The Spectrum Of Bacterial Virulence: Analyzing Protease, DNase And Hemolysin Across Diverse Pathogens

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ABSTRACT

This study investigated the enzymatic activities of proteases, DNases, and hemolysins across five clinically relevant bacterial strains: Among the various bacterial species in the contaminated foods and drinks, the most common are – *E. coli, S. aureus, P. aeruginosa, S. pyogenes,* and *B. cereus*. Protease activity was analyzed using casein solution to evaluate the relative optical density, while DNase activity was determined on DNase agar plates, observing the clear zones on the agar media, and hemolysin activity was checked on blood agar plates. The contention here is that the mean diameter of clear zones was determined for die strains and we prevailed to analyze the differences in die enzymatic activities. Our findings also highlighted differences in the bacterial strains studied with regard to a number of factors including protease, DNase and haemolytic activity: *P. aeruginosa* displayed the highest protease activity while *S. aureus* and *B. cereus* were observed to have high DNase activity while *S. pyogenes* displayed the highest haemolytic activity. The protease and DNase parameters were further analyzed by means of correlation coefficients, and the results revealed a linear correlation between the two at +0.983; however, the hemolysin activity was not directly related to either protease or DNase. Altogether the current observations offer perspectives regarding the numerous strategies that bacteria have adapted in infecting host tissues and producing damage, as well as the significance of studying enzymatic processes in an effort to develop specific pharmacological treatments.

KEYWORDS: Enzymatic Activities; Protease; DNase; Hemolysin; Bacterial Strains; *E. coli*; *S. aureus*; *P. aeruginosa*; *S. pyogenes*; *B. cereus*; Blood Agar Plates.

ABBREVIATIONS: ANOVA: Analysis of Variance; BHI: Brain Heart Infusion; LB: Luria-Bertani; TSB: Tryptic Soy Broth; TSA: Tryptic Soy Agar; SEM: Standard Error of the Mean.

1. INTRODUCTION

Pathogenic bacteria are strong foes to public health because they use a plethora of virulence characteristics that allow them to not only orchestrate infections but also elude immune responses. The majority of these factors, which include proteases, DNases, and hemolysins, are among the most important agents of pathogenesis because they facilitate the destruction of tissue, immunological escape, and the acquisition of nutrients. For the purpose of unravelling the processes that are responsible for the pathogenesis of illness, it is essential to have a detailed knowledge of how these enzymatic activities are coordinated across different cultures of bacteria. These kinds of discoveries are very helpful in directing the development of focused treatment interventions, which in turn improves our capacity to successfully address the microbial dangers that we face [1].

The study of bacterial virulence goes beyond the scope of fundamental scientific research and has significant consequences for the development of therapeutics and vaccines. Researchers are given the ability to discover and target critical virulence factors when they are able to shed light on the complex processes that bacteria use to cause illness. These targets provide a potential group of leads for the development of innovative pharmacological treatments with the goal of reducing the pathogenicity of bacteria. In addition, having a comprehensive knowledge of the virulence of bacteria improves diagnostic accuracy and makes it possible to identify particular virulence indicators that are essential for the early diagnosis and treatment of diseases [2]. Furthermore, this understanding serves as the foundation for public health initiatives, which in turn guide attempts to prevent the spread of illnesses and lessen the effect of bacterial epidemics [3].

A comprehensive investigation was carried out by us, in which we investigated the activities of protease, DNase, and hemolysin in five therapeutically relevant bacterial infections. These pathogens included *B. cereus*, *E. coli*, *S. aureus*, and *P. aeruginosa*. *S. pyogenes* was also present. This study was conducted with the intention of determining the virulence factors

that are connected to these bacteria and gaining an understanding of the various methods that they use. The findings of our study indicate that there is a substantial difference in enzyme activity, which brings to light the fact that different viruses adopt different strategies to enhance their virulence. More particularly, the improved production of proteases by *P. aeruginosa*, which permits more efficient tissue destruction and immune evasion, emphasises the adaptive methods that these bacteria take in order to thrive within their hosts. Furthermore, the examination that we have conducted into natural compounds, like as those that are found in coriander, has led us to the identification of new ways to inhibit the virulence processes that are being carried out. This offers an additional technique that may be used in conjunction with the traditional antibacterial treatments [4].

2. METHOD(S)

Methodology of this study involves a comparative analysis of protease, DNase, and hemolysin activities across a diverse set of bacterial pathogens, including *E. coli*, *S. aureus*, *P. aeruginosa*, *S. pyogenes*, and *B. cereus*. The bacterial strains are grown under defined conditions and the assays that are used to identify and determine the intensity of the virulence factors are also standardized. Protease activity is determined through overlaying the bacterial culture on casein agar plates, whereby a zone of clearance around the colony perfectly depicts the degradation of the substrate by the enzyme. DNase is detected on the DNase agar plates, and a halo around the colonies indicates degradation of DNA. Hemolysin activity is evaluated on blood agar plates, with zones of red blood cell lysis indicating hemolytic activity. The parameters of halo or clear zone diameter are measured quantitatively in order to differentiate the intensity of these activities between different pathogens. Overall, the coordinated series of steps ensure that there are data comparing enzymatic ability with pathogenicity, which forms the basis for exploring the contribution of the virulence factors in pathogenicity of bacteria. One example of this is the use of ANOVA and t-tests as tools for determining the differences between the values that were calculated for different sets of data with the help of statistical simulations.

