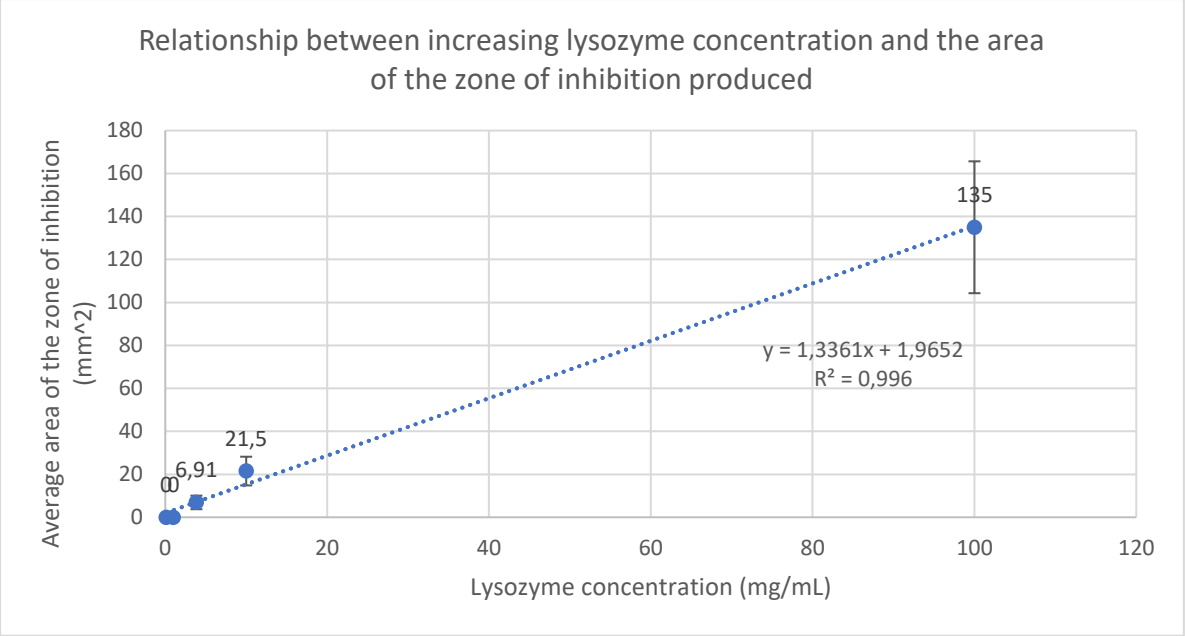

Figure 3 The effect of increasing lysozyme concentration on the average inhibition area. The linear regression passes through all error bars representing the standard deviation for the areas calculated. R^2= 0996 indicates a strong positive correlation between the lysozyme concentration and the average area of the zone of inhibition produced.

Lastly, to compare the antibacterial activity of different antimicrobial agents, the relative antibacterial activity value for each treatment was calculated. The relative antibacterial activity value for the average area of the zone of inhibition produced by 100µg/mL Kanamycin was set as 1. Then, the relative antibacterial activity values for other antimicrobial agents were calculated by dividing their average area of zone of inhibition with that of 100µg/mL kanamycin. Example below shows calculation of the relative antibacterial activity of 100mg/mL Lysozyme.

Relative antibacterial activity = $\frac{135mm^2}{108mm^2} = \mathbf{1.25}$

Table 7 Relative antibacterial activity values of each antimicrobial agent relative to 100µg/mL kanamycin.

Types of antimicrobial agent applied (50µL)	Relative antibacterial activity
Lysozyme (100µg/mL)	0
Lysozyme (1mg/mL)	0
Lysozyme (3.85mg/mL)	0.06
Lysozyme (10mg/mL)	0.20
Lysozyme (100mg/mL)	1.25
Kanamycin (100µg/mL)	1.00
Mixture of 25µL of Lysozyme (3.85mg/mL) and 25µL of Kanamycin (100µg/mL)	1.71

