Diabetic foot: microbiology, pathogenesis and glycan studies
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Diabetic Foot: Microbiology, Pathogenesis and Glycan studies

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Dedication

To my grandmother and my mother who passed away during the course of my PhD, may god bless their soul; I feel their presence through failure and achievement

ABSTRACT

Complications of type 2 diabetes mellitus are one of the major causes of morbidity and mortality around the world. Diabetic foot infections remain one of the major complications leading to a leg loss every 3 seconds due to amputations causing mental trauma and distress. In diabetic foot ulcers aerobes, anaerobes and fungus often interact with each other and form biofilms which is difficult to treat, enhancing antimicrobial resistance and lead to a non-healing ulcer. Co-existing peripheral vascular disease and neuropathy exacerbate the problems. In T2DM patients' minor cuts and wounds, often lead to hard to treat and chronic ulcers which can worsen to gangrene formation which may lead to osteomyelitis compromising the mechanics of the foot. It is necessary to identify the virulence factors of these clinically significant microbes and to identify the resistance patterns regularly to limit the antibiotic usage and target to the specific organisms.

A Cohort studies were carried out in India and in the UK to identify the risk factors among the diabetic foot patients along with their microbial aetiology and antibiotic resistance patterns from the tissue and pus samples. This part of the research has shown the presence of mixed cultures mainly from the Indian diabetic foot ulcer specimens with higher percentages of anaerobes than aerobes. Multi-drug resistant organisms were one of the peculiar characteristics of the diabetic foot ulcer profiles of Indian patients. As compared to the Indian patients, UK patients had few resistant organisms and the patients admitted to hospitals in India were at the last stage of foot ulcers whereas in the UK, surveillance and preventative strategies allow early detection and intervention.

Currently there is a lack of rapid, robust and an inexpensive diagnostic method for the rapid typing and identification of clinically significant anaerobes. Another part of the research focussed on utilising the glycan-lectin interactions by developing a simple enzyme linked lectin sorbent assay by employing biotinylated lectins to develop to an enzyme linked lectin sorbent assay (ELLA) on whole cells, Proteinase K treated cells and glycolipids of clinically significant aerobes and anaerobes. This study is concluded by utilising the glycan-lectin interactions and to develop a rapid typing method for clinically significant Methicillin resistant and sensitive *Staphylococcus aureus* and *epidermidis* species. The rapid identification of anaerobes and typing of *Peptostreptococcus* species was also by facilitated by the developed ELLA method.

Finegoldia magna is one of the most significant anaerobes from soft tissue infections and the Gas Chromatography – mass spectrometry (GC-MS) of the glycolipids of *Finegoldia magna* on composition analysis using show the presence of sialic acid which could be involved in pathogenesis. This sugar may be one of virulence factor employed by this organism in either attachment to the host or to other organisms.

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CHAPTER – 1

GENERAL INTRODUCTION

1.1 History of Diabetes

Diabetes has been known to be a potentially lethal disease for more than a thousand years (Dobson, 1968). In the past, different types of treatment were tried by Physicians from medications, concoctions to diets, which might prolong life.

The history of diabetes has involved contributions from many people from all over the world. It has evolved by step wise discovery from the identification of symptoms, to the recognition of the formal name for the condition, understanding the various types of diabetes and finally to the elucidation of the biochemistry of diabetes. The identification of the pancreas as the organ involved in causing this condition led to the discovery of Islets of Langerhans and subsequently the isolation of insulin: one of the most significant milestones in diabetes research (Leibiger, Brismar and Berggren, 2010). Most recently hopes have been raised that a cure is imminent with advances in the transplantation of beta cells, stem cell therapies and research into gene therapy (George, 2009; Robertson, Drexler and Vernillo, 2003 and Johnson and Luciani, 2010).

From the ancient times Physicians with different beliefs on the cause of diabetes tried a variety of treatments to take care of the condition. The first mentioned medical condition that was distinguishable as diabetes was found in an ancient papyrus, discovered in 1862 by German Egyptologist George Ebers (Papaspyros, 1982). From around 1500 B.C translations have revealed different concoctions that "*could remove the urine which runs very often among the people with such condition"*. A Roman writer, who lived during the times of Hippocrates, translated Greek writings on medicine and claimed that people with this medical condition urinated more than they drank. His view was accepted for hundreds of years, until Girolamo Cardano measured the amount of fluid consumed by such patients and then compared this to the amount of urine produced (Pyke, 1997). It was equivalent and hence the error was rectified. Aretaeus of Cappadocia in the

1st century AD were the first to describe the condition formally as diabetes and described the condition as the passing of copious amounts of urine and the loss of body flesh (Dobson, 1776). Another Greek Physician Galen said that this condition was "*a weakness of the kidney which cannot hold back water"* thus advising horse riding at the time as a good way to get rid of the excess urine. The Chinese physician Gang-ke suggested that the urine from diabetic people was sweet and that it attracted dogs.

In the fifth and the sixth century a reputed Indian Physician, Dr. Susruta, for the first time recognised the two primary types of diabetes, one affecting very thin people and another form most often seen among the obese and more sedentary individuals (Raghunathan, 1976). He recommended a moderate diet with low fat, salt and sugar along with exercise for overweight patients; a treatment still offered to people with diabetes (O'Gorman and Krook, 2008; Bluherm and Zimmer, 2010). Thomas Willis, a 17th century Oxford University Physician, believed that diabetes was the disease of blood and suggested that sugar found in the blood was found aberrantly in the urine. It was only in 1857 that Claude Bernard isolated a substance, glycogen, and illustrated the role it played in the metabolism of glucose (Young, 1957). Until this time however the organ involved was not known (Pyke, 1997). In 1869, German researcher Paul Langerhans highlighted a special cluster of cells within the pancreas. Although the cells were recognised, the role they played in diabetes was not established. In 1889, Oskar Minkowski performed experiments on removing the pancreases of dogs, thus inducing diabetes in the animals and definitively proving the significance of the pancreas (MacLeod, 1978; Leibowitz, 1972). This led to Miskowski and others to search unsuccessfully for the substance that differentiated diabetics from non-diabetics (MacLeod, 1978). In 1893, Edouard Laguesse noted the importance of the cells identified by Langerhans and in honour of Langerhans called them "Islands of Langerhans". They are now known as islets of Langerhans (Leibowitz, 1972).

In the early 20th century Rennie and Fraser used the extracts of islets of Langerhans from a special fish purchased from the Aberdeen fish market to treat patients with diabetes. Doctors ran out of the fish and hence the compounds could no longer be used to treat the patients (Rennie *et al*.,

1907). It was only in 1921 that Drs. Frederick Banting and Charles Best isolated insulin from the pancreas of an animal and used that to treat diabetes in humans. Throughout the years since the insulin from the animals were used the ingredients and forms of insulin have been changed and improved. Banting and Best's work on the discovery of insulin has overcome many potentially fatal symptoms of diabetes by controlling the blood sugar and acetone bodies (Allan, 1972 and Fulton, 1956). In the 21st century the treatment options for patients with diabetes include transplantations of beta cells, stem cell transplants as well as well transplants of organs such as pancreas or pancreas/kidney (Gunaselli and Doss, 2010 and Johnson and Luciani, 2010). Recombinant insulin identical to human insulin and modified human insulin with more rapid and much slower absorption are used thus replacing the traditionally used porcine insulin. Genetic engineering is now used to produce naturally occurring peptides that are able to stimulate the growth of insulin-producing cells in the pancreas (Garcia *et al*., 2001).

Advances in diabetes research are significant and much needed because diabetes is on the rise worldwide and is considered by some experts already to be at an epidemic level (Zimmet *et al.,* 2001 and Wild *et al.,* 2004).

1.2 Types of diabetes

It is clear from history that diabetes is a chronic incurable disorder which can be managed but not cured apart from pancreas transplantation. There are two main types, diabetes mellitus and diabetes insipidus (Rybka, 2010). Both diseases have the same symptoms of thirst and urination except that diabetes insipidus is caused due to changes in the antidiuretic hormone produced by the pituitary glands which directly affects water retention hence diabetes insipidus is called the 'water' diabetes. Diabetes mellitus on the other hand is often called the 'sugar' diabetes and is caused by the pancreas malfunctioning leading to insulin deficiency or a defect in the secretion of insulin or insulin resistance discussed in details in section 1.The research presented in this thesis is focussed only on diabetes mellitus. Diabetes mellitus is a common disorder affecting individuals world wide and can be broadly classified into 3 main types. Type 1 diabetes mellitus (T1DM) formerly known as insulin dependent diabetes mellitus is an auto-immune disease where the body's immune system destroys the insulin-producing beta cells in the pancreas. This type of diabetes is also

known as juvenile-onset diabetes and accounts for 10-15% of all people with the disease. It can appear at any age although most often below the age of 40. The second most significant type is the Type 2 diabetes mellitus (T2DM) previously known as non-insulin dependent diabetes mellitus. This type of diabetes is also known as late-onset diabetes and is characterised by insulin resistance and relative insulin deficiency therefore insulin concentrations in the blood are high. The pathophysiology, symptoms, prevalence and the complications arising due to T2DM are detailed later in this chapter. The third type of diabetes includes the gestational diabetes (GDM) which is often seen among pregnant women. GDM, or carbohydrate intolerance, is first diagnosed during pregnancy through an oral glucose tolerance test. Risk factors for GDM include a family history of diabetes, increasing maternal age, obesity, ethnic group with a high risk of developing T2DM (Landon, 2010 and Rybka, 2010). While the carbohydrate intolerance usually returns to normal after the birth, the mother has a significant risk of developing permanent diabetes while the baby is more likely to develop obesity and impaired glucose tolerance with diabetes later in life (Landon, 2010).