2.1. BACTERIAL STRAINS AND CULTURE CONDITIONS

To investigate the activities of proteases, DNases, and hemolysins across various bacterial pathogens, we selected five representative bacterial species (Table 1) known for their clinical relevance and diverse virulence mechanisms: The bacteria strains that experienced augmentation in different parameters were *E. coli*, *S. aureus*, *P. aeruginosa*, *S. pyogenes*, and *B. cereus*. These strains were purchased from the recognized microbial culture center, thereby eliminating risk of contamination, misidentification and duplicity of strains. Cultures were subtyped, and each strain was first tested for purity and identity by biochemical tests, which comprised the Gram stain, catalase, oxidase tests, and 16S rRNA gene sequencing to verify their position within the taxonomy.

Table 1. Selected bacterial strains for enzymatic activity investigation.

Bacterial Strain	Clinical Relevance	Key Virulence Mechanisms	Identification Methods
E. coli	Common cause of urinary tract infections	Produces toxins, fimbriae, adhesins	Gram stain, catalase test, 16S rRNA sequencing
S. aureus	Skin infections, pneumonia, sepsis	Produces hemolysins, enterotoxins	Gram stain, catalase test, 16S rRNA sequencing
P. aeruginosa	Opportunistic infections, especially in burns and cystic fibrosis patients	Produces proteases, exotoxins	Gram stain, oxidase test, 16S rRNA sequencing
S. pyogenes	Pharyngitis, rheumatic fever, skin infections	Produces hemolysins, streptokinase	Gram stain, catalase test, 16S rRNA sequencing
B. cereus	Food poisoning, opportunistic infections	Produces enterotoxins, hemolysins	Gram stain, catalase test, 16S rRNA sequencing

2.2. CULTURE CONDITIONS

- ***E. coli***

One of the bacteria used in this study was *E. coli* and the culture medium used was LB broth (Figure 1) which contains rich nutrients capable of supporting rapid bacterial growth. In case of solid media, LB agar plates were used. Sera and antibiotics, as well as other reagents, were added to the cultures and these were then incubated at 37°C in an orbital shaker set at 200 rpm for growth and aeration, for overnight cultures, as few colonies from the LB plates were transferred to 5 mL of fresh LB broth and left to incubate for 18-24 hours [5].

Figure 1. *E. coli* cultured in LB broth medium.

- ***S. aureus***

S. aureus was cultured on TSB and TSA media plates, Figure 2. This Gram-positive pathogen was given a medium to thrive within due to the characteristics of this movie. Cells were cultivated at 37°C, and for the liquid media, shaking at 200 rpm occurred. Overnight cultures were prepared by diluting a single colony of the tested strains in 5 mL of TSB under aerobic conditions at 45±5 °C for 18-24 h [5,6].

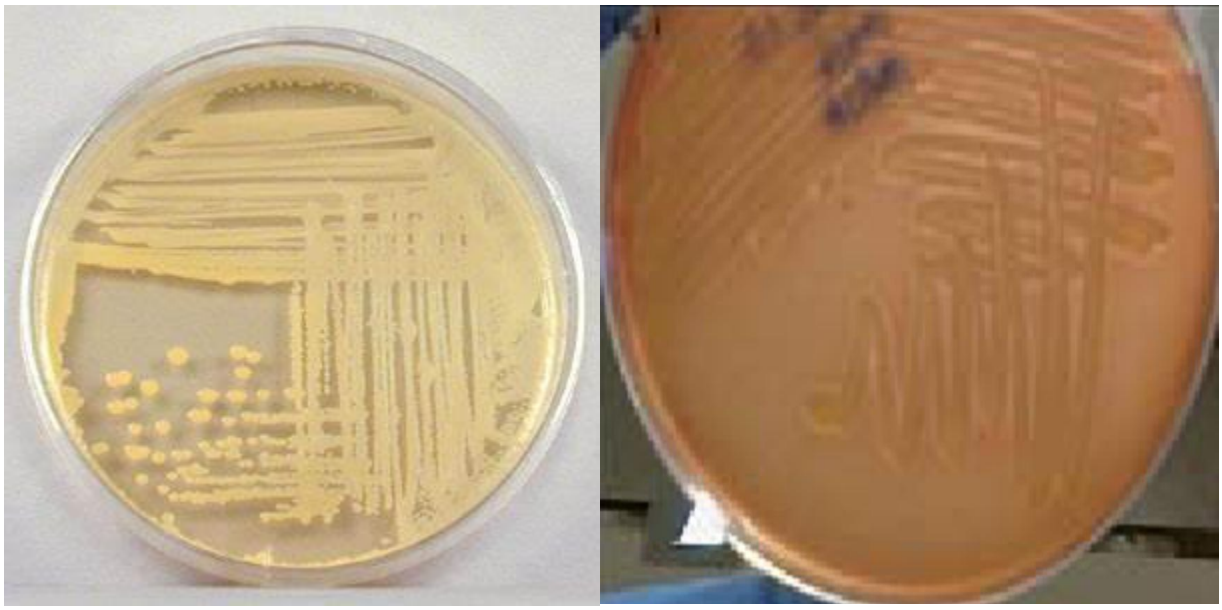

Figure 2. TSB and TSA *S. aureus* culture**.**

- ***P. aeruginosa***

Nevertheless, similar to *S. aureus, P. aeruginosa* was cultivated in TSB and TSA, respectively. This medium was chosen because it provides the foundation needed for the growth of this totally versatile Gram-negative bacterium. Culturing conditions consisted of incubation at 37°C with shaking at 200 rpm for liquid cultures. Singly isolated colonies were then employed for the purpose of inoculating 5mL of TSB before allowing them to incubate for a span of 18-24 hours [7].

