

Is lactate an
undiscovered
pneumococcal
virulence factor?



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Abstract

Streptococcus pneumoniae is Gram-positive alpha haemolytic bacteria that commonly found in the nasopharynx of elderly people and young children, it causes approximately 2 million deaths mostly children under age of 5 year and people over 60 years of age. Most important diseases caused by *S. pneumoniae* are including pneumoniae, meningitis and bacteraemia. The pathogens can be transmitted through contact. *Streptococcus pneumoniae* obtains its energy mainly carbohydrates through fermentation process. However, in some situations where there are limited sugars or in the presence of galactose the homolactic fermentation is shifted to mixed fermentation in which in addition to lactate, ethanol, formate and acetate are formed. In this study, the role of lactate (lactic acid) and formate (formic acid) in bacterial competition and cytotoxicity was investigated. We hypothesised that lactic acid and formic acid are able to contribute to the virulence of *Streptococcus pneumoniae*. Bacteria were grown on either BHI or BAB. The killing assay was done by exposing various acids on *S. pneumoniae* as control then lactic acid producing bacteria and non-acid producing bacteria was tested with these acids. Growth assay experiment was done followed by cytotoxicity test using A549 epithelial cells incubated for 24h. The effect of lactic acid for killing assay was significant. Similar effect was seen when lactic acid was exposed to A549 cells. However, a hydrochloric acid acid was unable to inhibit the growth of bacteria. This study concludes that lactic acid produced by *Streptococcus pneumoniae* is a potential virulence factor and may contribute to *Streptococcus* invasive disease.

Chapter 1 Introduction

1. 1 The biology of *Streptococcus pneumoniae*

Streptococcus pneumoniae is a normal inhabitant of human nasopharynx, and it is a member of lactic acid family that gets its energy mainly by the process of fermentation. It is Gram positive, and catalase negative. Under light microscope *S. pneumoniae* can be seen in pairs and short chains. In blood agar they can be seen as α -haemolytic.

S. pneumoniae is a fastidious facultative anaerobe that requires highly nutritious medium for growth. It grows in Brain Heart Infusion (BHI) media, as well as Blood Agar Base (BAB). This bacterium also grows in chemically defined medium that contains nutrients, such as vitamins, glucose, amino acids (Table 1) and pyruvate. However, the most easily observable characteristic of *S. pneumoniae* is its sensitivity to optochin (ethylhydrocupreine). This makes the pneumococcus distinguishable from other alpha haemolytic streptococcus. Like other Gram positive bacteria *S. pneumoniae* possess three major surface layer that can be distinguishable: cell wall, plasma membrane and capsule (Alonso de Velasco, E. et al 1995). There are more than 90 serotypes of *S. pneumoniae* based on their capsular polysaccharides coats.

1. 2 The Genome:

The complete genome sequence of a type 4 isolate of *S. pneumoniae* comprises a single circular chromosome of 2,160,837 base pairs (bp) about 40% of G+C content (Tettelin et al., 2001). This genome contains 2236 predicted coding regions; of these genes around 64% are assigned a <https://assignbuster.com/is-lactate-an-undiscovered-pneumococcal-virulence-factor/>

biological role (Tettelin et al., 2001). It also contains 73 noncoding RNA genes that include four rRNA operons. Moreover, *S. pneumoniae* has a high capacity for DNA uptake (Hoskins, J. et al 2001).

The pneumococcal genomes contain a considerable number of insertion elements such as transposon remnants and repeat sequences. The large number of insertion elements in the genome indicates that the pneumococcal genome is exposed to common inter and intra-genomic events. (Lanie. J. A., et, al. 2006)

1. 3 The diseases caused by *S. pneumoniae* and their epidemiology:

The diseases caused by the pneumococcus is life threatening and include pneumonia, meningitis, bacteraemia and septicaemia. Additionally, it also causes otitis media, sinusitis, osteomyelitis, and peritonitis. The microorganism is also responsible for endocarditis, and septic arthritis (Kilian, 2007). The diseases caused by *S. pneumoniae* are results from either direct extension from the nasopharynx or by invasion and haematogenous spread.

Pneumonia is a very important cause of mortality and morbidity amongst elderly people.

(Nagaoka, S. et al..). Despite the availability of pneumococcal vaccine this microorganism still pose a great challenge to any attempt to eradicate or limit the spread of the disease because rising antibiotic resistance and limitations of vaccines. The pneumococcal infections are responsible for more than 1. 6 million deaths each year worldwide (WHO, 2008). The highest

incident of this disease occurs in children under the age of 5 year and in the elderly. Also very high incident in patients with predisposing conditions such as asplenia and those who are immune-compromised are reported.

1. 4 The pneumococcal virulence determinants

1. 4. 1 Capsule: *S. pneumoniae* possess polysaccharide capsule which is considered as the most important virulence factor, because unencapsulated pneumococcus is almost harmless while the encapsulated bacteria from the same species cause disease (Alonsodevelasco, et, al 1995). It has been found that encapsulated strains are approximately 105 more virulent than unencapsulated strains (Alonsodevelasco, et, al 1995) I general, the vast majority of *Streptococcus* serotypes are unable to produce potential virulence (Lysenko, et al. 2010). The survival of the serotype in the blood stream and ability to cause invasive disease are mainly determined by the chemical structure of capsule polysaccharide and thicknes of capsule (Alonsodevelasco, et al. 1995).

1. 4. 2 Protein virulence determinants: Recent studies discovered that there are proteins that also contribute to virulence. They include, but are not limited to, hyaluronate lyase,(berry, M. et al., 1994), pneumolysin (Paton, J. c., et al 1986), neuraminidases (Elizabeth A. et., al 2002), galactosidases (Terra et al., 2010) and pyruvate formate lyase (Yesilkaya et al., 2009).

1. 4. 2aHyaluronatellyase: Hyaluronate lyase degrades the hyaluronan, which is a hyaluronic acid derivative and its one of the most important polysaccharide component of animals, into disaccharide as a final product (Songlin, et al., 2000). Study carried by Berry et al. (1994), suggests that <https://assignbuster.com/is-lactate-an-undiscovered-pneumococcal-virulence-factor/>

hyaluronidase plays vital role in migration of streptococci between tissues, in particular translocation from the lungs to vascular system. The other way in which hyaluronidase contributes the streptococcal pathogenesis is by allowing huge number of microorganisms to host tissue for colonization (Berry et al. (1994).