1.3 Prevalence of T2DM in the world: Projections for 2030

Type 2 diabetes mellitus (T2DM) has become a worldwide health problem that is almost certain to worsen. It remains an important cause of mortality and morbidity worldwide. Poor glucose control and presence of diabetes complications (detailed in this chapter in section 1.7) are commonly regarded as risk factors for mortality and morbidity. It has been predicted by the World Health Organisation (WHO) that Type 2 diabetes mellitus will soon become a serious health problem worldwide with an estimated 300 million diabetics in 2025 (Zimmet *et al.,* 2001 and Wild *et al.,* 2004)**.** According to WHO the top 10 countries with high number of diabetics are India, China, USA, Indonesia, Japan, Pakistan, Russia, Brazil, Italy and Bangladesh. The estimates for India include 31.7 million in the year 2000 to a drastic increase to 79.4 million diabetics by the year 2030. As per WHO

facts and figures in the UK the estimates include 1.7 million in the year 2000 to 2.6 million of diabetics by 2030 (WHO website).

1.4 Pathophysiology of Type 2 diabetes mellitus

Type 2 diabetes results from the body's ineffective use of insulin or defects in the synthesis of insulin and affects 90% of people with diabetes around the world (worlddiabetesday.org). The endocrine cells namely α and β cells produced by the islets of Langerhans in the pancreas are responsible for the production of hormones glucagon and insulin respectively (Spellman, 2007). About 60% of the endocrine mass comprises of the β cells which produce both insulin and amylin of which insulin is released in response to the elevated plasma glucose (Spellman, 2010). Thus the homeostasis in the body to control blood sugar relies on the production of the hormones insulin and glucagon. Insulin in a healthy individual converts the extra glucose in to glycogen and this is stored in the muscles. Insulin is essential for the entry of glucose into tissues and cells to provide energy. Glucagon is normally produced by α cells when the sugar levels in the body are low and this stimulates the liver to convert glycogen to glucose. Therefore the regulation of glucose involves the production of both hormones insulin and glucagon by the endocrine cells. The metabolic response to ingested carbohydrate is different in individuals with normal glucose tolerance in comparison to those with T2DM. After ingestion, there is a predictable increase in the plasma glucose levels consequently an increase in the insulin levels to dispose of the ingested glucose load in contrast however the glucagon secretion is discontinued (Spellman, 2010 and Unger and Parkin, 2010). This response keeps the plasma glucose within a homeostatic range despite the variation in food intake in healthy individuals. With the development of impaired glucose tolerance, and as the condition progresses, pre-diabetes is recognised with impaired fasting glucose (Wheatcroft *et al*., 2003). The metabolic responses to ingested glucose changes dramatically. Since insulin levels are not routinely measured in the clinical laboratory as a part of the standard biochemical measurements, insulin resistance is not clearly evident in a normal day to day life. Insulin resistance leads to a decrease in the uptake of glucose which results in postprandial hyperglycaemia Weyer *et al.,* 1999). Thus the pathophysiology of T2DM is mainly characterised by peripheral insulin resistance (an insensitivity of the tissues to the effects of

insulin), impaired regulation of glucose production and declining β-cell function, eventually leading to β-cell failure (Unger and Parkin, 2010).

There are many other factors which can lead to insulin resistance one of which is obesity. Body fat content in obese people produces insulin resistance by depositing the excess intra-abdominal fat within the adipose tissues which in normal healthy individuals would have held into liver and skeletal muscle cells (Matsuzawa, 2010). Due to this there are increasing levels of triglycerides by the release of fatty acids and the excess fat cells release chemicals called inflammatory cytokines which block the insulin receptors hence the pancreas produces more and more insulin eventually damaging the beta cells (Gabriely *et al*., 2002). In contrast, in low-birth weight individuals there is less adipose tissue to hold the excess fat leading to a similar increase in triglycerides levels leading to insulin resistance (Gabriely *et al., 2002).* Other studies have suggested that there are certain hormones such as leptin and resistin which play a significant role in increasing insulin resistance. In the absence of leptin, triglycerides accumulate in non-adipose tissues leading to insulin resistance while resistin similar to leptin plays a major role in pathogenesis of insulin resistance by virtue of its ability to oppose certain actions of insulin. Other studies have suggested that low adinopectin (an adipose tissue-derived protein) concentrations also can produce insulin resistance by stimulating the fatty acid oxidation (Rea and Donnelly, 2004 and Beltowski, 2003)**.**

Fatty acids levels are elevated in obese individuals, and this has a direct effect on carbohydrate metabolism such that fatty acid decreases the glucose uptake, glycogen synthesis and glycolysis: the effects that are normally promoted by insulin. Additionally the fatty acids also inhibit suppression of hepatic glucose production thus leading to increased hepatic glucose production (Boden, 2003).Another study suggests that islet amyloid could be involved in beta - cell failure as a result of T2DM (Andersson *et al.,* 2008 and Udayashankar *et al.,* 2009). Amyloid is a proteinaceous fibrillary deposit that is seen in certain pathologic processes. In T2DM, islet amyloidosis which is responsible for promoting beta cell damage consequently death is frequently observed (Hoppener *et al*., 2002). The regulation of glucose can also be altered by certain environmental factors such as sedentary lifestyle which can lead to obesity in turn leading to

insulin resistance. Genetic predisposition could be one of the factors which can cause beta cell damage and in turn ineffective production of insulin. The entry of glucose into the cells is necessary to provide energy and cell starvation can lead to an increased synthesis of ketone bodies which are a cause of a number of complications discussed below in section 1.6. Some of these complications however can be controlled by administration of insulin-secretory drugs such as sulfonylureas (Baron, 2002). If the insulin-secretory drugs can regulate the insulin levels in the blood then exogenous insulin would not be required as an essential part of the treatment.

1.5 Symptoms of Type 2 diabetes mellitus

To summarise from the above literature, the characteristic symptoms of diabetes mellitus are the result from abnormal glucose metabolism. Due to insulin resistance glucose does not reach the cells and hence glucose accumulates in the blood causing hyperglycaemia which is subsequently excreted in the urine (glycosuria). Glucose in the blood causes osmotic imbalance and hence causes frequent urination (polyuria). Because of the protein breakdown during gluconeogenesis in an attempt to provide more glucose to the cells there is weight loss. These symptoms are more common when there is severe insulin deficiency. T2DM often leads to severe complications described in this chapter in section 1.6.

1.6 Complications of Type 2 diabetes

Complication of diabetes affects almost all parts of the body. Patients with Type 2 diabetes mellitus (T2DM) are known to have an increased risk of microvascular coronary heart disease, myocardial infarction leading to cardiovascular disease, peripheral vascular disease, neuropathy, nephropathy, retinopathy, cerebrovascular disease and diabetic foot ulcerations. Progressive diabetes often leads to a depletion of vitamins and minerals excreted in the urine because of the kidneys attempting to remove the excess glucose. The loss of these nutrients prevents carbohydrate, protein and fat metabolism leading to electrolyte imbalance especially imbalance in salt and water that affect nerve protection, muscle relaxation and insulin regulation. Due to visceral fat and insulin resistance discussed earlier there is an increase in the production of triglycerides due to the conversion of extra glucose which further increases the viscosity of the

blood and cholesterol levels. Due to hyperinsulinemia, there is an increase in the blood pressure with thicker blood and narrow arteries. The Inflammatory mediators such as C-reactive proteins, fibrinogen, and lipoproteins are released due to T2DM and cause the formation of plaques in the artery walls which can over time lead to formation of blood clots. These inflammatory mediators can cause severe cardiovascular diseases such as heart attacks as well as strokes due to ischaemia (lack of blood supply). These inflammatory mediators can also cause damage to the proteins in the cells due to the increased production of advanced glycated products. When the blood glucose rises too high and hyperglycaemia is uncontrolled the glucose can attach permanently to the cells and is eventually converted to the toxic chemical sorbitol which destroys the cells (Wheatcroft *et al.,* 2003).This process along with other risk factors can lead to blurred vision, burning foot syndrome, tingling and loss of feeling in the limbs eventually to life-threatening conditions. The research presented in this thesis focuses on Type 2 diabetes mellitus and one of the complications, diabetic foot infections associated with it.

