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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 nosphrynax 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 pneumonaie, meningitis and bacteraemia. The pathogens can be transmitted through contact. Streptococcuspneumoniae 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 cytotoxicy 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 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.

Keywords: *Streptococcal, pneumonia, Gram-positive immunocompromised, microorganisms BAB, Lactate, fermentation*

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 and pyruvate.

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 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..).

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 10^5 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).

1.4.2a Hyaluronate lyase: 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 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).

1.4.2b Pneumolysin: 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 nasopharynax and also the development of otitis media (Anirban et al., 2010).

1.4.2d Galactosidases: 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 mucindegradation. 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)

1.4.2e Pyruvate formate lyase (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 (**Leppänen, 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/PFl-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).

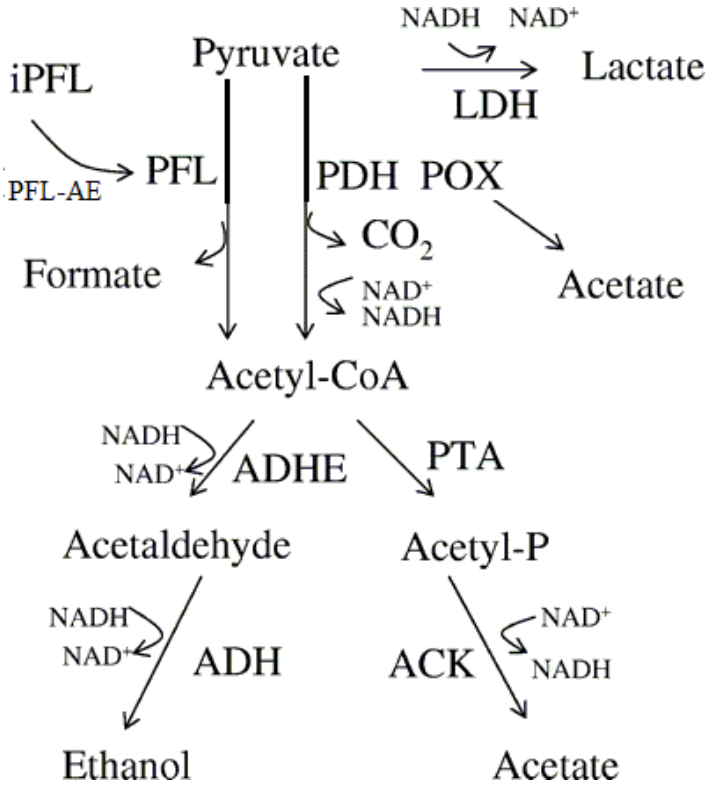

Figure 1: Schematic representation of the lactate pathway in lactic acid bacteria. LDH, lactate dehydrogenase, PFL, pyruvate formate lyase, iPFL, inactive pyruvate formate lyase, PFL-AE, pyruvate formate activating enzyme, PDH, pyruvate dehydrogenase, POX, pyruvate oxidase, ADH, alcohol dehydrogenase, ACK, acetate kinase, PTA, phosphotransacetylase. (Taken from Yesilkaya, 2009)

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 nasophrynax (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 catalyse 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 effects 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.

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 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 pnemoniae. For instance, both streptococcus pneumonia and Streptococcus suis causes meningitis and pneumonia via similar mechanisms.

2. Methods

2.1 Bacterial strains used:

Streptococcus pneumoniae serotype 2 strain D39 was mainly used in this experiment. Several different bacteria were investigated in this experiment. Some of the used bacteria were not explained in this study because I was unable to identify their own strain or they produce conflicting results. The 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. However, only some these of strains were discussed in this study.

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 increases the growth of these fastidious organisms. BAB also allows the detection of the haemolytic activity. The agar surfaces were dried before inoculation.

2.1.2 Bacterial growth and measurement of Optical density (OD_{500})

Stock cultures of *Streptococcus pneumoniae* strain, the wild type D39 was prepared by growing them in 10 ml BHI at 37^0 C under microaerophilic condition to optical density reached OD_{500} 0.4-0.5 at. Then the cultures were centrifuged at 3500 rpm for 10 min in Allegra^{TM} X-22, centrifuge (Beckman Coulter, CA, USA). inoculation were centrifuged at 1500g for 15 minutes and supernatant was discarded, thereafter, the pallet 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 -80c 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.