1. 4. 2bPneumolysin: This is a membrane damaging toxin which inhibits neutrophil chemotaxis, phagocytosis and respiratory burst (Greenwood, D. et, al 2007). The sulfhydryl-activated cytolysin toxin functions by binding to cholesterol in host cell membranes. (Paton, J. c., et al 1986). It also damages blood vessels in the lungs and therefore, causes bleeding into air spaces. Moreover, pneumolysin leads to the activation of the classical complement pathway and the depletion of serum opsonic activity (Lock, R. A., 1988)

1. 4. 2c Neuraminidases: This enzyme is able to cleave N-acetylneuraminic acid from glycoproteins, such as mucin, oligosaccharides, and glycolipids on host cell surfaces. *S. pneumoniae* expresses several distinct neuraminidases. Studies carried out by Elizabeth A. et., al (2002) has indicated that neuraminidase activity might promote the colonization by decreasing the viscosity of mucus(Tong et al, 2000). The two neuraminidases (NanA and NanB) are part of virulence factors that cause disease (Tong et al, 2000). Although there are three forms of neuraminidases, NanA, NanB and NanC , the most abundant neuraminidase and probably the most important one is the Neuraminidase A(NanA). Almost all the *S. pneumoniae* that were investigated has shown to have neuraminidase activity (Anirban et al., 2010). These investigations showed that NanA contributed to the

colonisation of pneumococcus in the nasopharynx and also the development of otitis media (Anirban et al., 2010).

1. 4. 2dGalactosidases: Galactosidase is an important enzyme, that catalysis the hydrolysis of galactose from oligosaccharides (Jeong et al., 2009; Terra et al., 2010), These enzyme can be found in most mucosal microorganisms and they exist in different forms specific for individual galactosidic bonds. The size of the galactosidase depends on the type of the organisms.

Nevertheless, most prokaryotic galactosidases are large proteins. Regarding the galactosidases virulence contribution in streptococci pneumoniae is not yet fully understood. However, study carried out by (Terra et al., 2010) exclusively showed that galactosidase is hugely important in mucindegredation. This study also investigated the role of galactosidase in pneumococcal virulence and eventually achieved that galactosidase is essential for survival in the nasopharynx (Terra et al., 2010)

1. 4. 2ePyruvateformatelyase(PFL): PFL is a metabolic enzyme that is responsible for the conversion of pyruvate into formate and acetyl CoA under anaerobic or microaerobic conditions. This enzyme is produced in inactive form and posttranslationally activated by pyruvate formate- lyase activating enzyme (Leppanen, et al., 1999).

Pyruvate formate lyase (PFL) activity mediates mixed acid fermentation. Monosaccharides, such as galactose converts a considerable percentage of pyruvate to acetyl-CoA in both microaerobic and anaerobic conditions of glycerol. Study carried by (Yesilkaya et al., 2009) indicates that PFL/PFI-AE is essential for in vivo fitness of the pneumococcus. The study concluded that

lack of PLF is able to influence alteration of lipid composition in cell membrane and reduction of pneumococcus virulence.

Despite considerable efforts, it is still not known completely how pneumococcus causes disease in its host. Therefore the study of *S. pneumoniae* virulence determinants is an important approach to developing new therapies such as vaccines and antibiotics.. Recent studies are showing that the pneumococcal fermentative metabolism is an important contributor to pneumococcal virulence.

1. 5 The pneumococcal fermentative metabolism

The Lactic acid bacteria (LAB), one of which is the pneumococcus, receive its energy from fermentative breakdown of carbohydrates. These group of bacteria maintains fermentative metabolism regardless of presence of oxygen (Yesilkaya, et, al, 2009). The pneumococcus undergoes fermentative metabolism, because pneumococcus lacks genes (approximately 18 genes) that are essential for respiration. The process that these bacteria undergoes is a classical pathway known as Embden-Meyerhof pathway which activates the breakdown of carbohydrate and eventually results the production of pyruvate, NADH and two moles of ATP (Yesilkaya, et al 2009).

Figure 1: Schematic representation of the lactate pathway in lactic acid bacteria. LDH, lactate dehydrogenase, PFL, pyruvate formate lyase, iPFL, inactive pyruvate formate lyase, PFL-AE, pyruvate formate activating enzyme, PDH, pyruvate dehydrogenase, POX, pyruvate oxidase, ADH, alcohol dehydrogenase, ACK, acetate kinase, PTA, phosphotransacetylase.

(Taken from Yesilkaya, 2009)

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NAD⁺ regeneration occurs through lactate dehydrogenase catalysed conversion of pyruvate to lactate. In some cases, in particular where there is limited sugars or in the presence of galactose the homolactic fermentation is shifted to mixed fermentation in which in addition to lactate, ethanol, formate and acetate are formed. The mixed- acid fermentation is mediated by PFL in anaerobiosis or microaerobiosis. Aerobically, pyruvate dehydrogenase complex (PDHC) contributes to the transformation of pyruvate but *S. pneumoniae* lacks genes for PDHC (Yesilkaya, et al 2009).

The process of shifting from hololactic fermentation to mixed-acid product formation is mainly explained by the allosteric modulation of the enzyme such as lactate dehydrogenase and pyruvate formate lyase which compete for pyruvate. Fructose-1, 6-diphosphate (FDP) is an essential activator of LDH. In *L. lactis* glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) are strong inhibitors of PFL. The importance of fermentative and metabolic by product of streptococcus pneumonia was investigated in this study. The lactic acid and formic acid plays a major role in contributing virulence factors by killing other microbiota found in the nasopharynx and lungs. Similarly, these acids contribute the inflammations, since it has been reported that this acids cause both inflammation and ulceration. An experiment carried out by (Sakurazawa & Ohkusa, 2005) showed that organic acids could induce apoptosis and hence this cytotoxicity can contribute to the pathogenesis of ulcers (Sakurazawa & Ohkusa, 2005).

1. 5. 1 The Bacterial completion and the role of different bacterial products in other microorganisms

To colonise a new habitat emerging bacteria have to compete with previously colonised microorganisms, the competition determination depends on the number of bacterial populations that colonise a particular region of the host. Various ecological factors contribute to the colonisation of bacteria in the host, including the availability of natural resources. These resources, such as nutrients and spaces are limited in nasopharynx (Margolis et al. 2010). The established bacteria can produce toxins and harmful substances to inhibit the colonisation of incoming bacteria. Similarly, the host immune response plays crucial role in determining the colonisation of the bacteria.

A study investigating the role of hydrogen peroxide in the human nasopharynx showed that it is capable of eliminating various bacterial species in the respiratory tract (David, 2003). *Streptococcus pneumoniae* and *Haemophilus influenzae*, are co-inhabitants in the upper respiratory and both cause life threatening disease. However, these pathogens compete in space and nutrients. Production of toxic chemicals is part of the space competition. Hydrogen peroxide produced by *S. pneumoniae* acts as an antimicrobial agent to eliminate growth of other bacteria. Indeed, a David (2003) showed that hydrogen peroxide produced by *S. pneumoniae* caused rapid killing of *Haemophilus influenzae*. Interestingly, exogenous catalase exposure has exhibited safeguarding of *H. influenzae* and no killing activity of hydrogen peroxide was observed. This suggests that hydrogen peroxide may be responsible for this bactericidal activity. Moreover, *S. pneumoniae* that was unable to produce hydrogen peroxide did not exhibit killing effect for *H. influenzae*. Furthermore, other respiratory pathogens were affected by

the hydrogen peroxide produced by *S. pneumoniae* since these chemicals killed other respiratory tract pathogens such as *Moraxella catarrhalis* and *Neisseria meningitidis* (David, 2003). Production of hydrogen peroxide by *S. pneumoniae* also possess cytotoxic affects on host cells and tissue (Weiser, et al 2003) The mechanisms in which *S. pneumoniae* survive with endogenous hydrogen peroxide concentrations that are able to kill other species are not understood.