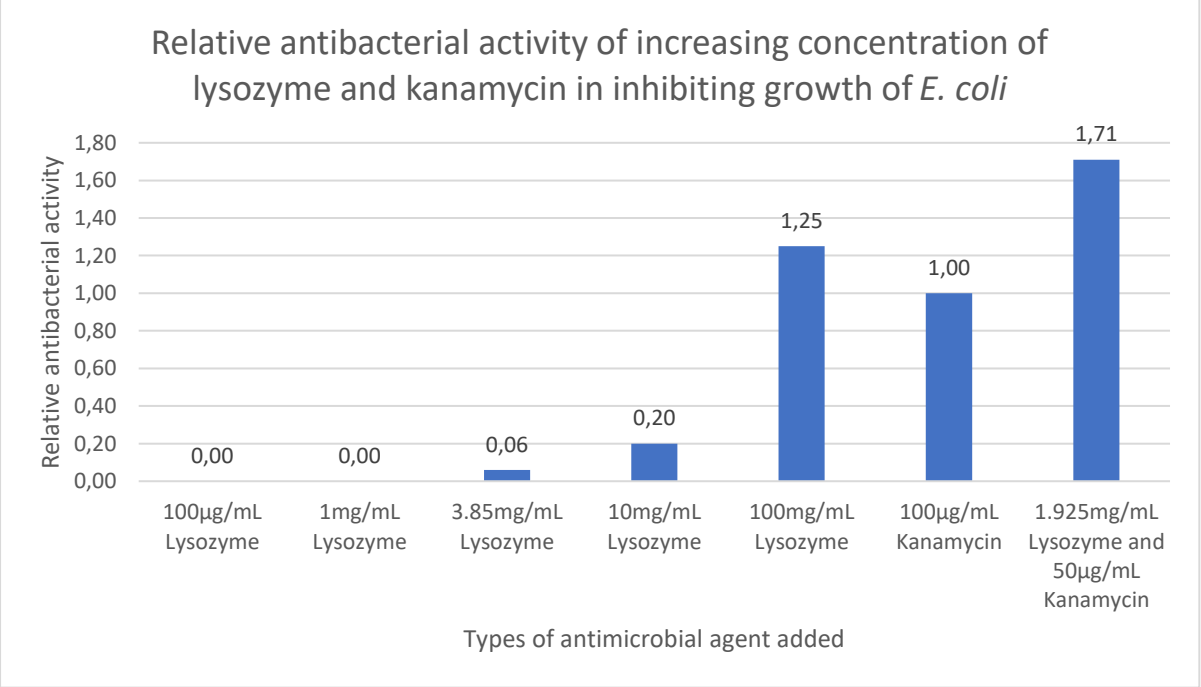

Figure 4 The relative antibacterial activity of lysozyme solutions and antimicrobial mixture compared to kanamycin set as a control.

Interpretation of data

As shown in table 5, there is a positive trend between the increasing lysozyme concentration and the diameter of the zone of inhibition formed in *E. coli* culture. While 100µg/mL and 1mg/mL of lysozyme did not produce visible antibacterial activity, 3.85mg/mL, 10mg/mL, and 100mg/mL of lysozyme demonstrated a progressively stronger effect in inhibiting the growth of *E. coli* by producing a larger average diameter of the zone of inhibition. As indicated by $R^2 = 0.996$ which is extremely close to 1, there is a strong linear positive correlation between the increasing lysozyme concentration and the area of zone of inhibition produced as shown in figure 3.

Assuming that the relationship between the lysozyme concentration and the zone of inhibition follows the linear regression model y = 1,3361x + 1,9652 presented in figure 3, one can interpolate that 79.4mg/mL lysozyme is required to produce 108mm^2 of inhibition zone – the average area of the zone of inhibition produced by 100µg/mL kanamycin. Since kanamycin demonstrates the same antibacterial activity as the 794 times more concentrated lysozyme solution, it can be interpreted that kanamycin is roughly 800 times more bactericidal than lysozyme is against *E. coli.*

The relative antibacterial activity values in figure 4 indicate that 3.85mg/mL lysozyme (the lysozyme concentration found in poultry egg white) has only 6% of the relative antibacterial activity that 100µg/mL kanamycin demonstrated against *E. coli*. Lastly, the antimicrobial mixture containing a lower concentration of lysozyme (1.925mg/mL) and kanamycin (50µg/mL) formed the largest zone of inhibition, demonstrating relative antibacterial activity 71% and 37% higher than in trials with standard 100µg/mL kanamycin treatment and 100mg/mL lysozyme, respectively. Most importantly, the T-test results shown in figure 2 confirms that there is more than 99% statistical significance that the inhibition area produced by the antimicrobial mixture is significantly larger than that of 100µg/mL kanamycin. This indicates that replacing half the volume of kanamycin with a lysozyme solution of the equivalent concentration as poultry egg white results in enhanced antibacterial activity against *E. coli*.

Conclusion

Unfortunately, the antibacterial activities of 100µg/mL and 1mg/mL lysozyme solutions were not observed when diffusion test was conducted to measure the diameter of the zone of inhibition formed on *Escherichia coli* culture after 24 hours of incubation at 37°C. However, the increasing diameter of the zone of inhibition for 3.85mg/mL, 10mg/mL, and 100mg/mL lysozyme solutions allow this research to conclude that a linear positive correlation exists between lysozyme concentration and the area of the zone of inhibition. As postulated in the hypothesis, the chance that lysozyme reaches the peptidoglycan layer through the amphipathic lipopolysaccharide outer membrane increments with the increasing lysozyme concentration (Macrophi Inc, n.d.). Hence, the increasing lysozyme concentration results in greater antibacterial activity, inhibiting *E. coli's* growth more.