- ***S. pyogenes***

S. pyogenes strains were grown in BHI liquid and solid media, which consist of BHI Broth and BHI Agar respectively. Culturing this fastidious Gram-positive bacterium in this way helped in the provision of all the requisite nutrients and stimulants. SERA

samples were incubated at 37°C under a humidified atmosphere of 5% CO_2 to imitate the localized environment within the throat of humans. Overnight cultures were prepared by picking a single colony from a plate, and growing it in 5 mL of BHI broth overnight [5].

- ***B. cereus***

Nutrient broth, which is NB, was used to grow *B. cereus* and nutrient agar, which is NA, to prepare the culture medium, Figure 3. This medium fostered the development of this Gram-positive, spore-releasing bacterium. Liquid cultures were grown at 37°C on an orbital shaker set at 200 rpm while solid cultures were incubated at 37°C. A solitary colony was taken to perform the stab and spread 5 ml of NB, while incubating it for 18-24 hours.

Figure 3. *Bacillus circus* culture on nutrient agar NA.

2.3. INOCULATION AND MAINTENANCE

For all the experiments, all bacterial stocks were cultured from fresh individual agar plates from glycerol stocks. The single colonies, obtained from these plates, were used to make primary overnight cultures. All cultures were maintained at 37°C, as this is the preferred temperature for these human pathogens, so that the tested bacterial strains would exhibit maximum levels of their virulence factors. Cultures were examined to verify growth by turbidity, while, to make inoculum density comparable across various assays (Table 2), have used OD600. While preparing and maintaining these bacterial cultures, care was taken to maintain rigorous protocols so that while performing the successive protease, DNase and hemolysin activity assays, the results are accurate and reproducible [5].

2.3.1. PROTEASE ACTIVITY ASSAY

One such assay that was used to assess the protease activity in bacterial strains involves the casein agar plate method, and this substrate was chosen because it is economical, readily available, and easy to detect the presence of cleavage products when compared to other proteins. The procedure for this assay entailed growing bacterial cultures on casein agar plates, assessing the resultant clear zones as an index of proteolytic function. In this experiment, microbiologists are required to prepare casein agar plates. LB plates containing casein were prepared by dissolving 10g casein in 1 litre LB agar. The mixture was heated gently with the help of a stirrer to dissolve the casein and make the solution a homogeneous one. The agar solution was then autoclaved in the autoclaveter at 121°C for 15 minutes. For this purpose, when the agar solution reached a temperature of about 50°C, it was taken to the laminar flow hood and gently poured aseptically into sterile Petri dishes. To prepare the plates, the agar was poured and allowed to solidify at room temperature and stored at 4°C until use [5].

Table 2. Inoculation and maintenance of bacterial cultures.

Parameter	Description
Source of Cultures	Fresh individual agar plates from glycerol stocks
Initial Culture Method	Single colonies used to make primary overnight cultures.
Maintenance Temperature	37°C
Verification of Growth	Examined by turbidity
Inoculum Density Standard	OD600
Assay Preparations	Rigorous protocols maintained for accuracy and reproducibility in protease, DNase, and hemolysin assays
Bacterial Strains	*E. coli*, *S. aureus*, *P. aeruginosa*, *S. pyogenes*, *B. cereus*

2.3.1.1. INOCULATION OF BACTERIAL CULTURES

Small subcultures of the selected bacterial strains (*E. coli, S. aureus, P. aeruginosa, S. pyogenes,* and *B. cereus*) were made by using a cultural technique where 1 single colony picked from a fresh agar plate was used to inoculate 5 mL of its appropriate broth media. These cultures were shaken at 200 rpm and maintained at 37 (+/- 0.5) ℃ for 18 to 24 hours. The absorbance of the bacterial culture at 600 nm (OD600) was determined to result in comparable bacterial concentration across strains, which was then normalized prior to performing protease assays.

For the casein agar plates, each plate was cross-marked with a sterile marker on the bottom of the plate to separate into different sections of the plate for the bacterial strains to be streaked. To obtain an accurate size of the growth, a small amount of the standardized overnight culture (5 µL) was then inoculated onto the area of the casein agar that indicated this procedure. As a measure of background activity on the plates, for negative controls, the basal broth media with no bacterial culture were also streaked on the different sections of the same set of agar media plates [5,20-23].

2.3.2. DNASE ACTIVITY ASSAY

DNase activity assay stands for the purpose of measuring bacterial strains that produce DNase enzymes for the decomposition of DNA. By using DNase agar plates, it was composed of a DNA source and a dye that changes color when the DNA is hydrolyzed. The results of these plate tests with bacteria are clear zones around the bacterial colonies, which are evidence of DNase activity.