2.14 The broth cultures used is Brain hearth 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 reach 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 aerus and Pseudomonas aeroginosa Luiria 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 37ºC without co_2 jar or jar with co_2.

2.1.5 Luiria Bertoni broth

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

2.2.1 Chemical 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.

2.2.2 Gram staining

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 2minutes, 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.

2.2.3 Preparing salt 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 µm 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 -20ºC for future usage.

2.2.4 Determination of organic and in organic 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 195µl of CDM and 5µl of bacteria. The other wells was added a solution containing salts at various concentrations and each well was put 195µl of the mixture. The cultures were incubated at 37 ºC in flat bottomed microtitter 96 wells for 2 h. After an incubation period,180µl Phospate buffered saline (PBS) was added to the empty wells to dilute the incubated samples by transferring 20µl of the incubated samples to next well. The dried blood agar plates were divided into six segments and 60 µl of the sample was put to each segment, plates were put near to the flame when dried plates were placed in CO_2 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 1moler. 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 pneumaniae and other bacteria employed in this project.

2.3. The Lactic acid and its effect on *S. pneumonoiae,* 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 co_2jar was used, the plates were sent to the incubator for overnight 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 resulted 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 nasophyrax that might compete with the natural resources. These species are include *Heamophilus influenza, Staphylococci aerus, Streptococci Suis, Group B bacteria, Streptococci 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 ($5X10^6$) 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 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 37^0 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'.

2.3.4 Maintenance of Cells (A549 cells and Hep-2 cells)

To thaw the frozen cells, the cells were immediately placed into 37^0 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 supernatant was discarded and the cell pellet was suspended in 1 ml of complete medium and then transferred into 25 cm^2 tissue culture flask with 15 ml of complete medium and incubated at 37 ^{0}C at 5 % CO_2. 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 37^0 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-2yl]-2-5-diphenyl tetrazolim bromide) is reduced to purple formazan with in mitochondria of living cells. The dead cells are unable to produce this enzyme. Using spectrophometer, 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.

2.4.1 Statistical analysis

Data are expressed as the means ± 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 <0.05.

3. RESULTS

3.1 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 ºC. 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.

3.1.1 Investigation of acids on pneumococcal survival and growth

To investigate the impact of organic and inorganic acids on *Streptococcus pneumoniae* D39 train, I have chosen the organic acids produced as the final metabolic product of *S. pneumonia* (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.

Streptococci 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 compare to control (p

3.1.2 Determination of impact of acids on *S. pneumoniae* D39 strain.

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.pneumonaie*. 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.

3.1.3 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 0.04h^{-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.pneumoniaewere observed*.

3.1.3.1 The effect of acids on survival and growth of Heamophilus influenzae

Haemophilus influenza was exposed to organic and inorganic acids as before. The result showed that LA was lethal at 10 mM onwards. At 5mM, 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 10mM a significant effect was seen. At this concentration 16% (SEM 2.84) of *Haemophilus infleunzae* were killed compare to control (Haemophilus *influenza*e 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.

3.1.4 Determination of impact of acids on *Haemophilus infleunzae*:

The affect 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 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-1 (± 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 ($p>0.05$).

The growth rate of *Haemophilus influenzae* exposed to various acids.

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 ($p<0.0001$). Regarding FA, 5,10, and 20 mM showed similar effect as LA.

At these concentrations the growth yield was 0.7311, 0.5047 and 0.4046 respecively. This reduction was statistically significant compare to control ($p<0.001$)

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

3.1.5 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 ($p<0.05$)

3.1.6 Determination of impact of acids on *Staphylococcus aeurus* survival.

***p* value below 0.05 was considered statistically significant**

The growth rate of untreated *S. aureus* was 0.540 h^{-1} ($\pm$ 0.013), but when the *S.aureus* was exposed to 5, 10 and 20 mM LA, the growth rates were 0.485 h^{-1}($\pm$ 0.034), 0.485 h^{-1} ($\pm$0.034) and 0.483 h^{-1} ($\pm$ 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} ($\pm$ 0.015), 0.489 h^{-1} ($\pm$0.073) and 0.488 h^{-1} ($\pm$ 0.095), respectively, relative to control (0.540 h^{-1} $\pm$ 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} ($\pm$ 0.080), 0.489 h^{-1} ($\pm$ 0.091) and 0.476h^{-1} ($\pm$0.089) for 5, 10 and 20 mM, respectively. However, this reduction was not significant relative to control ($p>0.05$).

