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Modulation of Virulent Factors in *Klebsiella pneumoniae* Exposed to Different Hydrogen Ion Concentrations

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ABSTRACT

The current investigation was aimed at determining the levels of some virulent factors that evolve in Klebsiella pneumoniae that are treated with different temperatures (-5, 4, 25 and 37 °C), pH (4 and 11.7) and oxygen level. The growth response of K. pneumoniae under these conditions was analyzed quantitatively by measuring the optical densities of the cultures. Biofilm formation was analyzed using the crystal violet method. Secreted metabolites were qualitatively observed on thin-layer chromatography while isolates were subjected to disk diffusion-based antibiotic susceptibility testing. Under the acidic condition, a significant reduction in bacterial growth in comparison to the control cultures were observed at all tested temperatures 37 °C (p=0.0088), 25 °C and 4 °C (p=0.0006) and -5 °C (p=0.0022). Similarly, there was a significant decrease in K. pneumoniae growth under basal conditions compared to 37 °C p<0.05. Biofilm formation was significantly reduced in acidic media under the aerobic condition (p=0.0259) while the basic media showed a similar significant reduction in the anaerobic condition (p=0.0150). Thin-layer chromatography showed the formation of bands at -5 °C and 4 °C with Rf of 0.6 and 0.9 both in acidic media. Antibiotic susceptibility tests demonstrated different patterns of the clearance zones with multiple antibiotic resistance (MAR) indices that fluctuate with increasing temperatures. Acidic and basic media also induced higher MAR indices of 0.6 while the control exhibited a lesser value of 0.0. The acidic medium under the anaerobic condition also induced a similar MAR index of 0.6 while basic media had 0.2. Conclusively, the exposure of K. pneumoniae to different conditions of pH, temperatures, and oxygen showed increases in growth, biofilm, secretion of metabolites, antibiotic resistance and susceptibility in some conditions, suggesting that these conditions promote virulence.

Keywords: *Culture, Oxygen, Biofilm, Antibiotic, Chromatography, Hydrogen, Ions, Water*

INTRODUCTION

Many microbes are exposed to constant stress, especially due to the host microenvironment. These stress factors could range from host defense mechanisms to external factors such as nutrients, temperature, oxygen conditions, food and herbal drugs [1] [2]. The cell wall acts as an important barrier that protects the cellular component of the bacterium from the exterior surroundings [3]. The cell wall is also key in communicating with the external environment through allowing for the transport of vital nutrients and molecules through signal transduction. At the same time, the cell wall insulates the bacterium from osmotic and oxidative stress as well as modulating the response to antimicrobial drugs [4].

Klebsiella pneumoniae is a prominent Gram-negative pathogen that causes a wide array of serious infections especially in immuno-compromised individuals. These infections include pneumonia, urinary tract infections, and bloodstream infections, with the potential for high morbidity and mortality [5]. The pathogenicity of *K. pneumoniae* is closely linked to its virulent factors, such as capsular polysaccharides, fimbriae, siderophores, and biofilm formation [2]. These factors play a crucial role in the ability of bacterium to evade host immune responses and adhere to host tissues to acquire essential nutrients for growth.

Environmental factors significantly influence the expression of these virulent determinants [6]. In order to survive the reduced nutrient content noted inside host cells, some *Klebsiella* species reduce their level of expression of capsule [7]. One such environmental factor is the hydrogen ion concentration (pH), which can alter microbial behavior by affecting bacterial metabolism, cellular integrity, and gene expression [8]. *Klebsiella pneumoniae* often encounters varying pH conditions in both its environmental and host-associated niches, such as during infection or within different compartments of the host organism [9]. These shifts in pH may trigger adaptive responses that modulate the bacterium's virulence, potentially enhancing its ability to survive hostile environments and establish infection.

pH and oxygen conditions can play different roles in the virulence of a pathogen. pH is known to influence bacterial growth and metabolic pathways [7], but the specific influence of pH on the expression of virulent factors in *K. pneumoniae* has not been extensively studied. Growth, biofilm, and antibiotic resistance were considered as virulent factors.