1.7 Prevalence of diabetic foot (DF) infections

Diabetic foot infections are one of the major long term complications of Type 2 diabetes mellitus which can result in gangrene and lower extremity amputation. Every year 4 million people around the world develop foot ulcers. Patients with diabetes are 25 times more likely to lose a leg than those without the condition, and up to 70% of all leg amputations occur in people with diabetes. The result is that a leg is lost to diabetes every 30 seconds somewhere in the world (Bakker, Houtum and Riley, 2005). The complications related to diabetic foot ulcers are so serious that a consensus document was produced in 1999 by the International Working Group on the guidelines on diagnosing and treating infected diabetic foot and most recently guidelines for the treatment of diabetic foot osteomyelitis in 2007.

1.8 Pathophysiology of diabetic foot infections

Foot infections in diabetic patients usually begin with the formation of the acute wound. A wound is defined as a break in the epithelial integrity of the skin; the disruption could be deeper, extending to the dermis, subcutaneous fat, muscle of even the bone. Healing in an acute wound involves a

sequential flow of overlapping processes which requires various cellular and inflammatory pathways including phagocytosis, chemotaxis, mitogenesis, collagen synthesis and the synthesis of other matrix components shown below in figure 1.1 (Clark, 1996).

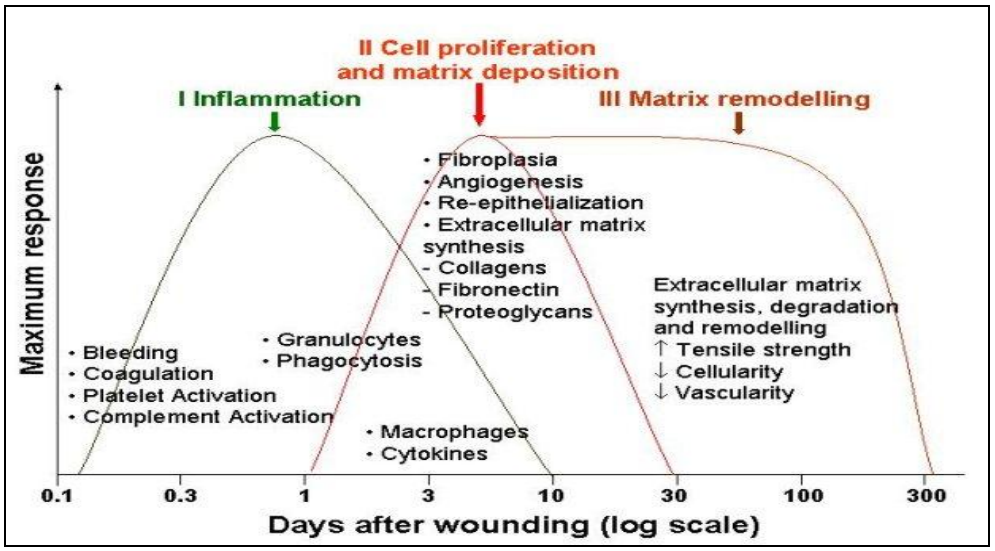


Figure 1.1 Cellular and Inflammatory responses to acute wound healing

http://www.worldwidewounds.com/2004/august/Enoch/Pathophysiology-of-Healing.html - reprinted with permission from the author Prof. Patricia Price.

A completely healed wound is one which restores its normal anatomical structure, function and appearance within a reasonable timeframe. In diabetic patients the cellular and the inflammatory pathways involved in wound healing are affected. In diabetes hyperglycaemia particularly through hyperosmotic effects influence neutrophils and fibroblasts breaking the healing cascade (Lazarus *et al.,* 1994). The faulty healing response is additionally affected due to the wound hypoxia caused by the microvascular and macrovascular conditions within the diabetic patients (Yamasaki *et al.,* 2010 and Kashiwagi *et al.,* 2010). The microvascular conditions lead to injury in small blood vessels often leading to vasoconstriction. As the condition progresses the vascular abnormalities affect the membranes of the blood vessels leading to macrovascular conditions mainly peripheral vascular disease affecting the leg artery. Neuropathy predisposes the foot to infection and angiopathy influences the outcome (Edmonds and Foster, 2004 and Armstrong and Boulton, 2007). Autonomic neuropathy causes dry skin and predisposes the skin to cracking. A foot with sensory neuropathy tends to suffer repeated injury, thus disrupting the skin integrity and providing a route for microbial invasion leading to an unhealed wound which

further develops into a chronic ulcer (Bjarnsholt *et al.,* 2008). Motor neuropathy causes atrophy of the intrinsic foot muscles, altering the foot architecture leading to osteomyelitis. Bacterial invasion results from the loss of integrity of the skin and causes trauma and results in infections (Tan and File, 1999). Due to the higher glucose levels and free fatty acid metabolism there is an oxidative stress in the wound often referred to as *ischemic wound* which renders a perfect environment for the anaerobes. Patients with these additional risk factors such as ischemia, neuropathy and peripheral vascular disease often have unsuccessful inflammatory responses and hence have weakened immune system. One of the peculiar characteristics often seen in the chronic wound within an immuno-compromised diabetic patient is replicating micro-organisms. Additionally the excess sugar lowers the resistance to infections which further leads to a gangrenous ulcer with lower limb amputations (Gadepalli *et al*., 2006). About 60% of the amputations are as a result of infection of the diabetic foot ulcer making infection the most important pathway of DF. Infection, however, is never the sole cause of DF and other risk factors include increasing age, sex, race, smoking habits, duration of diabetes and glycated haemoglobin (HBA1C) (Mavian *et al*., 2010). The history of previous hospitalisations for the same wound, duration of the wound, duration of hospital stay, osteomyelitis, previous antibiotic therapy and non-healing gangrenous foot ulcer can also affect the diabetic foot ulcer and its prognosis (Singh, Armstrong and Lipsky, 2005). Thus multiple factors contribute to the aetiopathogenesis of diabetic foot infections (Neu *et al*., 2001).

1.9 Rationale and objective of the research

An Infection plays a significant role in the worsening of the ulcers and can lead to the formation of gangrenous wounds which often can lead to amputations (Zayed *et al.,* 2009). The research presented in this thesis is an attempt to identify the aetiology of the varied microbial floras from two different diabetic foot patient's cohorts and understand if there are any antibiotic resistant strains present in the diabetic ulcers and understand the differences within the Indian and UK diabetic foot cohorts discussed in chapter 2 of this thesis. After understanding the microbial flora dominating in the DFI, the study was further extended to understand the pathogenic mechanisms utilised by these organisms to impart infections and worsen the

diabetic foot ulcer leading to amputations. There have been studies suggesting that glycan – lectin interactions can be utilised for cell – cell adherence or attachment of bacterial glycan and host lectin or glycans from one bacterium to the lectins of another bacterium facilitating their attachment and forming biofilms which often can be difficult to treat (Dowd *et al.,* 2008). The glycan – lectin interactions were then identified by an in house developed method discussed in chapter 3 of this thesis. The identification of anaerobes has always been a tedious and expensive procedure therefore there is a need to explore different methods that are easy and cost-effective for identification of the clinically significant anaerobes (Menon *et al.,* 2007 and Nagy, 2010). Since unique glycan – lectin binding patterns were obtained these were further explored for their potential in rapid diagnostics of microbial typing and identification. *Finegoldia magna* is one of the most dominant anaerobes cultured from DF ulcers (Wall *et al.*, 2002 and Stephens *et al*., 2003). It remains significant to understand the ways by which this organism can be pathogenic. Glycolipids have shown to be involved in imparting pathogenic characteristics to organisms (Lis & Sharon, 1998; Lloyd *et al*., 2007 and Schauer, 2000). Glycolipids were therefore extracted and glycan profiles were analysed using the in house developed method along with GC-MS and HPAEC that robust were tools for the characterisation of *Finegoldia magna* glycolipids discussed in chapter 4 of this thesis.

The aims of this research study were:

- To identify and compare the microbial aetiology and antibiotic susceptibility testing on the clinical isolates obtained from the diabetic foot patients from India and the UK.
- To investigate the use of glycan-lectin interactions as a robust tool in typing and identifying pathogens pre-dominantly present in diabetic foot infections.
- To characterise the glycolipids of *Finegoldia magna* one of the most dominant anaerobe cultured from the diabetic foot infections using robust methods such as GC-MS and HPAEC.