3.1.6a 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, 50mM 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).

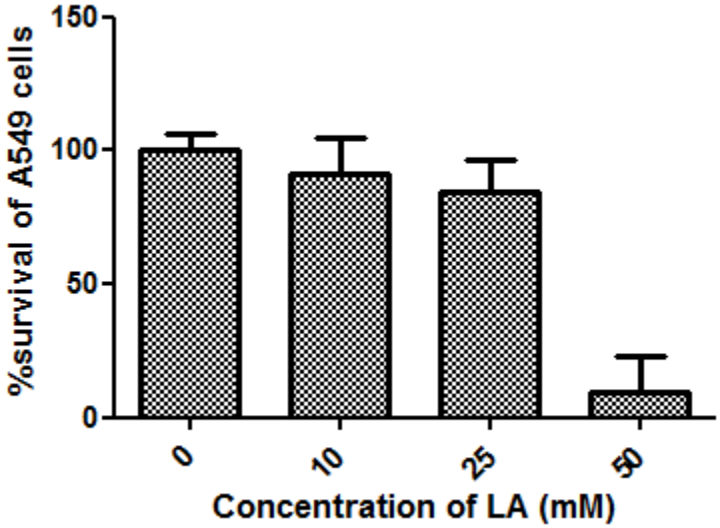

Figure 2. The effect of lactic acid on survival of A549 cells Human epithelial cells were incubated with lactic acid for 24h. The cells were grown in complete media that contain 1% (5ml) antibiotic [penicillin-streptomycin] and 10 % (50ml) Fetal Bovine Serum (FBS).

Regarding the formic acid **cytotoxicity test, significant effect was observed on all concentrations (**Figure.9). **At 10 mM formic acid the viability of cells was reduced significantly as 22% of them were killed after 24 of incubation. At 25 mM 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 50 mM. The p value shows that this killing effect was significant (0.0001) relative to control untreated cells which 100% survived.**

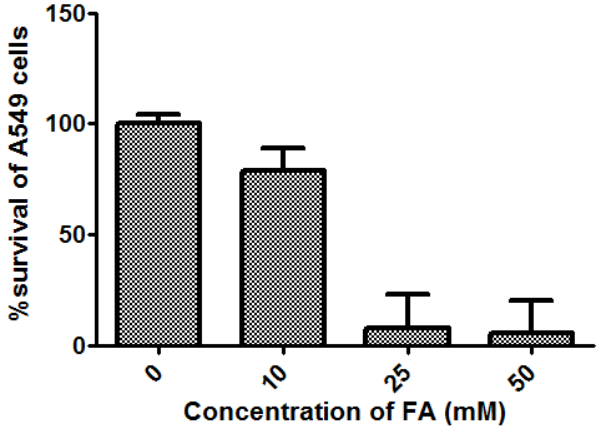

Figure.3. The effect of formic acid on survival of A549 cells. Human epithelial cells were incubated with FA for 24h. A549 was grown as explained in (2.4) A Significant reduction in viability was seen at 25 mM and 50 mM FA relative to untreated cells ($p < 0.001$).

Hydrochloric acid results shows that at 10 **mM 90% of the cells survived and at 25 mM 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 percentcytotoxicity was calculated versus the untreated control (see section 2.4 for formula).**

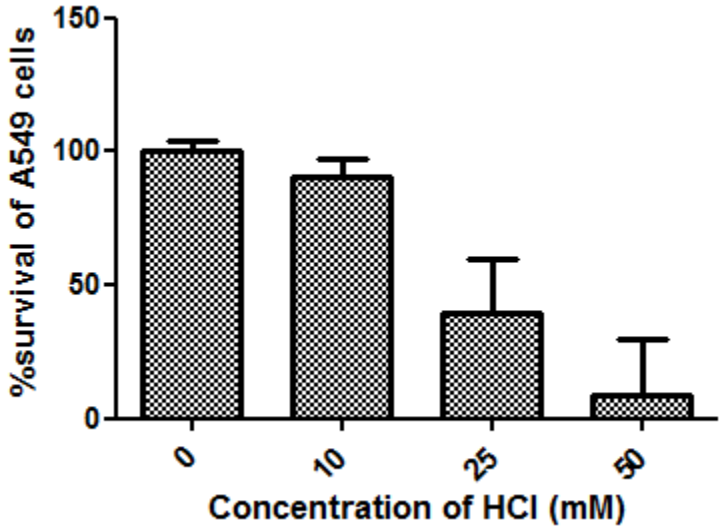

Figure .3. The effect of hydrochloric acid on survival of A549 cells

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 ($p < 0.0011$).