Understanding how different pH values modulate the expression of these virulence factors could provide valuable insights into the pathogenesis of *K. pneumoniae* and open new avenues for therapeutic interventions. Oxygen status was another factor considered in this study. Bacteria that are dependent on atmospheric oxygen utilize the molecular oxygen to perform respiratory functions as well as breakdown nutrient for energy utilization. Although reactive oxygen species (ROS) are essential for many physiological processes, oxidative stress (OS) results from an imbalance that favours ROS. When oxidants outnumber antioxidants, oxidative stress results. Oxidative stress is a disruption in the equilibrium between the environmental generation of reactive oxygen species, such as hydrogen peroxide (H_2O_2) and hydroxyl radical (OH), and biological systems' capacity to quickly identify, detoxify, or repair the damage they cause [10]. This is a widespread condition brought on by aerobic biological systems that prevent antioxidants from scavenging free radicals. Extremely reactive radicals oxidatively damage several macromolecules, including proteins, DNA, and lipids, resulting in function loss, a higher rate of mutagenesis, and ultimately cell death [10].

Klebsiella species undergo stress during host invasion as a result of host immunological defenses. The host mount defense mechanisms such as bactericidal peptides synthesized by epithelial cells. This mechanism may also be needed to adapt to other stress conditions such as oxygen limitation, hyperosmolarity and nitrosative stress. Looking at it from another perspective, a key significance of this study stems from the fact that microbes ingested via the mouth are exposed to the stomach acid content. As the bacteria migrate to the small and large intestine along with digested food, they become exposed to a more alkaline environment [11]. For the bacteria to survive in these harsh environments, they evolve stress response mechanisms to overcome these challenging factors.

The study aims to explore how varying hydrogen ion concentrations, temperature and oxygen conditions influence the virulent potential of *K. pneumoniae* by analyzing the expression of key virulence factors under different pH conditions. The findings of this research could advance our understanding of microbial adaptation and inform strategies to combat infections caused by *K. pneumoniae*.

Materials and Methods

2.1 Materials

Tryptic Soy Agar, Tryptic Soy Broth, ethanol, Bunsen burner, filter paper (Whatman no. 1), petri dish, automated pipette, wire loop, incubator, pH meter, glass rod, weighing balance, conical flask, measuring cylinder, bijou bottles, 24-well plate, antibiotic disks, hydrochloric acid, sodium hydroxide, potassium permanganate, ethyl acetate, methanol, beaker, thin layer chromatography plate, crystal violet, spectrophotometer.

2.2 Bacteria used in Study

This study used *K. pneumoniae* which has been identified using molecular methods at Lahor Research Laboratories, Benin, Edo state, Nigeria. 10% glycerol was used to preserve the pathogen at -20ºC.

2.3 Bacteria Media used in Study

The study worked with two types of media tryptic soy broth (TSB) and tryptic soy agar (TSA). These media were constituted by dissolving 28 g and 30 g of TSA and TSB media respectively using 1 L of distilled water.

The media were sterilized in an autoclave under 15 psi and 120 ºC for 15 minutes. The media were aliquoted into petri dishes aseptically. After the agar medium was solidified, the solid medium was stored at 4 ºC for subsequent use while the liquid medium was stored at room temperature to allow for microbial contamination to be detected.

2.4 Exposure Studies

The study used an experimental design approach. The exposure studies began by aliquoting 10 mL of TSB into a sterile bijou container under aseptic conditions. A single colony of overnight culture of *K. pneumoniae* pathogen was then inoculated in the TSB and incubated at 37ºC for 18 hours. The overnight inoculum was diluted to 1:500 of TSB. Two thousand micro-liters (2000 µL) of the 1:500 dilutions were put into a 24-well plate. 2 µL of hydrochloric acid of pH 4.0 was added into the well plate containing the 1:500 dilution of the organism. 2 µL of sodium hydroxide solution of pH 11.7 was added into a different well plate containing the 1:500 dilution of the organism. This was then incubated at the following temperature conditions; -5ºC, 4ºC, 25ºC, 37ºC. The oxygen conditions used were both anaerobic and aerobic for 48 hours.

2.4.1 Anaerobic Condition

Citrate solution was added to sodium bicarbonate and placed in a jar. This was sealed completely to prevent oxygen.

2.5 Antimicrobial Susceptibility Assay

After exposure to the different pH, temperature and oxygen conditions, 100 µL of the exposed culture from each of the conditions were inoculated on TSA and evenly distributed on the surface of the agar to allow for single layer of cell to grow. Antibiotics disks were put on the solid agar medium and incubated at 37ºC for 24 hours and the zone of clearance was measured in millimeters (mm).