Chapter 2

Pilot study on the microbiology and antibiotic susceptibility testing of clinical isolates from the diabetic foot patients from India and the UK

Rationale for the study:

In the UK the National Health Service provides a network of diabetic foot clinics resulting in early diagnosis of the diabetic foot infections (Home *et al.,* 1999). National diagnostic and treatment guidelines are set out by the Department of Health and the National Institute of Clinical Excellence (NICE). In India however due to the lack of guidelines, patient non – compliance to treatment, poverty and lack of health education, the diabetic foot patients often only reach the hospital at a very late stage of their infections (Jayasinghe *et al*., 2007 and Pradeepa *et al.,* 2002). In India alone there are 40,000 amputations a year and there are only 100 diabetic foot clinics all over India (Pendsey, 2010). Infections often play a significant role in causing amputations (Zayed *et al.,* 2009). It is important to have an effective knowledge of the principal bacteria, their local antibiotic sensitivities and the prevalence of resistant organisms. If clinicians could gain a better understanding of the wound's microbiota, and ecology, this would allow them to better manage healing of the wound improving the prognosis for patients.

2.1 Clinical aetiology of diabetic foot infections

A diabetic foot ulcer involves repeated infections due to aerobes, anaerobes or fungi individually or in combination (Zayed *et al*., 2009). The infection often starts locally with an ulcer affecting immediate surrounding skin with a purulent discharge and erythema. These signs can be missed due to the presence of neuropathy and ischaemia which are the two of the commonest risk factors to DFI (Boulton, 2007 and Pendsey, 2010). The infection can become subsequently a spreading infection as the sepsis progresses to cellulitis. This spreading infection can become severe causing extensive deep soft tissue damage. The deep tissue fills with pus and can cause abscess formation subsequently leading to tissue necrosis and severe bacteraemia. DF wound classifications is used to classify the stage of infection (Lavery, Armstrong and Harkless, 1996).

2.1.1 Different diabetic wound classification systems

Classification of ulcerations can facilitate a logical approach to treatment and can aid in the prediction of outcome (Frykberg *et al.,* 2002 and Armstrong and Lavery, 1998). Many wound classification systems which have been created based on the parameters such as extent of infection, neuropathy, ischaemia, depth of tissue loss, and location are discussed below.

2.1.1.1 Wagner's ulcer classification system

Wagner's ulcer classification system is based on the depth of penetration, the presence of gangrene and the extent of tissue necrosis shown below in table 2.1 (Wagner, 1987).

Table 2.1 Wagner's DFU classification

Wound grades	Description of grades
0	No lesion
1	Superficial ulcer
2	Deep ulcer
3	Abscess Osteitis
4	Gangrenous forefoot
5	Whole foot

The Wagner's grade classification is one of the most widely used classification system however the Wagner classification system does not consider two critically important parameters, ischaemia and infection (Frykberg, 2002).

2.1.1.2 University of Texas: diabetic wound classification

The University of Texas diabetic wound classification system assesses the depth of ulcer penetration, the presence of wound infection, and the presence of clinical signs of lower-extremity ischaemia (Oyibo *et al.,* 2001). The stages and grades of ulcers depth and ischaemia are discussed below

1) Stages

- stage A - No infection or ischaemia
- stage B - Infection present
- stage C - Ischaemia present
- stage D - Infection and Ischaemia present

2) Grading

- Grade 0 - epithelialized wound
- Grade 1 - superficial wound
- Grade 2 - wound penetrates to tendon or capsule
- Grade 3 - wound penetrates to bone or joint

This system uses the four grades of ulcer depth (0 to 3) and four stages (A to D), based on ischaemia or infection, or both thus covering both the significant co-morbidities of a DFU.

2.1.1.3 Curative health services (CHS) database

The CHS wound database classifies the wound on the basis of the rate at which the anatomy of the wound was affected to the progression of infection as seen in table 2.2 (Margolis *et al*., 2002).

Table 2.2: CHS wound grade scale

Wound grade	Description of grade
1	Partial thickness involving only dermis and epidermis
2	Full-thickness skin and subcutaneous tissues
3	Grade 2 plus exposed tendon, ligament and or joint tissue
4	Grade 3 plus abscess and or Osteomyelitis
5	Grade 3 plus necrotic tissue in wound
6	Grade 5 plus necrotic tissue surrounding the wound

Although the diabetic wound classification systems aid in the prediction of outcome and can help to initiate appropriate management, treatment is influenced by the nature of the aerobes, anaerobes and fungi affecting the DFU.

2.1.2 Predominant aerobes in diabetic foot infections

The microbiology of diabetic foot wound is complex. The Human body has a vast number of bacteria living as normal flora with skin harbouring many commensal. In a diabetic patient with poor immune responses even a normal skin commensal can cause significant infection. Common skin commensal such as *Staphylococcus aureus (S. aureus)* and *Staphylococcus epidermidis (S. epidermidis)* are seen as pathogens in diabetic wounds (Citron *et al*., 2007). Other nasal commensal such as *Streptococcus* species have also been cultured from the diabetic wound clinical specimens. *Streptococcus* species rarely cause infection but can, in rare cases cause severe blistering cellulitis and tissue destruction (Loan *et al*., 2005). Gram – negative aerobes such as the *Citrobacter sp., Escherichia coli (E. coli), Pseudomonas aeruginosa (P. aeruginosa), Acinetobacter* and *Serratia sp.* are examples of normal flora often cultured from a diabetic wound. There have been a number of studies showing the pathogenic nature of the Gram – negative bacteria in DFI (Bansal, 2008, Viswanthan, 2005, Citron *et al*., 2007, Abdulrazak *et al.,* 2005, Gadepalli *et al.,* 2006). Although these are normal flora of the gut and intestinal tract they

are isolated from the diabetic foot ulcer as the patient may lack good hygiene due to their age or obesity (Cowen and Steels, 2004). These organisms often act in synergy with other bacteria and can cause severe infections (Ambrosch, Lehnert and Lobmann, 2003). In the Microbiological laboratory the processing of the clinical specimens involves the identification of these clinically significant bacteria and often involves the use of biochemical tests which facilitate in their differentiation.

The tables below describe some of the distinguishing characteristics used for identification.

Table 2.3: Characteristics of Gram – positive aerobes from DFI

Genera	Gram nature	Family	Distinctive cellular morphology	Biochemical characteristics	Location Site	Common growth media	Colour of colonies or medium	Clinically significant species
Staphylo -coccus spp.	Gram-positive non motile, non sporing facultative aerobes	Staphylo - Coccaceae	spherical cocci	catalase (+ve), Coagulase (+/-ve),	Skin Commen-sal	Columbia blood agar, salt mannitol agar (7.5 10%), Nutrient agar	Golden yellow to cream buff pigmentation	*S. aureus,* *S epidermidis*
Streptococcus spp.		Strepto - cocaceae	cocci in pairs or chains	catalase, oxidase, VP and bile esculin (–ve)	Skin and throat commen-sal	Columbia blood agar	α, β and √ haemolysis	*S. pyogenes,* *S. agalactiae,* *S. anginosus,* *S. bovis*
Enterococcus spp.		Entero - coccaceae	cocci in pairs or chains	catalase and oxidase (–ve), VP and bile esculin (+ve)	Intestinal commen-sal	Columbia blood agar	√ haemolysis	*E. faecalis*

Table 2.3 presents the various characteristics of Gram – positive aerobes that are some of the clinically significant species isolated from the DFI. *Staphylococcus* species *S. aureus* and *S. epidermidis* can be further distinguished such that *S. aureus* is normally coagulase (+ve) and catalase (+ve) while *S. epidermidis* is coagulase (-ve) and catalase (+ve). Similarly bile esculin test aid in distinguishing *Streptococcus* and *Enterococcus* species with the former being bile esculin (+ve) and later being bile esculin (-ve). Other colony characteristics can also help in initial identification.