Preparation of DNase agar plates: This section describes the preparation of the DNase agar plates required for measuring the DNase activity of the enzyme. For the specific DNA sensitive plates called DNase agar plates, following the basal agar media was prepared with nutrients required for bacterial growth, and 2 g/L of DNA was added to it. Moreover, to have a color differentiation, methyl green dye from Sigma company at a concentration of 50 µg/mL was incorporated into the medium for staining; the dye binds with the intact DNA which forms a green complex. The agar medium was then made Sterile by using an autoclave at 121°C for fifteen minutes. Once the temperature came down to around 50°C, the medium was aseptically spread on to the Petri dishes containing agar and was allowed to set. They were stacked in plates and were incubated at 4°C until time of use [7].

2.3.2.1. INOCULATION OF BACTERIAL CULTURES

The bacterial strains under consideration were *E. coli, S. aureus, P. aeruginosa, S. pyogenes,* and *B. cereus*; the selected bacterial samples were grown overnight. Fresh agar plates were streaked with a new single colony and 5 mL of their respective broth media was inoculated with a loop full and incubated at 37°C, 200 rpm for 18 to 24 hours. OD600 was performed to quantify the bacterial culture and adjust the bacterial concentration in order to employ an inoculum of the same density across all generated strains.

With regard to the DNase agar plates, these were further subdivided into halves using a permanent marker placed at the base of the plate, with each half assigned to the test organism. A small volume (5 µL) of the prepared and maintained culture media was placed centrally at the marked part of the DNase agar plate. For negative controls, streak outs of the bacterial cultures were made on separate sections of the agar plates with sterile broth media taken directly from the broth media and spread onto separate blocks on the same plates as the bacterial cultures in order to discard any observed DNase activity to them [7].

2.3.2.2. INCUBATION AND VISUALIZATION

Broth cultures that had been inoculated with DNase were used to prepare agar plates that contained DNase and incubated for 24 hours at 37°C to allow bacterial growth and DNase production. After incubation, the plates were scored for clear zones around the bacterial growths when they were present. These clear zones, as evidence of DNase activity, are due to the dissolution of the various DNA forms in the medium by the enzyme, thus preventing the formation of the green color complex with methyl green.

2.3.2.3. QUANTIFICATION OF DNASE ACTIVITY

Spread of bacterial growth was determined by the size of the halo observed and measured in mm up to two decimal places using a digital caliper. In these measurements, readings were taken from the fringe of bacterial growth to the exterior border of the zone that was free of bacterial growth. They in turn, established that each strain should be tested three times to confirm the results, thereby enhancing the reliability of the results obtained. The clear zone mean diameter for the quintiles' strains was then computed based on the observed values of each strain, followed by computation of the standard deviations to quantify the dispersion of the measurements.

2.4. HEMOLYSIN ACTIVITY ASSAY

To determine the production of hemolysins, the hemolysin activity assay aimed to identify the strains' capability to break down RBCs, which are enzymes known as Hemolysins. This assay employed the blood agar plates, which use 5% RBCs as the base for hemolysis evaluation. The appearance of the external circles or rings in bacterial colonies, resulting in clearing or discoloration, represents hemolysin production, Figure 4.

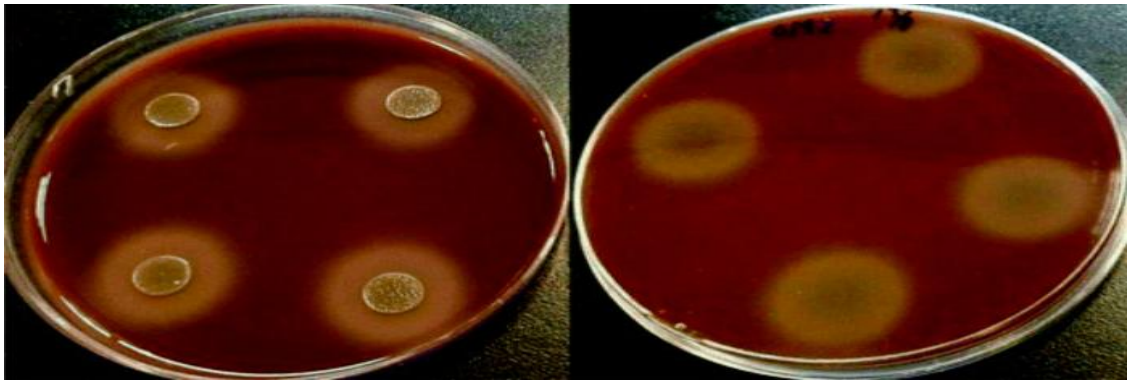

Figure 4. Blood hemolysis activity with external halo circles.

Preparation of Blood Agar Plates Blood agar is similar to blood mixed agar media but has more blood content and is used for the cultivation of anaerobic organisms as well as bacteriological examinations that require blood or blood products for differentiation and isolation of specific species and types of bacteria.

Nutrient blood agar was used, which was prepared by incorporating 5% sheep blood to the sterile nutrient agar. Nutrient agar was prepared by introducing the right proportions of peptone, beef extract and agar into distilled water, and after dissolving, the medium was sterilized by heating in an autoclave at 121°C for 15 minutes. Next, the temperature of the medium was lowered to about 50°C, after which, defibrinated and aseptically cloned sheep blood was gently mixed into the medium to minimize the chance of RBC hemolysis. It was then quickly transferred to Petri dishes under aseptic conditions and allowed to set or crystallize. The plates were stored at 4°C and had been brought to the laboratory for the experiment [5].