2.6 Biofilm Assay

The well plates were incubated for 48 hours, after which the broth was gently evacuated. The plates were then left to air-dry for approximately 8 hours. Extracellular polysaccharide was stained with 20% crystal violet, followed by resuspension in 1000 µL of ethanol. The absorbance was then detected using a spectrophotometer at 590 nm.

2.7 Spectrophotometric Analysis of Growth

One thousand micro-liter (1000 µL) of the 1:500 dilution was put into a 24-well plate and the various acid and base conditions were added and incubated under the various conditions for 24 hours. This was measured spectrophotometrically at 590 nm.

2.8 Preparation of TLC Developer and Mobile Phase

The study developed a mobile phase through the addition of 2 mL of ethyl acetate to 1 mL of methanol. The developer was formed by adding 2 g of potassium permanganate in 10 mL of distilled water.

2.9 Thin Layer Chromatography Assay

One thousand micro liter (1000 µL) of the 1:500 dilution was put on a 24-well plate and the various acid and base conditions were added and incubated under the various conditions for 24 hours. The various conditions were marked on the TLC plate with a capillary tube, and they were left to air dry for ten minutes. After that, the TLC plate was put in a flask with 10 millilitres of the mobile phase.

After that, it was fully sealed and let to rise to the designated point. After letting it air-dry, it was cleaned under water that was flowing and put in the developer.

2.10 Data Analyses

Results were presented as mean and standard deviation on a bar chart. The Student T-test was used to analyze the differences between the two groups. GraphPad Prism was used to analyze the data.

3. RESULTS

3.1 Growth Response of *K. pneumoniae* to Temperature and pH Conditions

The growth response of *K. pneumoniae* as shown in Figure 1 demonstrates a gradual increase in bacterial growth level with increasing temperature of 4-37 °C. This trend was observed in both acid and basic conditions. Compared to the control bacterial culture (neutral pH condition), all altered pH conditions (acidic and basic conditions) showed decreased levels of bacterial growth. Interestingly, the bacterial growth level at all tested temperatures was significantly reduced in both acidic and basic conditions were significantly reduced than the control conditions.

A holistic comparison of the bacterial growth conditions in the acid and basic conditions showed higher growth of the basic conditions compared to the acidic conditions. However, both pH conditions showed a low bacterial growth compared to control (Figures 2 a and b).

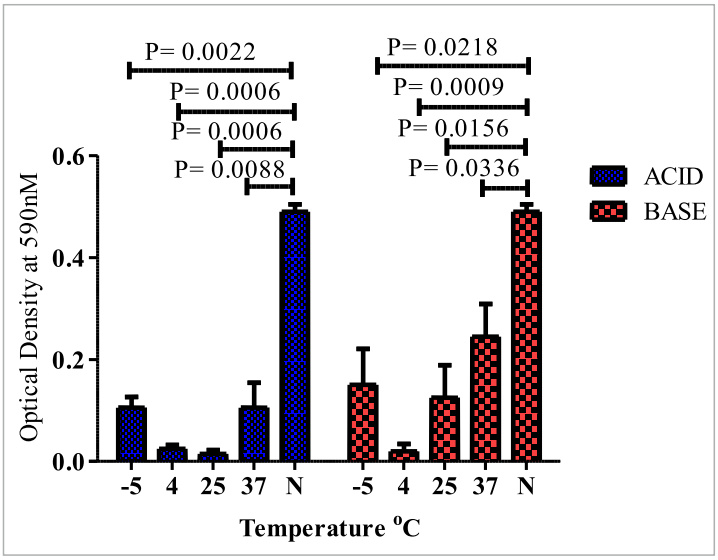

Figure 1. Growth responses of K. pneumoniae under different temperatures and pH. N signifies control condition. The outcomes are shown in the bar chart which represents the Mean ± SD.