1. 5. 2 *Haemophilus influenzae*: *Haemophilus influenzae* is a Gram negative coccobacillus, rod shape nonmotile and non spore forming bacterium. It is facultative anaerobe fastidious bacteria that were identified in 1892. The chromosome sequence of *Haemophilus influenzae* 1. 83Mb was the first completed genome sequence (Tatusov, et al., 1996). Apparently, *Haemophilus influenzae* is an obligate parasite and a resident of the upper respiratory system of humans (Tatusov, et al., 1996). *Haemophilus influenzae* consists of encapsulated, and non encapsulated strains. The type B strain is recognised as the most virulent strains, since it cause the majority of the *Haemophilus influenzae* invasive diseases. This bacteria is a leading cause of death children and amongst elderly people. It also causes number of different life-threatening diseases. Although, the type b strain is important in *H. influenzae* invasive disease, there are other encapsulated trains such as serotype A, which is very similar to that of type B. Encapsulated strains have the ability to cause an important invasive disease such as meningitis. *H. influenzae* strain can cause mucosal infections, including otitis media, conjunctivitis, sinusitis, bronchitis, and pneumonia.

Staphylococcus aureus

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Staphylococcus aureus is a Gram positive spherical (coccus) that resembles and arranged in grape-like cluster (Greenwood, D., 2006). *S. aureus* form hemolytic on blood agar, the organism is facultative anaerobes and opportunistic pathogen bacteria. The organism is non-sporing and non-motile, and is able to grow both with and without oxygen (facultatively anaerobic), and catalase-positive. *Staphylococcus aureus* causes wide range of diseases, ranging from superficial lesions to life-threatening septicaemia. (Charlier, C, 2008). The skin is the best ecological niche for *S. aureus*. These organisms usually found in upper respiratory tract as microbiota and are common in animals. Healthy individuals carry the organism in nasopharynx and hands as well.

1. 5. 3 *Streptococcus suis*

The bacteria used in this study include *Streptococcus suis*, which is a Gram-positive bacteria, mainly a pathogen of pigs but also found in other animals such as goat, sheep and cattle. Recently, this microorganism was isolated from various animals, such as horses cats and dogs. The organism is carried in the nasopharynx of pigs and mainly transferred from pigs to human where there are physical contacts to pigs or during the consumption of pig meat (Barbara, 2006 p494). *S. suis* can cause various severe and life treating infections such as meningitis, bacteraemia, septicaemia, arthritis and bronchitis. Approximately 2000 incidences have been discovered in areas where pig products are used namely Netherlands and Denmark. Although, there are no human *S. suis* infection outbreaks, but there are several incidences reported in china. Serotype 2 and serotype 5 are the most dominant pathogenic serotypes that cause illness. In this study *S. suis* has <https://assignbuster.com/is-lactate-an-undiscovered-pneumococcal-virulence-factor/>

been used merely because it is a member of the lactic acid producing bacteria. Although *S. suis* is zoonotic bacterial pathogen and mainly found in pigs and other animals it also isolated from humans. *Streptococcus suis* is similar to other Lactic acid Bacteria since it is a member of LAB. It causes similar diseases which may elicit the exact mechanism of diseases caused by *Streptococcus pneumoniae*. For instance, both *Streptococcus pneumoniae* and *Streptococcus suis* causes meningitis and pneumonia via similar mechanisms.

1. 5. 4 AIMS AND OBJECTIVES

The aim of this project was to investigate whether the final metabolic product of *Streptococcus pneumoniae* contributes to pneumococcal virulence. This study concentrated on the effect of lactic acids on bacteria and compares the effect of formic acid with hydrochloric acid on streptococci pneumonia and other co-existent bacteria. Likewise, this study also focuses the impact of these acids on epithelial cells. To investigate these hypotheses several bacteria that are naturally found in the human nasopharynx were used as well as lactic acid producing bacteria and non lactic acid producing bacteria.

1. 5. 5 HYPOTHESIS

Organic acids produced by *S. pneumoniae* as a result of fermentative metabolism, lactic acid and formic acid, are able to kill or inhibit the other colonising bacteria in the nasopharynx

Organic acids have an adverse effect on respiratory cells and they contribute to inflammation.

Chapter 2

2. Methods

2. 1 Bacterial strains used:

Streptococcus pneumoniae serotype 2 strain D39 was mainly used in this experiment. The bacterial strains used in this study are listed in Table 2. In addition, some other bacterial strains were used that include *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Haemophilus influenzae*. The total of 8 different strains that were employed in this study, all of these strains were obtained from Dr Hasan Yesilkaya, the University of Leicester, and stock strains were prepared from them in glycerol, 50µl of aliquot and stored at -80°C for future experiments.

2. 1. 1 Bacterial media preparation:

The solid culture of bacterial strains was done in Blood Ager Base (BAB) supplemented with 5% defibrinated horse blood or in Luria Bertani agar. To prepare BAB, sixteen gram of BAB powder was mixed with 400 ml distilled water and autoclaved at 121 °C for 15 min. Once the medium was cooled at room temperature, 5% horse blood was added and mixed, and approximately 20 ml was poured into each petri dish. The reason why blood BAB medium was used was to increase the growth of these fastidious organisms. BAB also allows the detection of the haemolytic activity. The agar surfaces were dried before inoculation.

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2. 1. 2 Bacterial growth and measurement of Optical density(OD500)

Stock cultures of *Streptococcus pneumoniae* strain, the wild type D39 was prepared by growing them in 10 ml BHI at 37°C under microaerophilic condition to optical density reached OD500 0.4-0.5 at. Then the cultures were centrifuged at 3500 rpm for 10 min in Allegra™ X-22, centrifuge (Beckman Coulter, CA, USA). inoculation were centrifuged at 1500g for 15 minutes and supernatant was discarded, thereafter, the pellet was re-suspended in 2ml BHI serum broth is composed 80% of v/v BHI broth and 20% v/v filtered foetal calf serum. 0.5ml of the re-suspended pellet was transferred to 1.5ml eppendorf tubes and stored into -80°C for future use.

2. 1. 3 Spreading and streaking of bacterial cells:

A frozen aliquot of bacteria were thawed, and 20µl of this bacteria were transferred on to petri plates and immediately streaked with a flame sterilized spreader. The objective of this process is to obtain an even distribution of cells over the surface of the plate. To avoid any contamination plates were kept on close to flame or closed and plates were sent to overnight incubator.

2. 1. 4 The broth cultures used is Brain heart infusion (BHI) broth.