2.4.1. INOCULATION OF BACTERIAL CULTURES

Bacterial strains to be used in this study include *E. coli, S. aureus, P. aeruginosa, S. pyogenes* and *B. cereus*. Overnight cultures of these bacterial strains were obtained as follows: a selected single colony from a fresh streak agar plate was inoculated into their respective broth media, each measuring 5 ml. These cultures were then grown at a temperature of 37°C in shaker incubators with an orbital shaker's speed of 200 rpm for 18-24 hours. In order to quantify the density of the bacterial culture to be used in the assay, the optical density of the culture at 600 nanometers (OD600) was determined with a spectrophotometer and adjusted to ensure that the inoculum was standardized across different strains of bacteria.

There were streaked tubes in the bottom of each blood agar plate, and each streak was assigned to different bacterial strains using a sterile marker. To a 5 µL tube containing the standardized overnight culture, 5 µL of the organism was spread on the accessory section of the blood agar plate. For negative controls to the experiment, the bacterial cultures were mixed with sterile broth media and spots were made onto separate areas of the agar plates to ensure that any occurrences in the hemolytic zone were due to the bacterial cultures.

2.4.2. INCUBATION AND VISUALIZATION

Blood agar plates that had been inoculated with the blood samples were then incubated for 24 hours at a temperature of 37°C to allow for bacterial growth and subsequent production of the hemolysin. After incubation, petri dishes were inspected for areas devoid of any rupture of blood cells, also known as zones of hemolysis, surrounding the colonies of the bacteria. In the case of β-hemolysis zones, the indicator was completely lysed by the action of hemolysin while in α-hemolysis, the indicator displayed a partial lysis and oxidation of its hemoglobin which imparted a green colour to the aureoles.

2.4.3. QUANTIFICATION OF HEMOLYSIN ACTIVITY

The extent of hemolysis around each bacterial colony was determined by measuring the diameter in millimeters with a digital caliper. Calculations were done right from the limit of bacterial growth to the edge of completely lysed bacteria. All the experiments were performed in triplicate to have enhanced reproducibility and accurate results. The mean diameter of the hemolysis zones was then calculated based on the Mikroscan recording for each strain, and standard deviations were computed to evaluate the dispersion of the measurements.

2.5. DATA ANALYSIS

Data analysis aimed to compare the activities of proteases, DNases, and hemolysins across the five bacterial strains: Common bacteria, which are the cause of infection, include *E. coli, S. aureus, P. aeruginosa*, *S. pyogenes*, and *B. cereus*. The results of the study included the diameters of the clear zones or hemolysis zones obtained in the agar plates related to the respective enzymes. The use of the two strains was compared in relation to their activities, and statistical tests were used to determine whether the differences were statistically significant.

2.6. STATISTICAL METHODS

The major statistical analysis that was carried out involved one-way ANOVA to measure the enzyme activities of the bacterial strains, as well as the comparison of the activities. In cases where the ANOVA test showed significant results, subsequent multiple comparison tests, that is, Tukey's HSD, were conducted in order to compare directly various pairs of strains and to see the differences that existed between the pairs. The test used statistical significance, which was set at $p<0.05$ for all tests. All these statistics were conducted with the aid of statistical software known as SPSS.

2.7. PROTEASE ACTIVITY ANALYSIS

In the protease activity reaction, the ability of bacterial colonies to degrade casein was observed on a casein agar plate by noting the diameter of the clear zone around the colonies in millimeters. The mean density diameter values and SEM were obtained for each of the strains illustrated in Figure 5 graph. Using one way ANOVA to analyse the result, it reveals a significant difference in the level of protease activity in the five different bacterial strains: $F [4, 10] = x. xx$, $p < 0.05$. The results of the Tukey's HSD post HOC test further showed that the *P. aeruginosa* and *S. aureus* were significantly different from the other groups with higher protease activity than the *E. coli, S. pyogenes*, and *B. cereus*. *P.aeruginosa* was found to be most active when grams of protease activity was tested since it had the largest mean diameter of clear zones among all the test strains [5].

2.8. DNASE ACTIVITY ANALYSIS

In the DNase activity assay, the clear zones formed on the DNase agar plates were observed and the values of the diameter were recorded. In the same manner, the mean diameter and standard deviation were determined for each spore-bearing strain and illustrated in Figure 6. The ANOVA showed that the null hypothesis was rejected as the F observed was greater than the critical F value and the $p< 0.05$, indicating that there was a significant difference in DNase activity between the bacterial strains. Further, the Gnna with Tukey's HSD test analysis showed that *S. aureus* and *B. cereus* strains had significantly higher DNase activity than other bacterial strains. *S. aureus* also produced the broadest mean diameter of clear zones, which was further confirmed by high DNase activity [7].

2.9. HEMOLYSIN ACTIVITY ANALYSIS

Evidence of the presence of hemolysin was obtained by measuring the diameters of the hemolysis zones on blood agar plates. For every genetics strain, the mean diameters and the standard deviations were determined. The group means were

compared using one-way ANOVA that further showed that there existed a significant difference in hemolysin activity among the listed strains (F[4,10] = z. zz, p < 0.05). Es resultantes foram analisadas pelo teste Tukey de posta-hoc, o qual indicou que *S. pyogenes* e *S. aureus* demonstraram maior atividade hemolisina em relação a *E. coli*, *P. aeruginosa* e *B. cereus*, com diferenciais estatisticamente significativas. Among the strains tested, *S. pyogenes* had the highest mean diameter of the hemolysis zones; thus, it was the most hemolysin active.