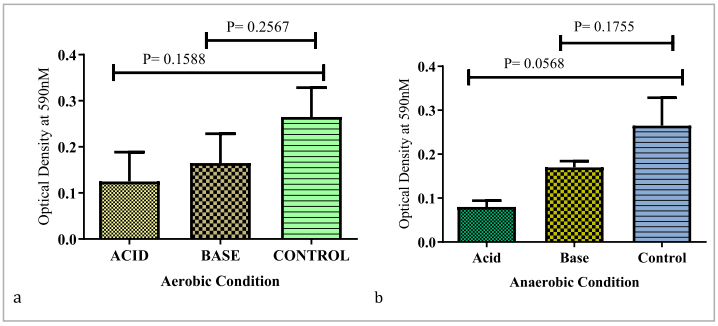

Figure 2. Growth responses of K. pneumoniae in different pH and oxygen conditions. The outcomes are shown in the bar charts which represent the Mean ± SD.

3.2 Biofilm Levels in different temperatures and oxygen conditions

Figure 3 represents biofilm production in *K. pneumoniae* cultures which were grown at different pH and temperature. Compared to 37°C, which is considered a control temperature, it was observed that the biofilm level was 1.7-fold lower at -5 °C, 1.4-fold lower at 4°C and 1.2-fold lower at 25 °C. No significant level of measurement was noted in these comparisons.

Figures 4 a and b show the biofilm formed by *K. pneumoniae* under aerobic and anaerobic conditions. The control was significantly higher (p=0.0258) than the acid by 2.25-fold but not significantly when compared to the base by 1.2-fold. The control was significantly higher than the alkaline condition by 1.8-fold (p=0.0150).

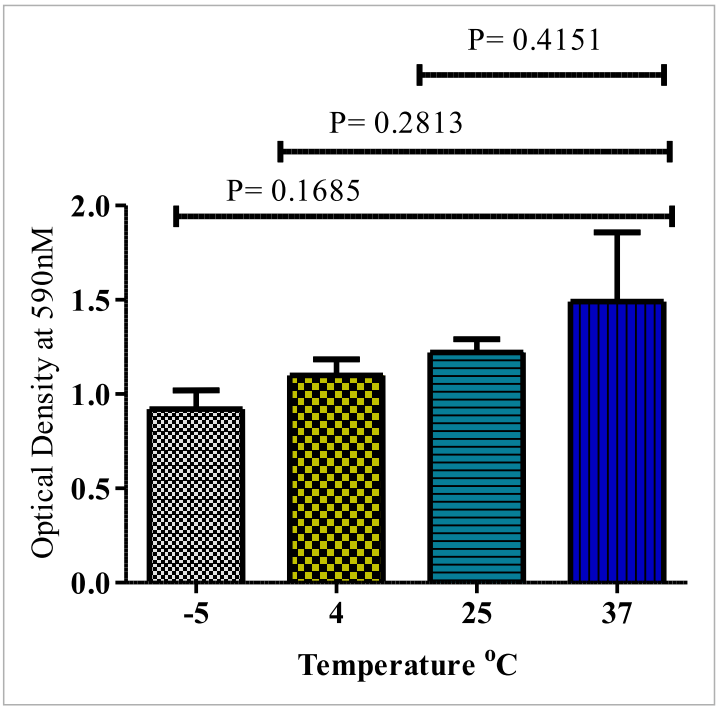

Figure 3. Effect of Temperature on Biofilm of K. pneumoniae. Isolates of K. pneumoniae were incubated at different temperatures (-5, 4, 25, and 37 ºC). The outcomes are shown in the bar chart which represents the Mean ± SD.

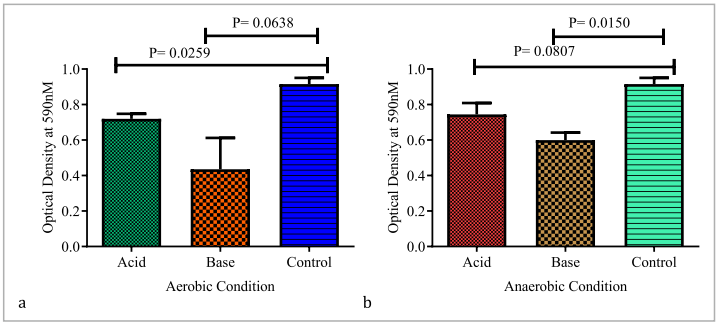

Figure 4. Effect of pH on biofilm of K. pneumoniae in different oxygen conditions. Isolates of K. pneumoniae were incubated under aerobic and anaerobic conditions. The outcomes are shown in the bar charts which represent the Mean ± SD.