3.1.6b The effect of D39 culture supernatant

After seeing the effect of different acids on *H. influenzaue*, I decided to test if S. pneumonia culture supernatant would have any effect on the survival of *H. influenzae*. To test this, *S.pneumoniae* was grown in CDM supplemented with galactose, which stimulates mixed acid fermentation. The bacteria were pelletd by centrifugation and supernate was filter sterilised. The supernate was kept at -20 until needed. *H. influenza* was resuspended in the culture supernate for 2 h at 37 C and the viability of bacteria was determined as before.

4.0 Discussions

In this work, I investigated how organic and inorganic acids contribute the pneumococcal virulence. Bacterial colonisation is essential step for the bacteria to cause disease to the host, the commensal bacteria such as Streptococcus pneumoniae and Haemophilus influenza colonise the nasophraynx and compete with natural resources. This competition can occur in species level as well as strain level (Margolis, 2010).

In upper respiratory tract, there are many other species and strains that are able to continuously compete for the natural resources that are available; these resources include nutrients, space and attachment space. As part of competition some bacteria are capable to secrete harmful substances that can kill or inhibit the growth of co-existent species. This inhibition or killing effect can hugely contribute the virulence factor for that species and thus significant reduction of the mircobiota in upper respiratory tract results in immune deficient.

To investigate the impact of pneumococcal metabolic end products on other species in nasophyranx I have examined whether the product of pneumococcal fermentative metabolism would kill other co-inhabiting bacteria. These acids, lactic and formic acids, were shown to be

secreted in culture medium. Here, the result showed that D39 strain is resistant to lactic acid and formic acid up to 25mM. In contrast, hydrochloric acids were unable to show any bactericidal effects even at 50 mM. To test whether the three species (Streptococci pneumoniae D39, Haemophilus influenzae and Staphylococci aeurus) that readily colonise the upper respiratory tract behave and elicit the same effect against the utilised acids, we evaluated the susceptibility of Heamophilus influenzae and Staphylococcus aureus on these acids (lactic acid, formic acid and hydrochloric acid). According to the above result, S. aureus is resistant to lactic acid in all concentration (Figure 6). However, the other two acids (formic and hydrochloric acids) are able significantly to kill or inhibit the bacteria. The reason for the efficacy of the formic acid and hydrochloric acid over lactic acid is not clear. Although, this question is beyond the scope of the present study it is possible to speculate that Staphylococcus aureus is lactic acid tolerant. Because acids possess different inhibition capacity and the effect of acids on S. aureus depends on the nature of the acid causing acidification (Charlier, C.2008). A previous study indicated that at a lower pH (4.5-4.4) lactic acid is able to completely inhibit S. aureus (Charlier, C.2008). It is important to note that the mechanism of acid antimicrobial activities appears to be multifactoral (Fayol-Messaoudi, 2005).

The level of pH can be very important and can influence the bacterial growth (Raftari, et al., 2009). Although, decrease of pH influences the inhibition of bacteria, yet lowering the level of pH alone will not be enough to inhibit bacteria. However, in this study, the pH level was close to neutral and organic acids were able to elicit bactericidal effect. Because acids possess different inhibition capacity, organic acids