Table 2.4: Characteristics of Gram – negative aerobes from DFI

Genera	Gram nature & Family	Distinctive Cellular Morphology	Biochemical Characteristics	Colour of colonies or medium	Location Site	Clinically significant species
Escherichia spp.	Entero – bactericeae	Motile	Catalase (+ve), oxidase and Citrate (-ve)	Pink on Mac Conkey's agar	Commensal of the gut	*E. coli*
Klebsiella spp.		Non-motile, polysaccharide capsule	Catalase, KCN and VP (+ve), oxidase (-ve)	Pink on Mac Conkey's agar	Normal flora of mouth, skin and intestine	*K. pneumoniae*
Enterobacter spp.		Motile	Catalase, VP, gluconate (+ve) and oxidase (-ve)	Pink on Mac Conkey's agar	Urinary and respiratory tract	*E. cloacae*
Citrobacter spp.		Motile	Catalase citrate (+ve), oxidase and lysine (-ve)	Occasionally Pink on Mac Conkey's agar	Normal intestinal flora	
Proteus spp.		Motile	Catalase, phenylalanine, (+ve) and oxidase (-ve), urea and gelatin hydrolysed	colourless on Mac Conkey's agar, Swarming growth on Nutrient agar	Normal intestinal flora	*P. mirabilis, P. vulgaris*
Salmonella spp.		Motile	Catalase, Simmons citrate, lysine decarboxylase (+ve), most of them are H2S producers, KCN and oxidase (-ve)	Colourless on Mac Conkey's agar	Transmitted by animals	*S. typhi, S paratyphi*
Serratia spp.		Motile,	Catalase, VP, gluconate (+ve), oxidase (-ve)	Red pigmentation by some strains on Mac Conkey's agar	UTI, RTI wounds	*S. marcescens*
Pseudomonas spp.		Motile,	catalase and oxidase (+ve)	Pyocin – flourescent yellow pigmentation on Nutrient agar, lactose non - fermentor	Immuno compromised	*P. aeruginosa*
Acinetobacter spp.	Gram - negative strict aerobe cocco bacilli Mora – xellaceae	Non - motile		Colourless colonies lactose non - fermentor	Open wounds immuno compromised	*A. baumannii*

The table 2.4 shows all the characteristics which are often used in microbiological laboratories to help distinguishing the clinically significant Gram – negative aerobes using traditional biochemical identification. Gram – negative aerobes from the DFI are mainly from the Enterobacteriaceae family and are mainly grouped as lactose fermentors and non fermentors using Mac Conkey's agar. *Proteus* species most significantly shows Phenylalanine test (+ve) while *Pseudomonas* species shows oxidase and catalase (+ve). *Serratia* species often show red pigmented colonies on the Mac Conkey's agar.

2.1.3 Predominant anaerobes in diabetic foot infections

Anaerobes are often present as normal flora of the skin and mucous membranes. In states of distorted host defence or where the skin's integrity is disrupted they can colonise and invade up to the vassal channels Finegold, 1993). Some of the anaerobes are rich in enzymes and toxins which help them in crossing the natural or anatomical barriers of the body causing toxaemic or septic syndromes (Gubina, 1997). In diabetic foot patients, due to ischaemia and neuropathy with vascular inefficiency, tissue anoxia occurs which lowers the redox potential and favours the growth of anaerobic bacteria (Armstrong *et al*., 2002 and Stefanopoulos and Kolokotronis, 2004). Within a wound environment, and particularly in the presence of dead tissue, obligate anaerobes are amongst the dominant groups of micro-organisms, despite the frequent exposure of the wound to air (Bowler, Duerden and Armstrong 2001). Anaerobes are able to cope with the toxic effects of oxygen by interacting with aerobes. As the aerobic bacteria grow they consume oxygen and create a more favourable environment for anaerobic bacteria. This has been demonstrated in laboratory studies involving communities of oral bacteria (Bradshaw *et al.*, 1996; and Bradshaw *et al.*, 1998). Many bacteria have a relatively narrow pH range for growth; but when present within a wound, they are able to survive within a broader pH range (Bradshaw *et al*., 1996). Therefore microbial communities overcome the constraints of the external macro-environment by creating, through their metabolism, microenvironments that enable the survival and growth of the component species. Similarly the breakdown of various nutrients within a polymicrobial community leads to the formation of a food chain where the product of one organism will become the substrate of another. This process leads to the development of microbial homeostasis within the community with one species relying on another to provide substrates for growth and development.

Most of the infections that harbour anaerobes have a foul odour, gas in the specimen and the location of infection is in proximity to a mucosal surface. There are specific major populations of bacteria evident in the diabetic wound. Gram - positive anaerobes include the Gram - positive cocci (*Peptostreptococcus* spp.) which form part of the normal flora of humans yet are the most frequently isolated from the clinical specimens others include

the Gram - positive spore forming cocci (*Clostridium* spp.) along with other Gram - positive non spore forming cocci (*Propionibacterium* spp) (Esposito *et al.,* 2008, Citron *et al.,* 2007 and Baines *et al*, 2008). Most common Gram – negative anaerobes cultured from the DFI are *Bacteroides fragilis*, *Prevotella* species, *Fusobacterium* species and *Veillonella* species (Fille *et al.,* 2006, Ge *et al*., 2002).

Anaerobes have recently undergone extensive taxonomic changes with the advances in molecular techniques. The genus of one clinically significant anaerobe *Peptostreptococcus* was shown, by 16S rRNA sequencing to be very heterogeneous and phylogenetically incoherent (Song, *et al.,* 2007a and 2007b). The table 2.5 below shows the classification of *Peptostreptococcus* spp. from past and present.

Table 2.5 Re - classification of gram positive anaerobes

Previous approved name	Current classification
P. asaccharolyticus	*Pst. asaccharolyticus, Peptoniphilus asacharolyticus*
P. glycinophilus	*Pst. micros, Micromonas micra, Parvimonas micra*
P. indolicus	*Pst. Indolicus*
P. magnus	*Pst. magnus, Finegoldia magna*
P. niger	*P. niger*
P. prevottii	*Anaerococcus prevottii*
P. anaerobius	*Pst. Anaerobius*
P. parvulus	*Pst. Parvulus*
P. productus	*Pst. Productus*
Newly proposed strains	*Peptoniphilus gorbachii*
	Peptoniphilus olsenii
	Anaerococcus murdochii

P = Peptococococcus, Pst = Peptostreptococccus

The changes in the nomenclature were made on the basis of one of the following: differing G+C ratios, differing atmospheric requirements, nucleic acid studies and saccharolytic activities. As a routine in a Microbiological laboratory identification of the anaerobes is not carried out or restricted to the genus level. Identification of species often requires tedious and expensive use of procedures which are not economical routinely in laboratories. The tables discussed below collate all the most commonly isolated anaerobes from DFI and the different characteristics that can be used to distinguish them.

Table 2.6: Characteristics of clinically significant *Peptostreptococcus* species from DFI

Anaerobes	Gram nature	Distinctive Cellular Morphology	Biochemical Characteristics	Distinguishing characteristics
Pst. anaerobius	Gram – positive, obligate anaerobe	Pairs, chains, elongated cocci	SPS sensitive , cells > 0.6 µm, glucose and Pro A (+ve)	Isocaproic acid sweet odour on blood agar
Finegoldia magna	Gram - positive	Single, pairs, clusters	SPS resistant, cells > 0.6 µm, indole, urease, glucose (-ve), Pyr A (+ve)	Clumping cells
Anaerococcus prevottii	Gram - positive	Short chains, clumps	Urease (+ve), indole, glucose (-ve), SPS resistant	
Parvimonas micra	Gram - positive	Pairs, short chains	SPS variable, cells < 0.6 µm diameter, Pyr (+ve), urease, indole, glucose and α glucosidase (-ve) and SPS resistant	Incomplete β haemolysis and milky halo around colonies on blood agar
Peptoniphilus asaccharolyticus	Gram - positive	Pairs, chains	Indole (+ve), regular cell size and shape, α glucosidase and Alkaline phosphatase (-ve)	butyrous odour on blood agar

SPS – sodium polyanethol sulfonate, clinical strains of *P. prevottii* are urease (-ve) (Jousimies - Somer *et al*., 2002). Presumptive identification on the basis of the colony morphology can help in speciation of the *Peptostreptococcus* species as shown in this table. Traditional manual methods using the biochemical tests shown helped in speciation of the *Peptostreptococcus* species.

Clostridium species have been most commonly cultured from a DFI (Baines *et al.,* 2008). These anaerobes are more often seen in severe infection often causing tissue gangrene and necrosis. The characteristics of some of the most commonly cultured *Clostridium* species are described below in table 2.7

Table 2.7: Characteristics of clinically significant *Clostridium* species from DFI

Properties of *Clostridium* spp.	Organisms	Gram nature	Distinctive Cellular Morphology	Biochemical characteristics
Saccharolytic Proteolytic	*C. difficile*	Gram - positive rod shaped obligate anaerobes, phylum firmicutes, family Clostriaceae		Gelatin, glucose, esculin and proline (+ve), lecithinase (-ve)
	C. botulinum			Gelatin, glucose , lipase and esculin (+ve)
	C. perfringens		Double zone haemolysis	Gelatin, glucose, lecithinase (+ve)
	C . putrificum			Gelatin and glucose (+ve)
Saccharolytic Non proteolytic	*C. butyricum*			Glucose and esculin (+ve)
	C. innocuum			Glucose, esculin (+ve) and proline (-ve)
	C. paraputrificum			Glucose and esculin (+ve)
	C. tertium	Gram-positive rod shaped aerotolerant, phylum firmicutes, family Clostriaceae	Anaerobically spores present aerobically no spores	Glucose, esculin and nitrate (+ve)
Assacharolytic	*C. histolyticum*			Gelatin (+ve)
	C. tetani		Tennis racket drum stick shaped, strong β haemolysis, thin growth on moist plates	Gelatin, indole (+ve) and lipase, α – glucosidase (-ve)
	C. limosum			Gelatin and lecithinase (+ve)

The *Clostridium* species of clinical importance have been subdivided based on proteolytic activity (gelatin hydrolysis), saccharolytic (glucose fermentation) or both. Egg yolk agar was the selective medium which allowed the detection of lecithinase and lipase activity (Jousimies - Somer *et al*., 2002).