3.3 Secreted metabolite by *K. pneumoniae* in different pH and acid conditions

Figures 5 A to E show the separation pattern of the movement of metabolites on the solid phase using a thin-layer chromatography. Only the two lowest temperatures used demonstrated the presence of metabolites that showed R_f values of 0.6 and 0.9 for -5 °C and 4 °C respectively. The anaerobic and aerobic conditions showed no distinguishable spots.

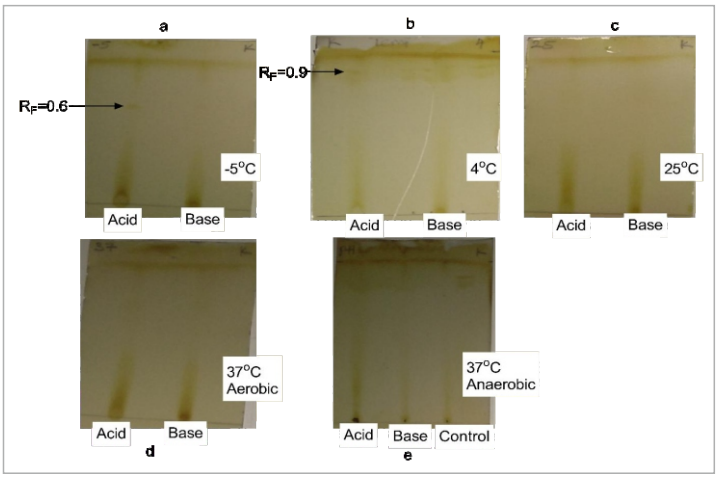

Figure 5. Secretory metabolites at different pH and oxygen conditions. a) -5 oC, b) 4 oC c) 25 oC d) 37 oC aerobic condition e) 37 oC anaerobic condition. TLC was done using the TLC plate, a mobile phase and a developer.

3.3 Antibiotic susceptibility pattern of *K. pneumoniae* in different pH oxygen conditions

Table 1 shows the antibiogram of *K. pneumoniae* which are exposed to different temperature conditions and different susceptibility levels when treated with known antibiotics. The 25 °C treatment conditions with a multiple antibiotic resistance (MAR) index of 0.4 had the greatest resistance, while the 4 °C had the least antibiotic resistance (MAR=0.0). For the aerobic state, the antibiotic resistance was the same under the acidic and basic conditions (MAR=0.6) while under the anaerobic conditions the resistance development was higher in the acidic conditions (MAR=0.6) than under the basic conditions (MAR=0.2).

Table 1. K. pneumoniae resistance profile at different temperatures, pH and oxygen conditions

	Zones of Clearance (mm)					MAR index
	AMC	CTX	CT	IPM	MEM	
Temperatures						
-5 ºC	32(S)	22(M)	20(R)	40(S)	34(S)	0.2
4 ºC	34(S)	32(S)	24(M)	32(S)	34(S)	0.0
25 ºC	28(S)	18(R)	18(R)	34(S)	34(S)	0.4
37 ºC	32(S)	26(M)	16(R)	32(S)	36(S)	0.2
Aerobic conditions						
A	14(R)	11(R)	20(R)	26(M)	29(S)	0.6
B	10(R)	15(R)	18(R)	29(S)	25(M)	0.6
C	36(S)	25(M)	27(S)	32(S)	33(S)	0.0
Anaerobic Conditions						
AN^A	12(R)	11(R)	13(R)	25(M)	28(S)	0.6
AN^B	34(S)	20(M)	10(R)	31(S)	32(S)	0.2
AN^C	23(M)	22(M)	26(M)	26(M)	27(S)	0.0

Key: AMC: Amoxicillin/Clavulanate, CTX: Cefotaxime, CT: Colistin Sulphate, IPM: Imipenem, MEM: Meropenem. S represents sensitivity, M denotes moderate resistance, and R indicates resistance. Resistance classifications: R (Resistant): Zone of inhibition ≤ 20 mm, M (Moderately Resistant): Zone of inhibition between 20-26 mm, S (Sensitive): Zone of inhibition > 26 mm Treatment conditions for *Klebsiella pneumoniae* include: A: Exposure to acidic conditions, B: Exposure to alkaline conditions, C: Control group (no treatment), AN^A: Anaerobic conditions with acid treatment, AN^B: Anaerobic conditions with alkaline treatment, AN^C: Anaerobic conditions without neutral pH,

4. DISCUSSION

K. pneumoniae is a common gut microbiota that is in constant touch with the gut surrounding. This gut surrounding possesses varied pH which ranges from the acidic stomach to the more alkaline large intestine [12]. The findings from this study showed varied response of *K. pneumoniae* to altered pH, temperatures, and oxygen conditions. Most importantly, varied antibiotics response was observed in the different conditions tested.