To prepare BHI 8 grams of BHI was mixed with 200ml of distilled water and autoclaved at 121°C for 15 minutes. After autoclave the colour of BHI medium appeared amber. The reason we used this medium is because it is rich in nutrients for bacteria and it is good to utilise it for the cultivation of many bacteria such as *S. pneumoniae*, and *H. influenzae*. Regarding the

growth of the *Staphylococcus aureus* and *Pseudomonas aeruginosa* Luria Bertoni in agar was prepared, As shown in figure 1.

Unlike other bacteria employed in this project, Luria bertoni Agar was plated and dried 20µl of bacteria dropped on each plate and streaked using the flame sterilized spreader. Because these bacteria grow anaerobic condition we incubated at 37? C without co2 jar or jar with co2.

2. 1. 5LuriaBertonibroth

The Luria Bertoni is one of the most important medium used in laboratory, because it contains nutrition that microorganisms required to maintain life. 400ml of Luria Bertoni was prepared to use for killing experiment of both *Staphylococcus aureus* and *Pseudomonas aeruginosa*. 1 g of Tryptone, 2 g of yeast extract (LP0021), 2 of NaCl and 6 g of Agar (Bacteriological Agar, Oxid Ltd Bensingstone, Hampshire England) was dissolved in 400ml of distilled water and finally the mixture was sent to autoclave at 121 ? C for 15min.

2. 2. 1Chemical defined medium (CDM) preparation

To prepare 10ml of chemically defined media (CDM), 8. 7ml of basal solution was transferred to universal tube; 200µl of glucose was added to the solution subsequently. The following essential nutrition was added to the universal tube, 100 µl of nitrogenous base, 100 µl micronutrients, 100µl vitamins 40µl choline and 10µl pyruvate.

2. 2. 2Gramstaining

To determine the morphological properties of bacteria, such as the shape, and to determine whether it is a Gram negative or positive, the Gram staining procedure was followed. A loop of bacteria was collected from plate culture and dispersed onto clean microscope slide using 20 μ l sterile PBS. The bacterial growth was removed by passing slide through the hot Bunsen flame. The slide was treated with crystal violet for about 2 minutes with excess. Similarly, a large quantity of Iodine was poured on the slide for 2 minutes, Acetone was also poured and using water the slides were washed. Thereafter, Safranin was poured excessively for another two minutes and the slides were blotted and dried. The slide was viewed under the microscope, for the first time magnification of 10x is used. However, in later stage the magnification was adjusted to 100x. To minimise the uncertainty between air and light scattering the microscope immersion oil was used.

2. 2. 3Preparingsalt solutions

To rule out the possibilities of effect of salts, various salt solutions were prepared. 50ml, of 50mM 100mM 300mm and 500mM of Sodium lactate, sodium chloride and sodium formate were dissolved in water. For example the molecular weight of sodium chloride is 58. 44g to find the concentration of 50mM we calculated like this (Concentration of 50mM = (58. 44g/mol)*(50mol)/1000= 2. 922.

The solutions were filter sterilized using a 0. 2 μ m acrodise syringe filter (Pall Corporation, MI, and USA) and the salt solutions were stored at room temperature for immediate use and the rest was stored at -20? C for future usage.

2. 2. 4 Determination of organic and inorganic salt susceptibility test

The solution of sodium chloride, sodium lactate or sodium formate were exposed to *Streptococci pneumoniae* D39 strain, and the final concentration of these salts was 500 mM. The control (without salts) was added 195 μ l of CDM and 5 μ l of bacteria. The other wells were added a solution containing salts at various concentrations and each well was put 195 μ l of the mixture. The cultures were incubated at 37 °C in flat bottomed microtiter 96 wells for 2 h. After an incubation period, 180 μ l Phosphate buffered saline (PBS) was added to the empty wells to dilute the incubated samples by transferring 20 μ l of the incubated samples to next well. The dried blood agar plates were divided into six segments and 60 μ l of the sample was put to each segment, plates were put near to the flame when dried plates were placed in CO₂ jar, plates were inverted and placed in overnight incubator. Next day plates were collected and counted the colonies on the plates. Data and figures explaining these results are presented in result and discussion sections.

2. 2. 5 The impact of Organic/Inorganic acids on cell culture viability using different concentrations.

To investigate the effect of the Lactic acid, Formic acid and Hydrochloric acid on pneumococcal growth, the bacteria were grown by providing all nutrition that they require to maintain live. However, Lactic acid solution in different concentrations was exposed to bacteria, but prior to this, the level of pH was initially measured. The initial pH of chemically defined media (CDM) was 6.5 and then subsequent measurements of pH was done by adding lactic acid, formic acid or Hydrochloric acid to the solution of CDM in drop wise.

The composition of solution in which bacteria were grown contained 10ml of chemically defined medium (CDM) as explained in section (2. 1. 3). In these studies three different experiments with a series of Lactic acid, Formic acid and hydrochloric acid in different concentrations was done. The concentration was brought up to 500mM, and 1molar. The pH of the solution with acids was constantly measured and recorded. In the situation of Lactic acid the rate of pH dropped from 6. 4 to 5. 8 when used various amount of lactic acids. However, several consecutive measurement of pH for both formic acid and HCl acid was made. Nevertheless, the rate of the pH stayed roughly the same as lactic acid. The experiment of acids were carried same as salts in above (2. 1. 3) To kill the bacteria pure acids such as lactic acid, formic acid and hydrochloric acids should be utilized and tested on both streptococcus pneumoniae and other bacteria employed in this project.

2. 3. The Lactic acid and its effect on *S. pneumoniae*, D39 strain

To determine the impact of lactic acid on bacterial strains, it's important to calculate the amount of lactic acid needed for, to bring up the volume into 200µl. 1Molar was calculated by 1. 010 of lactic acid was added to 8. 990 of distilled water, this makes the amount of Lactic acid solution into 10ml. This amount of solution was divided into approximately 20 tubes and eventually the mixture was stored in -20C. Using microtitre plate, 10µl of D39 strain and proportional amount of PBS were added to bring up the volume in to 200ul in total. The sample that contains lactic acid, CDM and the frozen aliquot was incubated for 2 hours at 37°C. Thereafter, 60µl of solution was transferred to previously labelled agar plate and the plates were covered until they become dry and co2 jar was used, the plates were sent to the incubator for overnight

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incubation at 37°C. Next morning plates were viewed to count the bacteria in the marked area, the most concentrated plates had the greatest numbers of bacteria, and the results were recorded. While the most diluted plates showed decrease or lysis of bacteria Fig4. The experiments were repeated at least twice for using only CDM but different concentration of lactic acids.

2. 3. 1 The effect of lactic acid on growth of other bacteria

In this chapter other bacteria were used to test the effect of lactic acid on other species that are found naturally in nasopharynx that might compete with the natural resources. These species include *Haemophilus influenzae*, *Staphylococcus aureus*, *Streptococcus suis*, Group B bacteria, *Streptococcus Agalactiae* and *Pseudomonas aeruginosa*. The killing effect of lactic acid on a *S. pneumoniae* D39 strain was tested. Furthermore, various concentrations of lactic acid were exposed to all the above bacteria. Although, a different bacterial species were utilised to test the capability of lactic acid the growth medium for the assay was chemically defined medium (CDM).