Eubacterium, Propionibacterium spp. and *Actinomyces* spp. are very occasionally isolated from DF clinical specimens (Goldstein *et al*., 2003 and Citron *et al*., 2007). They generally occur in association with other organisms and can be pathogenic. Some of the characteristics of the Gram – positive anaerobes are described below in table 2.8

Table 2.8: Characteristics of other Gram – positive anaerobes from DFI

Anaerobes	Gram nature	Family	Distinctive Cellular Morphology	Biochemical Characteristics	Location Site
Eubacterium spp.	Gram-positive obligate non sporing bacilli	Propionibacteriaceae	Non motile, variable morphology straight or curved, either single or in pairs or short chains	Catalase (-ve), indole and nitrate variable produces butyric, acetic acids	Normal Inhabitant of human skin and Cavities
Propionibacterium spp.	Gram-positive facultative non sporing bacilli	Propionibacteriaceae	Pleomorphic bacilli *P. acnes* – small irregular white colonies becoming larger with age	Catalase (+ve), indole, nitrate variable and motility (-ve) produces propionic and acetic acid. *P.acnes* nitrate and indole (+ve)	Commen-sal of the human skin
Actinomyces spp.	Gram-positive facultative non sporulating bacilli	Actinomycetaceae	Irregular branching fungus like hyphae	Catalase, indole and motility (-ve) nitrate (+ve)	Opportuni-stic Pathogen of oral cavity

Catalase and nitrate test were useful in the grouping of the non spore formers (Jousimies - Somer *et al*., 2002).

Some of the most significant Gram – negative anaerobes from DFI, their colony and biochemical characteristics are shown in table 2.9 and are often used in Microbiological laboratories for identification of Gram – negative anaerobes.

Table 2.9: Characteristics of Gram – negative anaerobes from DFI

Anaerobes	Gram nature	Family	Distinctive Cellular Morphology	Biochemical Characteristics	Location Site	Colour of colonies or medium
Bacteroides fragilis	Gram - negative bacilli	Bacteriodeaceae	2-3 mm diameter on blood agar	Sucrose, catalase, esculin, xylose (+ve), indole, trehalose, arabinose (-ve), growth on 20% bile	Normal inhabitants of oral, respiratory, Urogential cavities of human and Animals	Grey or white in colour
Bacteroides ureolyticus			Microaerophilic	Urease (+ve) and catalase and motility (-ve)		
Prevotella bivia	Gram - negative bacilli	Prevotellaceae		Lactose fermentation helps to distinguish species, proteolytic don't ferment sucrose	Host associated colonising mouth	Pigmented buff to black
Fusobacterium nucleatum	Gram - negative bacilli	Fusobacteriaceae	Thin long bacillus with pointed ends	Indole (+ve), bile sensitive, lipase (-ve)	Mucous membranes of humans serving as pathogens	Dry irregular Bread crumb-like colonies, greening of agar
Fusobacterium varium			Large rounded ends	Indole (-ve),bile resistant and lactose non-Fermentor		
Veillonella	Gram - negative cocci	Vellionellaceae	Small cells in pairs, chains or clusters	Nitrate positive, Catalase variable, lactate fermenting	Normal mouth, URT,GIT and vaginal flora	
Capnocytophaga	Gram - negative bacilli	Flavobacteriaceae	Smaller cells with tapered ends	Gliding motility, aerotolerant	Indigenous oral Flora	Yellowish Pinkish speckled Colonies

All Gram-negative bacilli are curved with pitting colonies (Jousimies - Somer *et al.,* 2002).

2.1.4 Predominant fungi in diabetic foot infections

Fungi are present as normal flora in the mucosal organs, skin, mouth and the digestive system. In certain situations, such as during illness, use of too many antibiotics, use of steroids, obesity and in immuno compromised hosts fungi are capable of multiplying. Moist skin or any damage to the skin can encourage fungal infections. These characteristics are often seen among the diabetic foot patients due to neuropathy and repeated trauma to the skin, fungi gain entry and multiply. One of the clinically significant fungi cultured from DF ulcers is *Candida* spp. *Candida* belongs to the family Saccharomycetaceae. In the laboratory *Candida* appears as large, round, white or cream dry colonies which under microscope show budding yeast cells. *Candida* is a clinically significant fungus and has been shown to cause bloodstream infections, oropharyngeal, superficial infections such as thrush and vaginal Candidiasis. *Candida* has also been shown to cause other deep seated yeast infections in immuno compromised diabetic foot patients (Bansal *et al*., 2008).

In one study carried out by Heald and his colleagues, (001) *Candida* was grown from the bone and deep fissures of an affected DF rather than superficial ulcers. Osteomyelitis was also seen due to *Candida* in one of the studies based on the clinical characteristics and radiographs. Some of the *Candida* species particularly *C. albicans* exist in more than one morphological form and have long been regarded as significant in pathogenesis, particularly in relation to the penetration of host tissues. One study has shown that morphogenesis helped in the formation and persistence of adherent cell populations. C. *albicans* strains are capable of dimorphism and form biofilms on catheter disks consisting of a basal yeast layer, which serves as an anchor to the substratum, and an outer hyphal layer, which has a more open architecture likely to facilitate the transport of nutrients (Baillie and Douglas, 1999). These dimorphic structures thus prove to an advantage to *Candida albicans* giving firm attachment and hence rendering an infection untreatable.

Tthe most significant species of *Candida* isolated from diabetic foot ulcers include *C. tropicalis, C. albicans, C. guilliermondii and C. pseudotropicalis* there are other species which in association with other organisms become opportunistic (Mlinaric *et al.,* 2005). There are many host factors in patients

suffering from diabetic foot ulcers which contribute to the pathogenicity of *Candida*. Hyperglycaemia is known to induce defects in the host granulocyte function, thereby leading to enhanced growth and tissue invasiveness, thus diabetic patients are at substantially higher risk of systemic *Candida* infections (Heald *et al*., 2001).

2.1.5 Mixed infections in diabetic foot infections

Diabetic foot infections are often complex and polymicrobial in nature (Bowler and Davies, 1999; Lipsky, Pecoraro and Wheat, 1990; Ge *et al.,* 2002; Vishwanathan *et al.,* 2002 and Hunt, 1992). One of the largest surveys carried out, on a total of 825 patients, investigated the microbiological profiles of patients with mild and moderately infected diabetic foot ulcers. They revealed an average of 2.4 organisms recovered per wound. Of the infected diabetic foot ulcers, 75% had multiple micro-organisms (Ge *et al.,* 2002). One of the most intriguing observations made in all microbiological studies of diabetic foot ulcers is that the obligate anaerobes were never found alone, they were always isolated with aerobes signifying a relationship with each other and the polymicrobial nature of the infection (Finegold and Wexler, 1988 and Hartemann *et al.,* 2004).

2.2 Antibiotic treatment of diabetic foot infections

The Infectious Diseases Society of America termed the 6 most feared pathogens as “ESKAPE” these included the *Enterococcus faecalis, Staphylococcus aureus, Clostridium difficile, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* species, often referred to in the press as the ‘Superbugs’ due to their resistance to almost all antibiotics (Rice, 2008). Choosing an antibiotic would depend on clinical diagnosis, Gram nature of the organisms , the aetiology of the organism and patient allergies to antibiotics, renal and hepatic insufficiency, local antibiotic susceptibility data, gastro intestinal absorption impairment and most importantly the severity of the infection.