cross cell membranes as non-dissociated form of organic acid, once inside cell the acids dissociate and releasing proton H+ .(Cotter and Hill,2003) This acidification results an increased loss of purines and pyrimidines from DNA. The excessive acidification might influence reduction of pH in cytoplasm and thus will lead to protein denaturation and the stimulation of DNA damage (Skrivanova, 2006). In this study, it was found that formic acid elicited the strongest antibacterial effect. Perhaps the formic acid activity is related to its structure. Formic acid is an organic acid that possess the shortest chain compare to other acids. This might contribute to the capability of formic acid to cross cell membrane and diffuse into the cell and eventually cause acidification of the cytoplasm (Raftari, et al 2009). Analysis of acids on H. influenzae revealed that lactic acid in particular is bactericidal against H. influenzae. Unlike S. aureus, H. influenzae was significantly killed by lactic acid at lower concentrations mM (10 mM) onwards (Figure 5). In contrast, formic acid also killed bacteria in considerable percentage at 25 mM. However, inorganic acids were unable to inhibit the growth of bacteria in any concentrations mM. The findings that lactic acid and formic acid which are pneumococcal by-products can affect Haemopilus influenzae suggests that the nasophyranx coinhabitants compete for natural resources in the host. As part of the process of competition, pneumococcal by-products reduce presence of other bacteria and data presented here supports this hypotheses. Although, acids alone may not act as virulence factors, they are undoubtedly contributed to pneumococcal virulence. To study the effect of acids on bacteria that live outside the nasophyranx, several other bacteria were also used. Streptococcus suis exposed to lactic acid formic acid revealed similar effect to that H. infleunzae. Although S. suis is a member of lactic acid bacteria similar to S. pneumoniae, it is not known why S. suis exhibited different phenotype than S. pneumoniae. The ability of culture supernatant from S. pneumoniae to kill or inhibit the growth of Haemophilus influenzae in vitro was investigated (Fig 11). The killing effect was significant and H.influenzae was eliminated in a time dependent manner. Another study carried out by (Pericone et al., 2000) showed that the

culture supernatant of S.pneumoniae killed Haemophilus influenzae. It is well established that S. pneumoniae secretes a soluble antimicrobial substance that can kill or inhibit the growth of other micro-organisms residing the upper respiratory tract of humans. Different studies documented that this antimicrobial substance was hydrogen peroxide (Pericone et al., 2000). The culture supernatant of S. pneumoniae exposed to H. influenzae showed that H.influenzae was eliminated. This elimination shows the effect of pneumococcal competition against other species co-existing in the upper respiratory tract. The substances produced by the pneumococcus are able to contribute to the pathogenesis of diseases and can exhibit cytotoxic effects on epithelial cells (Pericone et al., 2000). Lactate and formate are the by-products of carbohydrate metabolism, both acids are essential for generating energy. For formic acid cytocoxcicity study done by (Hayreh et al., 1980) have demonstrated that the metabolic formic acid posses cytotoxic effect on epithelial cells. The mechanism of this cytotoxicity is that formic acid disrupts mitochondrial electron transport and therefore energy production. In addition to that formate inhibits cytochrome oxidase activity when used at different concentrations particularly concentrations between 5 mM and 30 mM (Treichel, et al., 2004 and Hayreh et al., 1980). In this study, the cytotoxic effect of lactic, formic and hydrochloric acids on respiratory cells was investigated. Here we report that all acid show cytotoxic effect on epithelial cells A549 and apoptosis was found in all cell lines treated with the acids. For lactic acid the minimum concentration that able to inhibit the growth of A549 cells was 50 mM. The other two acids are capable of inhibiting the growth of cell lines at lower concentrations (25mM). Formic acid was more potent compared to other two acids. The results showed that all acids elicit a significant effect against epithelial cells however, the mechanisms that acids inhibit the epithelial cells was not clear. Other studies have shown that acids could kill cells by damaging epithelial cells and thus able to induce inflammation via secretion of tumor necrosis factor (TNF). Undamaged epithelial cells are important to prevent tissue from bacterial invasion (Maudsdotter, 2011). The experimental investigations showed that lactic acid and formic acid produced by lactic acid bacteria and inorganic hydrochloric acid are capable to induce apoptosis at physiological concentrations. This cytotoxicity may contribute to the pathogenesis and invasion of bacteria. In conclusion, Organic acids produced by S. pneumoniae as a result of fermentative metabolism are able to kill or inhibit the other colonising bacteria in the nasophynax. And they exhibit an adverse effect on respiratory cells and thus they are able to contribute inflammation. Future studies investigating the role of lactate to contribute pneumococcal virulence are required. Nevertheless, this investigation must consider the mechanisms of acids after crossing cell membrane and once penetrated inside cytoplasm.

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