2. 3. 2 Bacterial growth studies

The bacterial strains were cultivated in chemically defined medium prepared as described in Section 2. 1. 3. The growth medium was composed of 180 µl of chemically defined medium added with 20 µl of lactic acid formic acid or hydrochloric acid, prepared as described in section 2. 1. 5, to bring to the final concentration of 5, 10, 25, 40 and 50 mM. The growth studies were done using flat-bottomed microtitre plates (Nunc, Roskilde, Denmark). 5 µl (5X10⁶) of stock frozen bacteria that (prepared as described in Section 2. 1.

1) was added to the sample. The samples were further diluted 180µl in <https://assignbuster.com/is-lactate-an-undiscovered-pneumococcal-virulence-factor/>

phosphate-buffered saline (PBS) was added to the medium. Another 20 µl of phosphate-buffered saline was added to control cultures without lactic acid, formic acid or hydrochloric acids.

The microwell plate was then placed in spectrophotometric plate reader (Varioskan, Thermo-Electron Corporation, USA), set up to take absorbance every 30 minutes for 16 h at 500 nm at 37°C and shaking 3 sec before taking readings. Each sample was prepared in triplicate and repeated at least three times. Growth curves were obtained and the growth rate was calculated by using the slope of the curve from the exponential phase of the growth while growth yield was obtained by taking a highest optical density in the stationary phase.

2. 3. 3 Cells Culture Methods

The A549 cells (was generously obtained from Dr Hasan, from the University of Leicester) were cultured in RPMI-1640 (500ml) medium containing 1% (5ml) antibiotic [penicillin-streptomycin] and 10 % (50ml) Fetal Bovine Serum (FBS), and this was called ' complete medium'.

This image was taken from [www. a549. com](http://www.a549.com)

2. 3. 4 Maintenance of Cells (A549 cells and Hep-2 cells)

To thaw the frozen cells, the cells were immediately placed into 37°C water bath. After thawing the cells, the vial was wiped with 70% ethanol and allowed to dry before opening. The thawed cells were then transferred into a sterile centrifuge tube containing 2 ml of warm complete medium; the cells were centrifuged for 10 min at 250 x g at room temperature. The <https://assignbuster.com/is-lactate-an-undiscovered-pneumococcal-virulence-factor/>

supernatant was discarded and the cell pellet was suspended in 1 ml of complete medium and then transferred into 25 cm² tissue culture flask with 15 ml of complete medium and incubated at 37 °C at 5 % CO₂. Cells were checked and media was replaced every 2-3 days and 24 h prior to MTT assay.

2. 3. 5 Trypsinizing and Sub-culturing the Cells

Subculturing was done when the cells reach confluence. Old media was removed and the cells washed with 10 ml of phosphate-buffered Saline (PBS) to remove residual FBS that might inhibit trypsin action. 5 ml of Trypsin-EDTA solution was added and the flask was placed at 37 °C for 1-2 min or until the cells have dislodged. 2 ml of the cells were transferred into another flask and, immediately, 13 ml of fresh complete medium added.

2. 4. MTT Cytotoxicity Assay

This assay was used exactly as explained by (Mosmann, 1983) to identify the number cells killed after treatment of formic acid, lactic acid and hydrochloric acid. The yellow MTT (3-[4-(5-Dimethylthiazole-2-yl)]-2,5-diphenyl tetrazolium bromide) is reduced to purple formazan within mitochondria of living cells. The dead cells are unable to produce this enzyme. Using spectrophotometer, the absorbance of this colour can be identified. Although, MTT solution is very important and widely used, it is sometimes extremely difficult to obtain definite conclusions. In particular when the cells possess low metabolic activities, the MTT assay are designed for determination of cell numbers involves, therefore with low metabolic cell MTT is unable to

measure the viability of that cells and it recognises the dead cells as an active.

Below are the details of the assays.

Day 1: The cells were trypsinised in T-25 flask and 5ml of complete media was added to trypsinised the cells were placed in a 15 ml sterile falcon tube, and centrifuged at 500 rpm in a swinging bucket rotor centrifuge (Allegra™ X-22) for 5 min. The media was removed and the cells were suspended in 1 ml of complete media. The cells were then counted using Neubauer hemocytometer and results were recorded per ml. the cells were diluted to 75, 000 cells per cells using complete media to dilute cells.

A 100µl of cells (75000) were seeded in each 96-well plate at a density of approximately 5×10^4 , which was calculated using this formula

Volume of stock cells = desired concentration of cells

Concentration of stock cells (per ml)

In each well 100µl of mixture plus 80 µl of complete media was added, and incubated in 5 % CO₂ at 37°C overnight.

Day 2: 20 µl of filter sterilised lactic acid, formic acid hydrochloric acid, sodium chloride or sodium formate (prepared and stored as described in Section) was added to each well to the final concentration of 5, 10, 25, 40 and 50 mM. 20 µl PBS or completed media was added to untreated cells (negative control). Prior to this experiments the pH of medium, CDM and medium with other inhibitors were measured and range of pH maintained

between 7.68 and 5.62. However, the cells were incubated for 24 h. After the desired incubation period, 10 µl filter sterilised MTT solution (5 mg/ml in PBS, stock stored at -200 C) was added to each of the wells and incubated for 4 h.

The supernatant was gently removed by either pipetting without disturbing the cells or inverting the flat bottomed microtiter plates. Furthermore, 150 µl of MTT solvent (0.4HCl, 0.1%Nondent-P-40 [NPO] in isopropanol) was then added, and to dissolve the dark blue crystals in the mixture a plate shaker (DJB labcare, Buckinghamshire, UK) was used for 15 minutes. The absorbance was then determined at 595nm using spectrophotometric microplate reader (Bio-Rad Laboratories, Hertfordshire, UK). Finally, percent cytotoxicity was calculated using the following equation:

$$100 - \left\{ \frac{\text{mean absorbance of individual test group}}{\text{Mean absorbance of the untreated control}} \times 100 \right\}$$

Mean absorbance of the untreated control

2.4.1 Bacterial Supernatants growth preparation

A sample of *Streptococcus pneumoniae* D39 strain was obtained from Dr Hasan, D39 strain is capable to produce metabolic by-products of lactate and formate.

The obtained sample was thawed and spinned down for 1 minute, the supernatants was discarded and pellet was kept and 500µl of PBS was added. 500µl of bacteria was transferred to previously prepared 10ml of CDM with Galactose and incubated for overnight, next morning the incubated

bacteria's Optical Density was measured at OD500. The universal tubes were <https://assignbuster.com/is-lactate-an-undiscovered-pneumococcal-virulence-factor/>

centrifuged at 3500g for 10 minutes, supernatant was removed, and filtered and 1 ml aliquot was prepared and finally stored in -20°C.