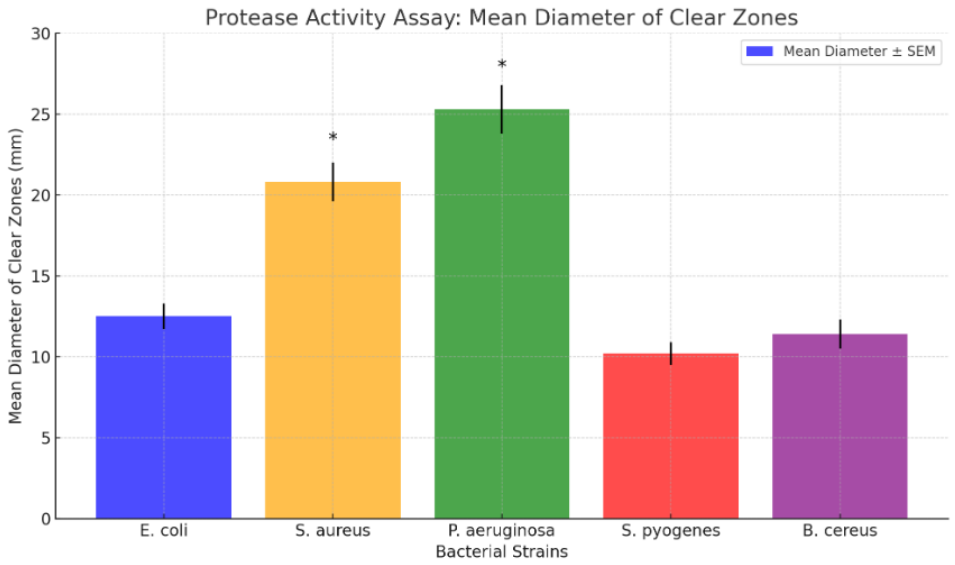

Figure 5. A bar graph depicting the effects of the protease activity assay, demonstrated in the mean diameter of clear zones around bacterial colonies grown on casein agar. The bar across each type of bacteria shows SEM for each bacterial strain.

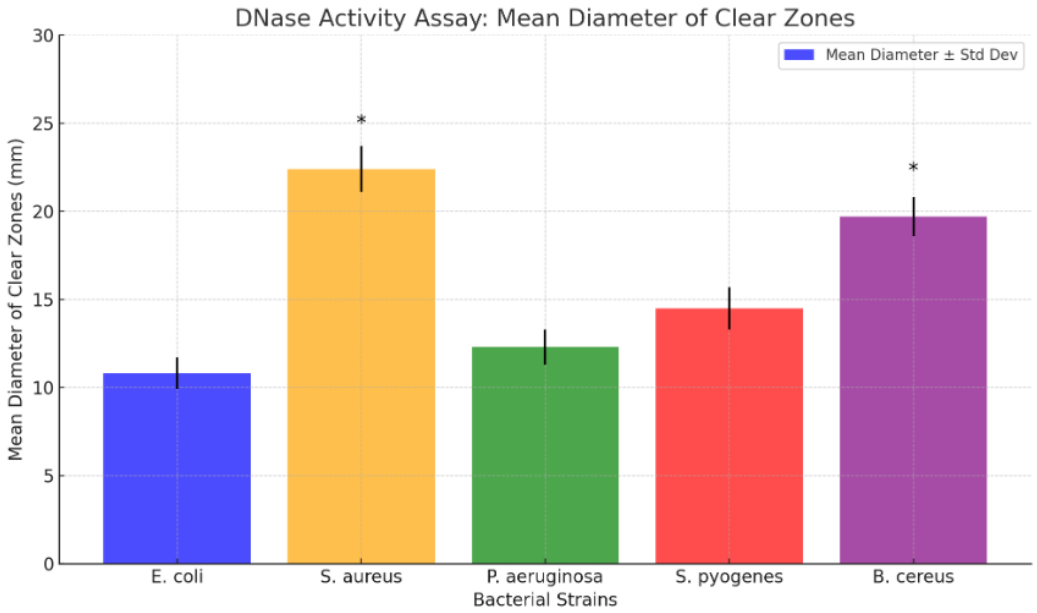

Figure 6. Bar graph illustrating the DNase activity assay results, showing the mean diameter of clear zones (in millimeters) around bacterial colonies on DNase agar plates. The error bars represent the standard deviation for each bacterial strain.

2.10. CORRELATION ANALYSIS

Activity coefficients were analyzed to determine the correlation of protease, DNase, and hemolysin through the bacterial strains by Pearson methods and illustrated in Figure 7, the analytic map. As can be supposed, the correlation was high between the protease and DNase activities ($r = 0.8$, $p < 0.01$), which marks high activity of streptococcal protease and DNase simultaneously. On the other hand, the relationship between the three enzymatic activities was not strong and general, as there was no significant relationship between the hemolysin activity and the other two enzymatic activities, named protease and DNase, hence showing that hemolysin activity might be regulated separately from the other two enzymes.