Since DF ulcers often start with localised infection topical agents such as antiseptic lotions with antimicrobial properties can be initially used to irrigate or cleanse the wounds. These can only be helpful for a brief contact time with the wound surface. Antiseptic lotions in the form of hypochlorite or potassium permanganate are used for localised infections. Some preparations can stay in contact with the wound surface for a longer period of time in the form of dressings these mainly include creams, ointments or impregnations. Silver based products have recently shown to have broad – spectrum effect against both the Gram – positive and Gram – negative organisms along with the yeasts and fungi (O’Meara *et al.,* 2000). Oral antibiotics have most commonly been used as treatment on an outpatient basis (Lipsky *et al.,* 2004). Clinicians normally use systemic antibiotics as the last resort when topical interventions fail to initiate healing of the DF ulcer. Systemic antibiotics mainly including the penicillins, cephalosporins, aminoglycosides and quinolones can be used against a wide variety of Gram – positive and Gram – negative organisms (Vuorisalo, Venermo and Lepantalo, 2009). The penicillins interfere with the development of bacterial cell walls and cross linkages. Some of the broad - spectrum antibiotics such as ampicillin and amoxicillin are active against both the Gram – positive and Gram – negative organisms. These are often inactivated by penicillinases and cephalosporinases produced by organisms such as *S. aureus* and *E. coli* (Bush, 1989). To overcome resistance, combination antibiotics such as amoxicillin/clavulanic acid and piperacillin/tazobactam can be used as β –

lactamase inhibitors. Aminoglycosides and tetracylines inhibit protein synthesis and bind to the conserved sequences within the 16s rRNA of the 30s rRNA subunit while macrolides and chloromphenicol act on the 23s rRNA on the 50s rRNA subunit (Livermore, 2003). Bacterial growth depends on the DNA and RNA synthesis. The aminoglycosides are active against many Gram – negative organisms. There are some antibiotics such as fluoroquinolones which can disrupt the nucleic acid synthesis directly, whereas sulfonamides and trimethoprim act indirectly on folic acid metabolism (Mascaretti, 2003). The quinolones are active against both the Gram – positive and Gram – negative organisms. When the number of the possible pathogens causing infections is small the antibiotic susceptibility is predictable and empirical treatment could be justified. It covers the most common pathogens with some modifications according to the severity of the infection is being considered (Frykberg, 2002). For spreading and severe infections the patients admitted to the hospital can be given antibiotics with wide spectrum intravenously. Recent clinical trials have shown that patients treated with ertapenem gave clinical outcomes equivalent to those for patients treated with piperacillin/tazobactam in a randomised trial in adults (n = 586) with diabetes and a foot infection classified as moderate to severe and requiring intravenous antibiotics (Lipsky *et al.,* 2005).

The primary goal in the treatment of diabetic foot ulcers is to obtain wound closure. Along with antibiotic treatment the management of the foot ulcer is largely determined by the severity of grades and vascularity, along with the infections present. A multidisciplinary approach needs to be used because of the multi factorial nature of foot ulcers and the numerous co morbidities that can occur in the DF patients. Treatment of peripheral neuropathy and ischaemia is critical as these impair the delivery of oxygen, leucocytes and other host defence factors along with the antibiotics (Singh, Armstrong and Lipsky, 2005 and Steed *et al*., 2008). This can lead to deformities due to abnormal pressure and therefore it becomes imperative to tackle vascular supply. Many vaso-dilator drugs have been proved to be beneficial in promoting the healing of lesions (Caputo *et al*., 1994b). Since surgical methods would not restore the normal host factors, adjunctive therapy using hyperbaric oxygen therapy (Cimsit, Uzun and Yildiz, 2009) and the use of maggots (fly larva) for bio surgical debridement of calluses (Frykberg, 2002)

can prove advantageous in the healing of diabetic ulcers (Steed *et al*., 1996). These calluses further increase the pressure and cause a non healing ulceration. Debridement promotes re-epithelialisation from the edge of the ulcer with a cast applied on the wound enabling the patient to walk freely but still keeping the pressure off the lesion (Frykberg, 2002). A continuous monitoring for the degree of neuropathy using a widely accepted instrument known as nylon Semmes-Weinstein monofilament is essential for closed progression of the foot (Steed *et al*., 2008).

2.2.1 Trends in antibiotic resistance among the aerobic bacteria

Organisms belonging to the *Enterobacteriaceae* family and the species from the *Staphylococcus* genus are the 2 most common organisms isolated from the diabetic foot ulcers. Antimicrobial resistance first emerged due to the production of β-lactamases; these are plasmid or chromosomally encoded bacterial enzymes that catalyse the hydrolysis of the β-lactam C-N bond of the β-lactam antibiotic to give the corresponding β-amino acid devoid of antibacterial activity (Nikaido, 1998). Resistance to β-lactam antibiotics was known even before penicillin was clinically used. In 1940 Abraham and Chain first described a bacterial enzyme in E*. coli* that destroyed penicillin and termed it as penicillinase as the structure of β-lactams were unknown (Mascaretti, 2003). There are 5 classes of the β-lactamases from the Gram - negative bacteria mainly named after their substrate profiles. β-lactamases are often the penicillinases, cephalosporinases and carbapenemases (Bush, 1989). Many species of the Enterobacteriaceae family are ESBL (extended spectrum β-lactamase) producers and are a global problem. One study has highlighted the number of ESBL producing organisms from DFI. Of the total 134 isolates of *E. coli* and *K. pneumoniae* 31 isolates showed ESBL production: 15 E*. coli* and 16 *K. pneumoniae* (Varaiya *et al*., 2008). These are a group of enzymes that have the common property of providing resistance to extended-spectrum β-lactamases antibiotics such as cefotaxime, ceftazidime, ceftriaxone, cefpime and cefpirome (Rossi *et al*., 2006). *AmpC* genes acquired by the *Pseudomonas aeruginosa* are responsible for imparting ESBL resistance**.** *Pseudomonas aeruginosa* exhibits high intrinsic resistance to penem antibiotics such as faropenem, ritipenem, sulopenem, and sulphonamides due to the permeability barrier to the compound (Okamoto, Gotoh and Nishino, 2001). *K. pneumoniae*

carbapenemase (KPC) and metallo- β-lactamases (MBL) are some of the other ESBL seen among the *Enterobacteriaceae* family imparting high antibacterial resistance (Jacoby, 2009).

Penicillin resistance emerged in *Staphylococcus aureus* in the early 1940s, shortly after its introduction (Thornsberry, 1988). Resistant *Staphylococci* have been a clinical problem since 1944 when sulphonamide-resistant strains appeared among the wounded during the 2nd world war (Massad *et al.,* 1993). By the 1948 it was reported that in over half of the hospital *Staphylococcus* strains were penicillin resistant. Streptomycin and erythromycin, tetracycline and chloromphenicol resistance had also developed by the 1950s. Within 10 years of the introduction of methicillin in the 1960 resistance to methicillin was discovered. *Staphylococcus* species have been shown to possess the penicillin binding protein PBP2a which is encoded by the *mec A* gene. The PB2a protein has a low affinity binding for the beta lactam antibiotics (Grubb, 1998). This *mecA* gene thus has rendered many of the *Staphylococcus* species resistant to methicillin. By the 1980s epidemic strains of MRSA emerged (Enright, 2002). In 2002, there were various studies reporting some strains of *Staphylococcus* showing resistance to Vancomycin. The *Vanco* gene *Van A* was co- transferred from the resistant organism *Enterococcus faecalis* NCTC 12201 to *Staphylococcus* (Chang *et al*., 2004). Most predominantly favoured organisms in DFI are the methicillin resistant *Staphylococcus aureus* (MRSA) followed by methicillin resistant *Staphylococcus epidermidis* (MRSE) (Citron *et al.,* 2007).

2.2.2 Trends in antibiotic resistance among the anaerobic bacteria

Susceptibility testing of anaerobes originated after the methods had been used for the aerobic bacteria. Early in the 1950s antimicrobial testing included the tube dilution, plate dilution and disk method, all revealing the minimum inhibitory concentration (MIC) but without any standardisation for the inoculum, period of incubation, choice of complete or partial inhibition as an endpoint. The testing varied in different locations; different media and even different methods were used (Hecht, 2002). In 1985, a standardised method was published for carrying out antimicrobial susceptibility testing. However, until today it is undergoing continuous refinement. A survey carried out by Smith and his colleagues (2010) as a part of the UK Clinical

Pathology Accreditation (CPA) scheme based in the NHS suggested that the methodology and reproducibility of the susceptibility testing of anaerobes in routine diagnostic laboratories had been diverse in different laboratories. Literature on the antimicrobial susceptibility of anaerobes is very limited as compared to the aerobes, and most often the antimicrobial agents are tested against very few isolates as anaerobes are difficult to isolate and identify; thus the frequency of testing at the hospitals is extremely low. This is evidenced by one study carried out in New Zealand by Roberts and her colleagues (2006), where she reported that over 15 years only 3 surveys had been carried out looking at the susceptibility patterns of the clinical isolates (Roberts *et al*., 2006). In Belgium a 3rd multi-centre antimicrobial susceptibility survey was carried out on 443 anaerobes collected over a period of 2 years and their antimicrobial susceptibility patterns remained stable except for reduced susceptibility to clindamycin (Wybo *et al*., 2007).