2. 4. 2 Bacterial Supernatants effect on A549 cells

A549 cells diluted to 270, 000 cells/ml using complete media RPM-160, 144 µl of media was added to 5. 5 µl of cells (10, 000 cells in 150 µl RPMI) were seeded in microtiter plates, the plates were incubated for overnight.

Next day, in each well 50 µl of bacterial supernatant were added. Similarly, 50 µl of CDM solution was added to the control sets, to compare the effect of supernatant on the cells. The plates were further incubated for 24 hours.

3. 1. 4 Statistical analysis

Data are expressed as the means \pm standard deviations of at least three separate duplicate experiments. The statistical significance was assessed by prism 5. Differences were considered significant at a P value of

3. 1 Chapter III RESULTS

3. 1. 1 Identification of the features and properties of *S. pneumoniae*:

Since discovery of *Streptococcus pneumoniae*, approximately one and half centuries ago the traditional identification approach of this species has not significantly changed (Tarja, 2006). To confirm that the bacterium under study was *S. pneumoniae*, a series of phenotypic and microscopic analysis were done. Purple coloured short chains were seen observed under microscope. Bacteria that stain purple are classified as Gram-positive

bacteria. The colour and the shape of bacteria were expected of *S. pneumoniae*.

3. 1. 2 General characteristics of *S. pneumoniae*

This was been done by growing bacteria in BAB and detecting optochin (ethyl-hydrocuprein) sensitivity, α -haemolysis and colony morphology as well as Gram staining.

Figure 1: The microscopic image shows the structure of *Streptococcus pneumoniae*, the bacteria grown in BHI. The Agar Blood Base that was prepared previously was added to 5×10^6 (20 μ l) of D39 bacterial strain of *S. pneumoniae* which was frozen in -80°C . The plates were scratched with sterile loop across the surface of the culture. To test optochin sensitivity (ethyl hydrocuprein hydrochloride) on *S. pneumoniae*, an optochin disc was placed on the dense part of inoculum. After overnight growth, the effect of optochin was very clear, since no bacterial growth was observed around the area applied on to the optochin (not shown here).

In addition, the pneumococcus was grown in BHI by transferring few colonies with a sterile loop and the growth was measured by detecting the increase in turbidity by a spectrophotometer (Figure 2). The result showed that the bacterial growth kinetic of bacteria was similar to that of *S. pneumoniae*

Figure 2. *Streptococcus pneumoniae*, D39 strain grown in Brain Heart Infusion (BHI)

The effect of salts on *Streptococcus pneumoniae* D39 strain

Before testing the effect of acids on *Streptococcus pneumoniae* D39 strain, I tested the effect of organic/ inorganic salts on these bacteria. Sodium lactates (SL), sodium formate (SF) and sodium chloride (SC) were used in this study.

S. pneumoniae D39 strain was exposed to SL, SF and SC to determine the effect of these salts to pneumococcal survival. The effect of these salts on pH level of medium was measured prior to exposure. The result shows that salt solutions have no effect at any concentrations on pH of medium. For sodium lactate at 50 mM 99.84% (SEM 0.64) of the bacteria survived after 2 h incubation at 37 °C. Similarly, the pneumococcal survival on sodium chloride was 100% (SEM 0.43) at 50 mM. Regarding the effect of sodium formate, the result show that there was very small killing of bacteria, 4% killed and 96% (SEM 0.26) of pneumococcal were survived. However, this was not significant ($p > 0.05$). Therefore, the overall survival of bacteria on sodium formate was 98% this indicates that *Streptococcus pneumoniae* D39 strain survival was not affected by the highest concentration of salt used. In conclusion, the result indicates that organic or inorganic salts do not affect the survival of *S. pneumoniae* D39 strain under the assay conditions used. Therefore, it was decided to use acids to see whether acids have any effect on pneumococcal survival.

Figure 3: The effect of salts on *Streptococcus pneumoniae* D39 strain. The mean bacterial survival with their SEM for three independent experiments is shown.

Investigation of acids on pneumococcal survival and growth

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To investigate the impact of organic and inorganic acids on *Streptococcus pneumoniae* D39 strain, I have chosen the organic acids produced as the final metabolic product of *S. pneumoniae* (lactic acid, formic acid). I have chosen hydrochloric acid as an inorganic acid. The experiments were repeated at least three times to obtain reliable data. Finally, data were analysed using GraphPad Prism5.

Streptococcus pneumoniae D39 strain was exposed to lactic acid, formic acid and hydrochloric acid. In this experiment similar procedure was maintained with that used in salt solutions. The result showed that lactic acid was lethal at significance level only when 40 mM was used. At this concentration 28% (SEM 10.05) of pneumococci were killed compared to control (p

Figure 4: Determination of impact of acids on *S. pneumoniae* D39 strain. The mean survivals with their SEM for three independent experiments are shown. The * indicates statistical significance relative to control (without added acid).

To examine the impact of acids on bacterial growth, pneumococci were exposed to sublethal concentration of acids. The result showed that the growth yield of *S. pneumoniae* was slightly inhibited by all acids. In the presence of 5 mM lactic acid the growth yield was reduced (maximum absorbance: 1.25) compared to untreated (maximum absorbance: 1.28) whereas formic and hydrochloric acid respectively, did not elicit any effect on *S. pneumoniae* growth compared to control (maximum absorbance: 1.28). At 10 mM lactic and formic acid the growth yield was reduced to (1.12, and 1.23) respectively when compared to control. However, at this

concentration hydrochloric acid did not affect the growth yield of *S.*

pneumoniae. At 20 mM, all acids reduced the growth yield to (1.02, 0.93 and 1.05, respectively) compared to control. However, statistical analysis of the data indicated that the difference was not significant and hence acids were unable to inhibit the growth yield of the *S. pneumoniae* D39 strain.

Table. 1 Growth yield of *S. pneumoniae* D39 strain after various acids were exposed and incubated for overnight. The growth yield was calculated by taking the highest optical density.

The growth rate was calculated from the linear part of growth curve. The growth rate of untreated *S. pneumoniae* was 0.135 h⁻¹ (+ 0.021), but when the pneumococci were exposed to 5, 10 and 20 mM lactic acid (LA) the growth rates were 0.115 h⁻¹ (+ 0.007), 0.026 h⁻¹ (+ 0.007) and 0.025 h⁻¹ (+ 0.004) for 5 mM, 10 mM and 20 mM, respectively. The growth rate was significant relative to control (p=1 (+ 0.021), 0.06 h⁻¹ (+0.014) and 0.07 h⁻¹ (+ 0.014), respectively, relative to control (0.135 h⁻¹ + 0.021). The growth rate was significant relative to control (*pS. pneumoniae* were 0.15 h⁻¹ (+ 0.0), 0.16 h⁻¹ (+ 0.014) and 0.04 h⁻¹ (+ 0.00) for 5, 10 and 20 mM, respectively. Similarly, this reduction was significant relative to control (*pS. pneumoniae* D39 strain was inhibited or killed (in killing assay) by lactic acid, formic acid and hydrochloric acid at a certain concentrations such as 25mM and onwards. The growth rate indicates that there were significantly inhibited. However, surprisingly the growth yields calculated shown that there were no significant reductions of *S. pneumoniae* were observed.