Overall, the growth response of *K. pneumoniae* across a temperature gradient from 4 to 37 °C reveals a consistent increase in growth with rising temperatures under both acid and basic conditions. However, the ideal growth temperature for *Klebsiella pneumoniae* was 37 °C, while the ideal pH level was about 7.2. Under ideal temperature, pH, and oxygen levels, organisms thrive and grow efficiently. However, when exposed to extreme conditions, such as very high or low temperatures, bacterial cells respond by selectively producing specific proteins that help them adapt and survive these adverse environmental changes [13]. The altered pH conditions significantly inhibited bacterial growth compared to neutral pH controls, corroborating findings from previous studies that extreme pH environments can disrupt microbial homeostasis [14]. These growth results further corroborate that the pathogen is a neutrophile [15] as neither the acid nor basic conditions supported favorable growth of the pathogen.

It is well established that many bacteria acquire virulent genes are affected due to limited oxygen conditions, and they try to adjust their metabolic pathways appropriately [16]. *Pseudomonas aeruginosa* has shown a deletion in the sicX gene in reduced oxygen conditions in order to ensure its survival in vitro [17]. This reduced oxygen condition is proposed to confer a survival advantage to pathogens using the quorum sensing system [18]. Whenever there is a change from aerobic to anaerobic condition affects the cellular NADH/NAD+ balance of bacteria indicated by the repressor Rex in *Staphylococcus aureus* and other Gram-positive bacteria [19]. Thus, another focus of this research was to determine quantitatively the levels of siderophore in *Klebsiella* under different temperatures and pH.

The growth and survival of *K. pneumoniae* under these conditions could as a result of biofilm formation and the secretion of molecules and as such further analysis was carried out.

To withstand unfavorable conditions, bacteria produce extracellular polysaccharides or capsules which serve as a barrier between the external environment and the bacteria [7]. The formation of biofilm, a key virulence factor [20], was significantly influenced by variations in pH and temperature. At suboptimal temperatures (-5 °C, 4 °C, and 25 °C), biofilm levels were markedly lower than at 37 °C, though these differences were not statistically significant. This temperature serves as control because it is the normal body temperature. These findings suggest that biofilm production in *K. pneumoniae* is optimized at physiological temperatures, consistent with its adaptation to host environments [21] [22].

Compared to the 37 °C, all other temperatures observed a reduced level of biofilm production in *K. pneumoniae* in a temperature-dependent manner with the lowest level of biofilm formation being observed at 4°C. However, no significant level of biofilm was noted implying that temperatures may not play a crucial role in the formation of biofilm in *K. pneumoniae.* This contradicts the overwhelming data that temperature affects biofilm production [23]. However, Hostacka et al. [24] did not notice any significant biofilm level in *Pseudomonas aeruginosa, Klebsiella pneumoniae,* and *Vibrio cholera*. These contrasting observations point to the fact that an intrinsic genetic factor may be the playmaker in biofilm production. Significantly lowered biofilm levels were detected in the acidic cultures under aerobic conditions while basic cultures showed significantly reduced biofilm levels. This portrays the oxygen conditions acting in a toxic manner at these different pH conditions to the bacteria which are confirmed in other studies for obligate anaerobes [25].

When organisms experience stress, they release specific molecules known as bacteriocins, which help them endure harsh conditions. This study examined these molecules using thin-layer chromatography. The secreted molecules were tested under varying temperatures, pH levels, and oxygen conditions. Under temperature and pH treatments, a band with a retardation factor (Rf) of 0.6 appeared at -5°C in an acidic environment but was absent in a basic environment. At 4°C, a band was observed in both acidic and basic conditions, with an Rf of 0.9. However, under anaerobic conditions, no bands were detected on the TLC plate, including in the control (without pH treatment). The presence of bands suggests the secretion of specific molecules by *Klebsiella pneumoniae*, potentially including proteins, amino acids, or siderophores, which may aid in stress survival. The optimal level of bacteriocin production in bacteria have been observed to be within 4-5°C which is similar to the result obtained in this study [26]. Conversely, the absence of bands in certain conditions suggests that secretion was significantly inhibited.