Interpolation using the linear regression model indicates that 79.4mg/mL lysozyme is required to produce antibacterial activity corresponding to 100µg/mL kanamycin. Since kanamycin demonstrates equal antibacterial activity as 794 times more concentrated lysozyme solution, kanamycin is roughly 800 times more bactericidal against *E. coli.* As predicted by the hypothesis, kanamycin demonstrates superior antibacterial activity owing to its polycationic aminoglycosides that displace divalent cations from the lipopolysaccharides and inner membranes, enabling kanamycin to freely enter *E. coli's* cytoplasm (Gallego, n.d.). Unlike lysozyme that relies on simple diffusion, such a self-promoted uptake mechanism allows kanamycin to easily cross through the outer membrane, allowing kanamycin to exert bactericidal pressure by interfering with the protein synthesis even at a low concentration (Gallego, 2023).

When kanamycin enters the bacterial cytoplasm and disrupts protein synthesis integral for the outer membrane's structural integrity, the peptidoglycan cell wall of gram-negative bacteria previously hidden under the lipopolysaccharide layer becomes exposed (Gonzalez, 1998). Consequently, the synergy between lysozyme and kanamycin enables lysozymes in the antimicrobial mixture to freely digest gram-negative bacterial cell wall by hydrolyzing glycosidic linkage connecting peptidoglycan's polysaccharide. While kanamycin continues to disrupt *E. coli*'s protein synthesis, lysozyme's simultaneous breakdown of peptidoglycan cell wall imposes three bactericidal pressures on *E. coli*: accelerating cell death through translation errors, leakage of intracellular contents, and cell lysis (Gonzalez, 1998). Such

synergic antibacterial activity explains the largest zone of inhibition observed from the diffusion test with an antimicrobial mixture composed of 25µL of 3.85mg/mL lysozyme and 25µL of 100µg/mL kanamycin. Even when mixing diluted the concentration of lysozyme and kanamycin into 1.925mg/mL and 50µg/mL, joint application of lysozyme and kanamycin demonstrates a higher bactericidal effect against *E. coli*.

Comparison of the relative antibacterial activity values indicates that lysozyme concentration in egg-white (3.85mg/mL) demonstrates only 6% of the antibacterial activity that kanamycin demonstrates at a concentration as low as 100µg/mL against *E. coli*. Thus, it is evident that lysozyme in egg-white is not sufficient to suppress the growth of gram-negative bacteria in poultry products, and supplementary antibiotics are necessary to aid poultry embryo's defense against infectious diseases. As shown in trials with the antimicrobial mixture, a joint application of lysozyme with kanamycin produces synergic antibacterial activity that is significantly more bactericidal against *E. coli* than when either of them is applied separately. The antimicrobial mixture demonstrated 37% and 71% higher antibacterial activity compared to trials with 100mg/mL lysozyme and 100µg/mL kanamycin, respectively.

Hence, rather than relying upon kanamycin as the sole antimicrobial agent in poultry farms, this research concludes that adding lysozyme together with kanamycin as an antimicrobial mixture can more successfully inhibit the growth of gram-negative bacteria by resolving the structural challenge posed by the outer membrane on lysozyme's activity. As the T-test shows, there is more than 99 percent statistical significance that antibacterial activity produced by the antimicrobial mixture is higher than that of kanamycin. Hence, although administration of additional antimicrobial agents is necessary to aid poultry product's defense against gram-negative bacteria, antibiotic overuse can be mitigated by administering lysozyme as an alternative supplementary antimicrobial agent alongside kanamycin; their synergic antibacterial activity elevates the antimicrobial mixture's bactericidal effect on *E. coli* even with a lower concentration of lysozyme and kanamycin. Such a joint application can further reduce the selection and spread of multidrug-resistant bacteria from poultry farms as it is harder for bacteria evolve resistance against peptidoglycan-degrading enzymes (P. Ferraboschi, 2021).

Evaluation

Evaluation of the hypothesis

Measurement of the zone of inhibition with the increasing concentration of lysozyme aligns with the prediction that solutions with higher lysozyme concentration will demonstrate stronger antibacterial activity against *E. coli*. However, this positive trend of lysozyme concentration and diameter of zone of inhibition constitutes only three data sets as no zone of inhibition was measured for 100µg/mL and 1mg/mL lysozyme solutions. Therefore, the antibacterial activity of a greater variety of lysozyme concentrations must be measured to fully support the hypothesis and validate the positive correlation between lysozyme concentration and its area of the zone of inhibition. The comparison of relative antibacterial activity values strongly supports the hypothesis that kanamycin is more bactericidal than lysozyme against gram-negative bacteria strains like *E. coli* found on poultry products.