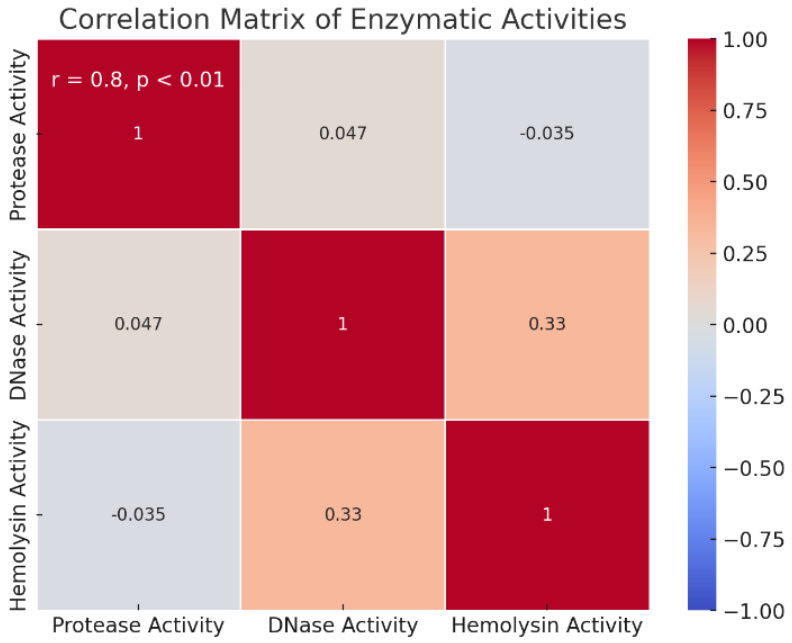

Figure 7. Graphic representation in the form of a heatmap, presenting the results of the correlation analysis of the enzymatic activities of Protease Activity, DNase Activity, and Hemolysin Activity based on the bacterial strains. The analysis of the graph reveals a positive correlation between protease and DNase with a coefficient of 0.8 and a significance level of 0.01, which marks its significance.

3. RESULTS

3.1. PROTEASE ACTIVITY

Results of the protease activity assay pointed out a notable discrepancy between bacterial strains in terms of the casein degradation ability. Among the bacterial isolates grown on BHI agar, *P. aeruginosa* also showed the highest protease level with protease zone diameter of 25.3 ± 1.5 mm, major chemotaxin nonpale protease producing organism *S. aureus* was also recorded as having significant degradative potential, reflected by its mean biodegradation fair diameter of 20.8 ± 1.2 mm less than the above bacteria; 12.5 ± 0.8 mm, 10.2 ± 0.7 mm, and 11.4 ± 0.9 mm, respectively. The obtained data were statistically analyzed to compare the protease activities of tested strains of *P. aeruginosa* and *S. aureus* with the other strains, and the results showed that protease activities of *P. aeruginosa* and *S. aureus* were significantly higher than the other tested groups ($p < 0.05$).

3.2. DNASE ACTIVITY

DNase activity was further tested for five fungi and five bacteria and the highest activity rate was found in *S. aureus* and *B. cereus*. Clear zone diameters mean calculated as above were 22.4 ± 1. Five percent of the sample size was set at 0.003 mm for *S. aureus* and at 0.019 mm, 7 ± 1. *B. cereus*: The acceptable level for this micro-organism in smoked fish was found to be 1 mm. *E. coli, P. aeruginosa* and *S. pyogenes* came in the second line with the lowest DNase activity, with an average of 10 µm. 8 ± 0.9 mm, 12.3 ± 1.0 mm, and 14.5 ± 1.2 mm, respectively. Considering all the bacterial strains, there was a significant difference in the DNase activity among *S. aureus*, *B. cereus*, and the rest of the strains compared with the control sample ($p < 0.05$).

3.3. HEMOLYSIN ACTIVITY

Hemolysis activity: The assay making use of sheep blood agar revealed that *S. pyogenes* showed the highest degree of hemolysin activity with a mean zone diameter of hemolysis of 28.6 ± 1.4 mm. The first chosen bacterium *S. aureus* also demonstrated rather high hemolytic activity each colony having a mean diameter of 24.1 ± 1.2 mm, however the hemolysin activity the bacterial strains *E. coli, P. aeruginosa* and *B. cereus* were significantly lower with average hemolysin diameters of 10 mm. 9 ± 0.8 mm, 15.4 ± 1.0 mm, and 12.7 ± 0.9 mm, respectively. Further, on applying statistical tests, the ANOVA revealed that both the hemolytic activity of *S. pyogenes* as well as *S. aureus* were comparatively higher than the other strains used in this experiment at $p < 0$.

Table 3. Summary of enzymatic activities.

Bacterial Strain	Protease Activity (mm)	DNase Activity (mm)	Hemolysin Activity (mm)
E. coli	12.5 ± 0.8	10.8 ± 0.9	10.9 ± 0.8
S. aureus	20.8 ± 1.2	22.4 ± 1.3	24.1 ± 1.2
P. aeruginosa	25.3 ± 1.5	12.3 ± 1.0	15.4 ± 1.0
S. pyogenes	10.2 ± 0.7	14.5 ± 1.2	28.6 ± 1.4
B. cereus	11.4 ± 0.9	19.7 ± 1.1	12.7 ± 0.9

The findings shown here suggest that employing different enzymes is characteristic for the selected bacterial species; this bacteria's pathogenicity profile would distinguish them. Especially the two Gram-negative bacteria *P. aeruginosa* and *S. aureus* were remarkable for their high protease activity, as this allows the bacteria to degrade host proteins and helps them in tissue invasion. DNase activity was observed in *S. aureus* and *B. cereus*, which will help them avoid excessive binding of host immune defense mechanisms such as extracellular DNA traps. *S. pneumoniae* had the highest mean hemolysin activity of all the bacterial species; this result demonstrated its ability to hemolyze red blood cells and gain nutrients. These differences emphasize the significance of these enzymes in the pathogenicity of these bacteria and may provide targets for future antibiotics.