The antibiogram results revealed variable antibiotic resistance levels, influenced by both temperature and pH conditions. The 25°C treatment exhibited the highest resistance (MAR=0.4), indicating that suboptimal temperatures could favor the expression of resistance genes, potentially through the activation of stress-induced regulatory mechanisms [27]. In particular acidic conditions under anaerobic settings showed a MAR index of 0.6, higher than their basic counterparts (MAR=0.2). This indicates that the oxygen limitation in combination with low pH may exacerbate resistance development, possibly by enhancing the expression of efflux pumps or stress-related enzymes [28].

Conclusion

The growth of *Klebsiella pneumoniae* was significantly reduced in both basic and acidic conditions. Under the aerobic conditions, the pathogen that was exposed to the acidic condition showed significantly decreased the biofilm level while in the anaerobic condition, the pathogen exposed to the basic condition significantly reduced the biofilm level. Secretory molecules were present only at -5 °C and 4 °C. The MAR index was highest at 25 °C but none at 4 °C. Both pH conditions increase the AMR potential of the pathogen.

REFERENCES

1. Monsi, T.P., Wokem, G.N. and Aleruchi, P.C., 2017. Development of antibiotic resistance in herbal drug-sensitized Staphylococcus aureus isolate. Journal of Advances in Microbiology, 7(4), pp.1-7.

2. Monsi, T.P., Abbey, S.D., Wachukwu, C.K. and Wokem, G.N. (2018). Levels of biofilm expression in Klebsiella pneumoniae isolates exposed to herbal drugs. *Journal of Advances in Microbiology*, *12*(1), pp.1-7.

3. Nikolic, P. and Mudgil, P., 2023. The cell wall, cell membrane and virulence factors of Staphylococcus aureus and their role in antibiotic resistance. Microorganisms, 11(2), p.259.

4. Bucher, T., Oppenheimer-Shaanan, Y., Savidor, A., Bloom-Ackermann, Z. & Kolodkin-Gal, I. (2005). Disturbance of the bacterial cell wall specifically interferes with biofilm formation. *Environmental Microbiology Report,* 7, 990–1004.

5. Paczosa, M.K. and Mecsas, J., 2016. Klebsiella pneumoniae: going on the offense with a strong defense. *Microbiology and molecular biology reviews*, *80*(3), pp.629-661.

6. Diard, M. and Hardt, W.D., 2017. Evolution of bacterial virulence. FEMS microbiology reviews, 41(5), pp.679-697.

7. Buffet, A., Rocha, E.P. and Rendueles, O., 2021. Nutrient conditions are primary drivers of bacterial capsule maintenance in Klebsiella. *Proceedings of the Royal Society B*, *288*(1946), p.20202876.

8. Anga, O.G., Monsi, T.P., Konne, F.E. and Mike-Ogburia, M.I., 2020. In Vitro Quantitative Assessment of Some Virulence Factors Produced by Escherichia coli in Different pH, Temperature and Oxygen Conditions. *Advances in Microbiology*, *10*(12), pp.647-662.

9. Abuhelwa, A.Y., Williams, D.B., Upton, R.N. and Foster, D.J., 2017. Food, gastrointestinal pH, and models of oral drug absorption. *European journal of pharmaceutics and biopharmaceutics*, *112*, pp.234-248.

10. De Oru`e Lucana, D. O., Wedderhoff, I., Groves, M. R. (2013). ROS-mediated signaling in bacteria: zinc-containing cys-x-x-cys redox centres and iron-based oxidative stress. *Journal of Signal Transduction And Targeted Therapy,* P.9

11. Fang, C. T., Chuang, Y.P., Shun, C. T., Chang, S. C. & Wang, J.T. (2004). A novel virulence gene in *Klebsiella pneumoniae* strains causing primary liver abscess and septic metastatic complication. *Journal of Experimental Medicine*, 199, 679–705.