Strengths of the research

Finding the average zone of inhibition from five repeated trials reduces the influence of random error and allows the research to distinguish outliers by collecting a sufficient replicate of the dependent variable. Furthermore, all variables apart from the independent variable were controlled successfully. This allows the experiment to produce consistent measurements of the zone of inhibition when replicated, ensuring the reliability of the research. As the *E. coli* strain used in this experiment was ampicillin resistant, treating the *E. coli* culture with ampicillin ensured that there were no other bacteria present on the agar medium to interfere with the measurement. This allows the results to present accurate relative antibacterial activity values of different antimicrobial agents against *E. coli*, increasing the validity of the research. Also, the linear regression model presented in figure 3 passes through all the error bars that represent the standard deviation of the inhibited area. This indicates that experimental errors associated with the apparatus (ruler with ±0.5mm absolute uncertainty) or human errors when measuring the diameter of the zone of inhibition are smaller than the standard deviation, further increasing the reliability and the validity of the data collected. Lastly, considering how lysozyme and kanamycin are water-soluble, the use of diffusion test suited for determining the antibacterial activity of polar antimicrobial agents can be evaluated as an appropriate choice in methodology (Microchem, 2022).

Limitations of the research

This research has performed the diffusion test by boring a cylindrical well on the agar plate. However, filling a well with antimicrobial agents allows them to diffuse throughout the entire cross-section of the agar medium when *E. coli* can only grow on the surface of the agar plate. Consequently, only a fraction of the antimicrobial agents supplied into the wells would have acted against *E. coli*, with most being wasted within the agar medium. This fault in the methodology could have reduced the diameter of the zone of inhibition that would otherwise have been observed and measured.

Furthermore, there was a lack of quantity and consistency of the variation of the independent variable – the concentration of lysozyme used in the experiment. Though five different lysozyme concentrations were chosen (including the lysozyme concentration found in egg white), antibacterial activity could not be measured for trials with 100µg/mL and 1mg/mL of lysozyme. Consequently, antibacterial activity was measured for only three different lysozyme concentrations, which may not be sufficient to validate the positive trend between independent and dependent variables with a complete certainty. Also, increasing the concentration by tenfold creates unequal lysozyme concentration differences between each treatment.

Suggestions of improvement

To prevent antimicrobial agents from being wasted in the agar medium, diffusion discs can be used instead of boring cylindrical well. Placing diffusion disc containing antimicrobial agents on top of the agar medium will allow antimicrobial agents to mainly diffuse across the surface on which *E. coli* grow. Consequently, performing disc diffusion test can improve this investigation's methodology by measuring the diameter of the zone of inhibition more accurately with minimal loss of antimicrobial agents into agar.

In addition, making equal and more numerous variations of the lysozyme concentration (like 20mg, 40mg, 60mg, 80mg, and 100mg per mL) can increase the reliability and validity of the linear regression (best-fit line) used to model the positive correlation between lysozyme concentration and its antibacterial activity. Though the R^2 value calculated implies a strong

positive trend, this research cannot confidently conclude whether the antibacterial activity of lysozyme increases linearly or non-linearly throughout the concentration tested owing to the large gap between 10mg/mL and 100mg/mL lysozyme. With a best-fit model that provides a complete picture of the relationship between lysozyme concentration and the zone of inhibition, the optimal lysozyme concentration that produces the maximal bactericidal effect against *E. coli* can also be determined.

Lastly, imaging software can be utilized instead of a ruler to reduce human errors in measurements of the zone of inhibition, which can improve the reliability of data and increase accuracy in relative antibacterial activity values calculated.

Suggestions of extension

Due to the lack of non-pathogenic *Staphylococcus* strains in the laboratory at the University of Oslo, this research merely assumed that lysozyme would be able to exert its full bactericidal effect against gram-positive strains found on poultry products owing to their lack of lipopolysaccharide outer membrane. To quantify the relative antibacterial activity of lysozyme and kanamycin associated with gram-positive bacteria detected in poultry products, future studies should attempt to replicate this experiment using *Staphylococcus* cultures.

Furthermore, as there are other major antibiotics used in the agricultural industry, it is worth comparing the antibacterial activity of lysozyme with different types of antibiotics. While kanamycin targets bacteria's protein synthesis, there are other antibiotics like penicillin and vancomycin used in agriculture that disrupt the synthesis of bacterial peptidoglycan cell wall to neutralize bacteria (Romaniuk, 2015). Hence, investigating the antibacterial activity of lysozyme in a larger context will allow future research to determine whether lysozyme can be used as an alternative antimicrobial agent in agricultural practices to mitigate the global spread of multi-drug resistant bacteria strains.

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