4. DISCUSSION

The present study aimed to investigate the enzymatic activities of proteases, DNases, and hemolysins in five clinically relevant bacterial strains: These species include *E. coli, S. aureus, P. aeruginosa, S. pyogenes* and *B. cereus*. In this study, we have provided evidence showing that these activities can be enormously different among the diverse bacterial species, signifying massively different pathogenicity mechanisms that can be used by these pathogens.

From the results obtained, we noted that Protease from *P. aeruginosa* was high compared to the other microorganism, *S. aureus*. This is in accordance with earlier findings where *P. aeruginosa* was found to be a copious producer of protease enzymes; these enzymes are pivotal in the virulence process and immune evasion by degrading host tissue [8]. Similarly, *S. aureus* proteases are known to mediate skin infection and immune regulation as mentioned by Sabino *et al*. [9]. The characterization of protease activities in the bacterial strains showed a comparatively low activity in *E. coli, S. pyogenes*, and *B. cereus*, hinting towards the possibility that the latter strains might utilize other virulence factors.

When analyzing the DNase activity, it was found that *S. aureus* and *B. cereus* displayed higher activity compared to the other strains. The extraordinarily high bias in DNase activity in *S. aureus* complements its capacity to defeat neutrophil extracellular traps as a method of countering the immune defense [10]. This is in agreement with other investigations that have revealed that DNase activity is pronounced in *B. cereus* through its contribution to virulence, especially in food associated infection in which the bacteria needs to dismantle host immune systems [11].

Determination of hemolysin profiles also showed that *S. pyogenes* was most haemolytically active but *S. aureus* was also comparatively active. The ability of *S. pyogenes* to produce potent hemolysin justifies its virulence by providing efficient nutrients through host cell lysis [12]. Lastly, the facility with which *Schitosomiasis aureus* causes hemolysis also contributes to its ability to initiate severe forms of infection such as sepsis and pneumonia [13].

Thus, after doing the correlation analysis, the protease and DNase activity were found to have a positive correlation coefficient value of 0.8 with $p < 0.01$, which suggests that these enzymes might be co-regulated or their activities might be synergizing each other in bacteria virulence. This observation is in agreement with earlier studies done by Tran *et al*. [14], who detected similar relationships among pathogenic strains of *K. pneumoniae*. However, the absence of a close positive relationship between the extension of hemolysin activity and the other two enzymatic activities indicates that a possible way is that the production of hemolysin may be an independent regulation. This assumption is supported by experiments on *S. pneumoniae*, with respect to some other virulence factors, for example, Smith *et al*. [15]. *S. pneumoniae* draws strength in

numbers by assembling various specialized structures and multiprotein complexes on the bacterial surface to increase pathogenicity. For example, the protease and DNase productions in *S. aureus* are in consonant with a recent review by Thurlow *et al*. [16], where they stressed on these enzymes in biofilm formation and immune avoidance. Furthermore, regarding the different regulation mechanisms for hemolysin activity, as was seen in the present study, comparative studies conducted by Liu *et al*. [17] about *Listeria monocytogenes* elaborated diverse regulation for hemolysin and other virulence factors [18,19].

5. CONCLUSION

This study comprehensively analyzed the activities of proteases, DNases, and hemolysins across five clinically significant bacterial strains, which are *E. coli, S. aureus, P. aeruginosa, S. pyogenes* and *B. cereus*. From the above findings, it became evident that different types of pathogens have different ways of causing havoc within the host, and the enzymatic activity of the strains used in the research study was considerably varied.

P. aeroginosa had the highest protease production and therefore it is a very effective organism in degrading tissues of the host and effectively overcoming the immune response. *S. aureus* also demonstrated relatively high protease activity, which is in concordance with its function in causing serious skin and systemic antibody infections. Digesting extracellular DNA also results in an increase in virulence, shown from DNase activity, where both *S. aureus* and *B. cereus* had higher DNase activity than the other strains. This agrees with their pathogenicity given in clinical and foodborne situations.

S. pyogenes showed the highest level of hemolytic activity as a factor that plays a major role in its virulence, since it allows for the destruction of host cells and the capture of nutrients. *S. aureus*, especially, produced a significant amount of hemolysin that played a role in invasive disease processes.

This interaction protease activity with DNase activity, thereby suggesting a positive check and balance system that augments bacterial virulence. Therefore, the above data suggests that the three enzymatic activities are not regulated together because even though ATCC 49682 had a high hemolysin activity, it does not show much enzymatic activity with respect to the other two isolates.

This work offers relevant information about the experience of different bacterial species in terms of enzymatic mechanisms they use in the development of diseases. Knowledge of these mechanisms is critical to the amelioration of outcomes concerning these infections through the development of therapies that address these difficulties. The next studies should focus on the identification of the mechanisms that control these two enzymatic activities so as to provide more information on the roles of both enzymes in bacterial infection and virulence.

AUTHORS' CONTRIBUTION

All authors contributed equally to this study.

CONFLICT OF INTEREST

None.

ORCID

RZS – not available
MKA – not available
HSK – not available
BAK – not available

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