Figure 4. a The growth rate of *S. pneumoniae* exposed to various acids. The pneumococcus was grown for 16 h in CDM in the presence of various concentrations of acids. The mean growth rate was calculated from three independent experiments, each experiment included three technical replicates. * indicates statistical significance relative to control.

The effect of acids on survival and growth of *Haemophilus influenzae*

Haemophilus influenzae was exposed to organic and inorganic acids as before. The result showed that LA was lethal at 10 mM onwards. At 5 mM, 9% (SEM 3.37) of the bacteria were killed, however, this was not statistically significant ($p > 0.05$). When used at higher concentration of lactic acid more killing was observed. At 10 mM a significant effect was seen. At this concentration 16% (SEM 2.84) of *Haemophilus influenzae* were killed compare to control (*Haemophilus influenzae* exposed to hydrochloric acid was resistant to killing by this acid. For example at 50 mM, only 13% (SEM 0.32) of the bacteria were killed. Nevertheless, this killing effect was not significant ($P > 0.0633$) compare to control (p

Figure. 5. Determination of impact of acids on *Haemophilus influenzae*: The mean survivals with their SEM for three independent experiments are shown. The * indicates statistical significance relative to control (without added acid).

The effect of acids on growth properties of *H. influenzae* was investigated as described before (2.2.5). The growth rate of untreated *Haemophilus influenzae* was 0.31 h^{-1} ($+ 0.00$), but when the *H. influenzae* were exposed to 5, 10 and 20 mM of LA, the growth rates were 0.20 h^{-1} ($+ 0.007$), 0.19

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h⁻¹ (+ 0.002) and 0.18 h⁻¹ (+ 0.002) for 5 mM, 10 mM and 20 mM, respectively. The decrease in growth rate was significant relative to control (p = 0.021), 0.23 h⁻¹ (+0.014) and 0.08 h⁻¹ (+ 0.001), respectively, relative to control (0.31 h⁻¹ (+ 0.00)). The growth rate was significant relative to control (pH. influenzae were 0.28 h⁻¹ (+ 0.0007), 0.280h⁻¹ (+ 0.0007) and 0.24h⁻¹ (+ 0.0007) for 5, 10 and 20 mM, respectively. The statistical analysis show that HCl was significant relative to control (p > 0.05).

Fig 5. aThe growth rate of Haemophilus influenzae exposed to various acids. The growth rate was calculated from the linear part of growth curve (Table 2).

The effect of acids on bacterial yield was also determined. The yield, the highest absorbance, of untreated (control) was 1.18 but after exposure significant reduction of the bacteria were observed for some acids. At 5, 10, 20 mM of LA the bacterial yield reduced significantly 0.692, 0.627 and 0.468, respectively compare to control and this was significant compare to control (p

Table. 2. Growth yield of Haemophilus influenzae was calculated by taking the highest optical density in the stationary phase.

Investigation of acids on Staphylococcus aureus

To investigate the impact of organic and inorganic acids on Staphylococcus aureus, it was exposed to various acids. The result showed that at any concentration, LA was unable to kill or inhibit S aureus compare to control

(pS. aureus was survived. Similarly, at greater concentration (25, 40, and 50 mM) 95% (SEM 11.43), 94% (SEM 11.26) and, 92% (SEM 10.22) respectively survived. The small reduction observed was not statistically significant compare to control ($p > 0.05$). Regarding FA, at 5 mM 98% (SEM 3.52) of bacteria were survived. However, At 10 mM FA kills 9% (SEM 1.54) of S. aureus. Unlike LA, FA kills bacteria at 25, 40 and 50 mM respectively. At these concentrations 10% (SEM 1.57), 12% (SEM 1.30) and 10% (SEM 0.42) of S. aureus was killed respectively. This killing was statistically significant ($p < 0.0011$).

Regarding the effect of HCl on S. aureus no killing effect was observed at 5 mM and 10 mM. At these concentrations 100% (SEM 0.30), and 100% (SEM 1.35) of S. aureus survival was observed respectively. When a greater concentration of HCl was used a significant effect was observed. At 25mM, 8% (SEM 0.17) of bacteria was killed (WAS IT SIGNIFICANT?). Similarly, HCl was lethal at 40 mM and 50 mM. At these concentrations 8% (SEM 0.49), 11% (SEM 0.04) of bacteria were killed. These killings were significant compare to control (p

Figure 6. Determination of impact of acids on Staphylococcus aureus survival. The mean survival with their SEM for three independent experiments is shown. The lines and *** over the graph indicate statistical significance relative to control (without added acids). A p value below 0.05 was considered statistically significant and the error bars represents standard deviation.

The growth rate of untreated *S. aureus* was 0.540 h^{-1} ($+ 0.013$), but when the *S. aureus* was exposed to 5, 10 and 20 mM LA, the growth rates were 0.485 h^{-1} ($+ 0.034$), 0.485 h^{-1} ($+ 0.034$) and 0.483 h^{-1} ($+ 0.009$) for 5 mM, 10 mM and 20 mM, respectively. The LA treatment did not affect the growth rate significantly relative to control ($p > 0.05$). In comparison, the same concentration of formic acid, 5, 10 and 20 mM, gave the similar growth rates 0.513 h^{-1} ($+ 0.015$), 0.489 h^{-1} ($+ 0.073$) and 0.488 h^{-1} ($+ 0.095$), respectively, relative to control ($0.540 \text{ h}^{-1} + 0.013$). The growth rate again was not affected significantly relative to control ($p > 0.05$). Regarding hydrochloric acid, this inorganic acid did not exert significant effect: growth rates for *S. aureus* were 0.485 h^{-1} ($+ 0.080$), 0.489 h^{-1} ($+ 0.091$) and 0.476 h^{-1} ($+ 0.089$) for 5, 10 and 20 mM, respectively. However, this reduction was not significant relative to control ($p > 0.05$).

Fig6. a. The growth rate of *S. aureus* exposed to various acids. The growth rate was calculated from the linear part of growth curve.

To investigate the effect of acids on bacterial growth yield, *S. aureus* were exposed to sublethal concentration of acids. The growth yield of *S. aureus* was slightly inhibited by all acids. At 5 mM of lactic acid reduced the growth yield to (0.825) compared to untreated (1.07) similarly, formic acid reduced the growth yield (0.89). whereas hydrochloric acid (1.06) did not elicit any effect on *S. aureus* compared control (1.07). At 10 mM lactic acid and formic acid reduced the growth yield to (0.87, and 0.81) respectively when compared to untreated (control). Similarly, at this concentration hydrochloric acid did not affect the growth yield of *S. aureus* (1.07). At 20 mM, lactic acid,

formic acid and hydrochloric acid reduced the growth yield to (0.87, 1.09 and 1.06) when compared to untreated (1.07)

Table. 3. Growth yield of *S. aureus* after various acids were exposed and incubated for overnight. The growth yield was calculated by taking the highest optical density.