12. Grubor, B., Meyerholz, D.K. and Ackermann, M.R. (2006) Collectins and Cationic Antimicrobial Peptides of the Respiratory Epithelia. Veterinary Pathology, 43, 595-612.

13. James, P.D.A., Edwards, C. and Dawson, M., 1991. The effects of temperature, pH and growth rate on secondary metabolism in Streptomyces thermoviolaceus grown in a chemostat. *Microbiology*, *137*(7), pp.1715-1720.

14. Krulwich, T.A., Sachs, G. and Padan, E., 2011. Molecular aspects of bacterial pH sensing and homeostasis. *Nature Reviews Microbiology*, *9*(5), pp.330-343.

15. Jin Q and Kirk MF (2018) pH as a Primary Control in Environmental Microbiology: 1. Thermodynamic Perspective. *Front. Environ. Sci*. 6:21. doi: 10.3389/fenvs.2018.00021

16. Hibbert, T.M., Whiteley, M., Renshaw, S.A., Neill, D.R. and Fothergill, J.L., 2024. Emerging strategies to target virulence in Pseudomonas aeruginosa respiratory infections. *Critical reviews in microbiology*, *50*(6), pp.1037-1052.

17. Cao, P., Fleming, D., Moustafa, D.A., Dolan, S.K., Szymanik, K.H., Redman, W.K., Ramos, A., Diggle, F.L., Sullivan, C.S., Goldberg, J.B. and Rumbaugh, K.P., 2023. A Pseudomonas aeruginosa small RNA regulates chronic and acute infection. *Nature*, *618*(7964), pp.358-364.

18. Clay ME, Hammond JH, Zhong F, Chen X, Kowalski CH, Lee AJ, Porter MS, Hampton TH, Greene CS, Pletneva EV. 2020. Pseudomonas aeruginosa lasR mutant fitness in microoxia is supported by an Anr-regulated oxygen-binding hemerythrin. Proceedings of the National Academy of Sciences. 117(6):3167–3173.

19. Enany, S., 2020. Impact of low PH on microbial growth rate, ATP production, and NADH to NAD+ ratio. *Egyptian Journal of Medical Microbiology*, *29*(3), pp.121-128.

20. Surgers, L., Boyd, A., Girard, P.M., Arlet, G. and Decré, D., 2019. Biofilm formation by ESBL-producing strains of Escherichia coli and Klebsiella pneumoniae. *International Journal of Medical Microbiology*, *309*(1), pp.13-18.

21. Piperaki, E.T., Syrogiannopoulos, G.A., Tzouvelekis, L.S. and Daikos, G.L., 2017. Klebsiella pneumoniae: virulence, biofilm and antimicrobial resistance. *The Pediatric infectious disease journal*, *36*(10), pp.1002-1005.

22. Guerra, M.E.S., Destro, G., Vieira, B., Lima, A.S., Ferraz, L.F.C., Hakansson, A.P., Darrieux, M. and Converso, T.R., 2022. Klebsiella pneumoniae biofilms and their role in disease pathogenesis. *Frontiers in cellular and infection microbiology*, *12*, p.877995.

23. Çam, S. and Brinkmeyer, R., 2020. The effects of temperature, pH, and iron on biofilm formation by clinical versus environmental strains of Vibrio vulnificus. *Folia Microbiologica*, *65*, pp.557-566.

24. Hostacka A, Ciznar I, Stefkovicova M (2010) Temperature and pH affect the production of bacterial biofilm. Folia Microbiol 55:75–78

25. Boyanova, L., Boyanova, L., Hadzhiyski, P., Gergova, R. and Markovska, R., 2024. Oxygen tolerance in anaerobes as a virulence factor and a health-beneficial property. *Anaerobe*, p.102897.

26. Abiom, L.A., Ire, F.S. and Ahaotu, I., 2024. Production and characterization of bacteriocin from lactic acid bacteria isolated from fermented food products. *Scientia Africana*, *23*(3), pp.101-114.

27. Zhang, Y. and Gross, C.A., 2021. Cold shock response in bacteria. *Annual Review of Genetics*, *55*(1), pp.377-400.

28. Mojica, E.A. and Kültz, D., 2022. Physiological mechanisms of stress-induced evolution. *Journal of Experimental Biology*, *225*(Suppl_1), p.jeb243264.