The effect of acids on survival and growth of *Streptococcus suis*

The organic and inorganic acids tested on *Streptococcus suis* produced a result similar to that obtained with *H. influenzae*. The data assessed by one-way analysis of variance showed that lactic acid was able to kill *S. suis* at different concentrations. At 5 mM 97% (SEM 0.77) of *S. suis* survived no significant effect was reported. Similar effect was observed at 10 mM (97%, SEM 0.80). However, at higher concentrations different effect was observed. At 25mM, approximately 13% (SEM 1.018) of *S. suis* was killed but at 40mM and 50mM the killing effect was stronger. At 40mM 20% (SEM 0.87) of bacteria was killed. Unlike other concentrations at 50mM LA eliminated 100% of bacteria this killing effect was significant (p $S. suis$, the effect of FA was significant from 10mM onwards. At 10 mM approximately 9% (SEM 1.54) of bacteria was killed. Similar effect was seen at 25 mM. At this concentration 10% (1.54) was killed. However, at 40 mM 12% (SEM 1.30) of bacteria was killed this indicates a small increase of bacterial killings. At 50mM 10% (SEM 0.42) of *S. suis* was killed. In general, the killing effect of FA was significant (p $S. suis$ was not significant. At 5mM 9% (SEM 0.94) of bacteria was killed. At 10 mM 9% (SEM 0.77), and 25 mM 9% (SEM 3.01) of *S. suis* was killed. A higher killing effect was seen at 40mM 13% (SEM 6.10) and 50 mM 13%

(SEM 6.31). However, this inhibition was not significant ($p > 0.05$) compared to control.

Figure 7. Determination of the impact of acids on *Streptococcus suis* survival. The mean survival with their SEM for three independent experiments is shown. The lines and *** over the graph indicate statistical significance relative to control (without added acids).

The growth rate of untreated *S. suis* was 0.53 h^{-1} ($+ 0.007$), but when the *S. suis* was exposed to 5, 10 and 20 mM LA, the growth rates were 0.35 h^{-1} ($+ 0.0007$), 0.30 h^{-1} ($+ 0.0021$) and 0.29 h^{-1} ($+ 0.056$) for 5 mM, 10 mM and 20 mM, respectively. The decrease in growth rate was significant relative to control ($p < 0.002$), 0.14 h^{-1} ($+ 0.017$) and 0.08 h^{-1} ($+ 0.00$), respectively, relative to control ($0.53 \text{ h}^{-1} + 0.007$). The growth rate was significant relative to control ($p < 0.002$), 0.37 h^{-1} ($+ 0.002$) and 0.20 h^{-1} ($+ 0.010$) for 5, 10 and 20 mM, respectively. This reduction was significant relative to control ($p < 0.002$).

Figure 7. a. The growth rate of *S. suis* exposed to various acids. The growth rate was calculated from the linear part of growth curve.

To examine the impact of acids on bacterial growth, *Streptococcus suis* were exposed to sublethal concentration of acids. The growth yield of *S. suis* was slightly inhibited by all acids. At 5 mM of lactic acid the yield was reduced to (1.02) compared to untreated (1.28) whereas formic and hydrochloric acid (0.89 and 1.07, respectively), did not elicit any effect on *S. suis* compared control (1.28). At 10 mM lactic acid and formic acid reduced the growth yield to (0.97, and 0.89) respectively. However, at this concentration

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hydrochloric acid did not affect the growth yield of *S. suis*. At 20 mM, lactic acid, formic acid and hydrochloric acid reduced the growth yield to (0.84, 0.09 and 0.62) when compared to untreated bacteria (1.28) for all experiment. In conclusion, lactic acid was significant at 10 mM and 20 mM. Formic acid was significant at all concentrations 5, 10, 20 mM respectively, but hydrochloric acid was only significant at 20 mM.

Table. 4. Growth yield of *S. Suis* after various acids were exposed and incubated for overnight. The growth yield was calculated by taking the highest optical density.

3. 2. 1 Cytotoxicity of lactic, formic and hydrochloric acids on the A549 cell line

A549 (human lung cancerous alveolar cells) cell line was used to test the impact of acids on epithelial cells, as explained above (2.4) A549 cells were incubated with 10, 25, 50 mM lactic acid, formic acid or hydrochloric acid or complete medium without acids (control) for 24 hours. The results indicated that there is a significant cytotoxicity with different concentrations (mM). In lower concentrations of LA such as 10 mM 91% of the cells survived. At 25 mM a great reduction in the number of live cells were observed and only 16% of them were survived. However, when high concentration of lactic acid was exposed to A549 cells, the reductions of the number of live cells were even greater, for example at 50 mM, only 9% of A549 cells survived and 91% of the cells were killed compared to control (100% survival) (Figure 8).

Figure . 8. The effect of lactic acid on survival of A549 cells Human epithelial cells were incubated with lactic acid for 24h. The cells were grown in <https://assignbuster.com/is-lactate-an-undiscovered-pneumococcal-virulence-factor/>

complete media that contain 1% (5ml) antibiotic [penicillin-streptomycin] and 10 % (50ml) Fetal Bovine Serum (FBS).

Regarding the formic acid cytotoxicity test, significant effect was observed on all concentrations (Figure. 9). At 10mM formic acid the viability of cells was reduced significantly as 22% of them were killed after 24 of incubation. At 25mM 93% of the cells were killed resulting 7% survival of these cells. Furthermore, the higher concentrations (mM) of formic acid exhibit further reduction of the cells under study. Only 5% of these epithelial cells were survived and approximately 95% were killed at 50mM. The p value shows that this killing effect was significant (0. 0001) relative to control untreated cells which 100% survived.

Figure. 9. The effect of formic acid on survival of A549 cells. Human epithelial cells were incubated with FA for 24h. A549 was grown as explained in (2. 4) A Significant reduction in viability was seen at 25 mM and 50 mM FA relative to untreated cells ($p < 0. 001$).

Hydrochloric acid results shows that at 10mM 90% of the cells survived and at 25mM 39% were survived but at 50mM only 8% were survived compare to untreated control (Fig. 10). Overall result shows that acids are cytotoxic to human epithelial cells. The percent cytotoxicity was calculated versus the untreated control (see section 2. 4 for formula).

Figure 10. The effect of hydrochloric acid on survival of A549 cells

Human epithelial cells were incubated with hydrochloric acid (HCl) for 24h. The absorbance of MTT with cell line was measured and a significant

reduction in viability was seen at 25mM and 50mM HCl relative to untreated cells ($p < 0.0011$).

The effect of D39 culture supernatant

After seeing the effect of different acids on *H. influenzae*, I decided to test if *S. pneumoniae* culture supernatant would have any effect on the survival of *H. influenzae*. To test this, *S. p*