Since the early 1970s antibiotic resistance among the anaerobes has become evident. *Bacteroides fragilis* was one of the most frequently isolated resistant anaerobes. *Bacteroides fragilis* groups have shown complete resistance to penicillin G and clindamycin due to the presence of the *ermF, ermG* and *ermS* determinants (Aldridge *et al*., 2001). Many of the *Clostridium* species have also shown resistance to clindamycin due to *ermQ, ermP* determinants. All these determinants were shown to be encoded by genes on the chromosome, plasmid or transposons and are transferable by conjugation (Brazier *et al*., 2008). Piperacillin had been effective in the treatment of infections against anaerobes until the 1990s. No more than 25% of the Gram - negative anaerobes showed resistance to piperacillin alone (Behra-Miellet *et al.,* 2003a). Most of the anaerobes have been shown to produce zinc metallo- β-lactamases encoded by the *ccrA, cfiA* genes that hydrolysed the carbapenems imipenem, meropenem and ertapenem (Yang *et al.,* 1992). β-lactamases are inhibited by the classical β-lactamase inhibitors such as clavulanic acid, sulbactam and tazobactam and hence these are used in combination with other antibiotics (Maiti *et al.*, 1998). Metronidazole resistance has been more common among the Gram - positive anaerobes and is caused due to the presence of the *nim* genes (Mory *et al*., 1998). Resistance to quinolones, mainly the fluoroquinolones,

is seen due to the presence of the *gyrA* and *gyrB* genes (Brazier *et al*., 2008). These antibiotics are generally given in combination with aminoglycosides or cephalosporins. Very few anaerobic strains have shown resistance to carbapenems such as imipenems. Most of the anaerobes are susceptible to piperacillin/tazobactam, ampicillin/sulbactam, meropenem and vancomycin (Brazier *et al*., 2008; Behra-Miellet *et al.,* 2003b and Citron *et al*., 2007). One of the studies highlights the use of tetracycline showing there is less resistance seen among the anaerobes in the UK because tetracycline had not been routinely prescribed in the past (Hecht, 2004). Similarly fusidic acid is not frequently used in Asia hence very few resistant strains are seen. Although Gram – negative organisms except *Bacteroides* species are intrinsically resistant to fusidic acid but can be effective against the Gram – positive organisms mainly the *Staphylococcus* and *Streptococcus* species (Chinedum, 2005). Both these studies highlight the need to limit the use of antibiotics and use other mechanisms to combat infections (Brazier *et al*., 2008 and Behra-Miellet, *et al.,* 2003b). There have been a number of new antimicrobial agents used for the treatment of infections due to *Clostridium difficile* these include the lipoglycopeptides, macrocyclic esters, nitrothiazoles and diarytdiamines (Koeth *et al.,* 2004; Brook, 2007 and Caputo, 1994a).There have been very few new antimicrobials developed for the treatment of Gram - negative anaerobes which could be a problem in the future.

2.2.3 Biofilms in DFI and their resistance to antibiotics

Biofilms are found to be present in two-thirds of chronic wounds and they are often been resistant to antibiotics (Dowd *et al*., 2008). In many animal models combinations of anaerobic and aerobic bacteria have been shown to produce levels of sepsis or disease that could not be induced by individual species (Bradshaw *et al.*, 1996). Such synergy has been demonstrated between wound pathogens such as: *Prevotella melaninogenicus*, *Porphyromonas asaccharolytica* and *Parvimonas micra (*Devine *et al.*, 1999 and Sundqvist *et al.*, 1979) *Porphyromonas asaccharolytica* and *Klebsiella pneumoniae*; *Escherichia coli* and *Bacteriodes fragilis* and *Staphylococcus aureus* (Mayrand *et al.*, 1980).

There are number of familiar mechanisms of antibiotic resistance, such as efflux pumps (Nikaido, 1996), modifying enzymes (Livermore, 2003) and target mutations (Hall and Partridge, 2001). However, in biofilms even the most sensitive bacteria with no genetic basis for resistance can have greatly reduced susceptibility to antibiotics. Biofilms are defined as communities of microbial cells growing on a surface and embedded in a self-synthesized matrix composed of extracellular polymeric substances (Laue *et al.*, 2006). The major components of these are the exopolysaccharides with DNA, protein and lipids making up a significant proportion of the matrix. These exopolysaccharides have been reported to play a very important role in the attachment of bacterial cells to the surface and/or in building and maintaining the three–dimensional, complex structure of the biofilms. Exopolysaccharides have been utilised, for example, for the initial attachment to a surface of the *Vibrio cholerae* and for the development of *Pseudomonas*, *Staphylococcus epidermidis* and many other pathogenic biofilms (Laue *et al.*, 2006). These biofilms protect organisms from phagocytosis and facilitate antibiotic resistance, acting as a barrier, decreasing the diffusion of antibiotics, antimicrobial proteins, lysozymes and small molecular antimicrobials such as defensins. Biofilms concentrate bacterial enzymes that degrade antibiotics for example β-lactamases (Wilson and Devine, 2003). Antimicrobial agents kill rapidly growing bacterial cells more effectively than those of the slower growing ones**.** Some antibiotics, in order to be active, require certain rates of bacterial growth, for example penicillin and ampicillin fail to kill non-growing bacterial cells. Other new antibiotics such as aminoglycosides and fluoroquinolones can kill non-growing bacterial cells, but they are more effective at killing fast growing cells (Wilson and Devine, 2003). In biofilms, the bacterial cells experience limited diffusion of nutrients and oxygen and also accumulation of metabolic waste. These conditions favour anaerobic metabolism producing acidic compounds that act on the deeper regions of the biofilms further reducing the activity of certain antibiotics such as aminoglycosides and quinolones (Stewart and Costerton, 2001).

2. Materials and Method

2.1 Research design for Indian diabetic foot Cohort study

Ethics approval for this study was obtained for the patients from the UK and India (appendix I). Patient's consent was obtained (appendix II) and a questionnaire (appendix III) was designed. In collaboration with the 'All India Institute of Diabetes' at Raheja hospital in Mumbai a pilot study was carried out over a period of 3 months on 89 patients with diabetic foot ulcers admitted at Raheja hospital. This was funded by the Society for General Microbiology – President Fund (appendix IV). Diabetic patients were interviewed by their doctors about diabetes mellitus type, duration along with their treatment profile, level of glucose control and presence or absence of chronic DM complications including previous ulcer/amputation of the lower limbs (appendix III). Both feet were also examined for signs of peripheral vascular disease and neuropathic symptoms of tingling, numbness, and burning sensation including skin status (colour, thickness, dryness, cracking), nail status (colour, thickness) and the presence of deformities (such as claws and hammer toes), oedema, infection, ulceration, callus and blistering. Gangrene and amputation were also noted and if a wound existed it was classified on the Wagner's based classification system (appendix V).

Additionally, the research nurse took measurement of patients' blood pressure, using a standard mercury sphygmomanometer. The WHO definition of hypertension was used in this study: systolic blood pressure 160 mmHg or more and/or a diastolic blood pressure 95 mmHg or more and any on going treatment with anti-hypertensive drugs was recorded. Height, weight measurements, electrocardiogram, insulin usage and duration of insulin use were recorded for all patients. Other clinical parameters such as fasting blood sugar, lipid profile, serum electrolytes, haemoglobin, cell count and packed cell volume (PCV) were measured in the Biochemistry department of the hospital. Any other complications or diseases were also recorded. Clinical specimens such as tissue biopsies and pus samples were obtained and were processed for microbiological study. Antibiotic susceptibility testing was carried out on the clinical isolates obtained from tissues and pus specimens.

2.1.1 Identification of clinical isolates

Following superficial pre-cleansing of wounds with iodine, tissue biopsies in case of a necrotic wound or amputated toe(s) were placed in sterile saline and send immediately to the Microbiology laboratory located on the same site as the collection ward by the duty doctor. Pus samples were aspirated after pre – cleansing of the wounds with iodine or in some cases swabs were taken by targeting the moist and necrotic areas of the ulcer which are likely to contain aerobic and anaerobic microbial populations.

2.1.1.1 Clinical processing of tissue specimens

A total of 73 tissue biopsies were cultured to record their microflora. On obtaining the tissue biopsies or an amputed toe, the necrotic or gangrenous part was teased and sectioned using forceps and scalpel and then placed on the slide and gram stained. Small gangrenous tissue sections were placed on one corner of the series of selective and non - selective media agar plates shown in figure 2.1, and incubated under aerobic and anaerobic conditions. To obtain anaerobic conditions an anaerobic gas jar with anaerobic gas pack – Anaerogen sachet (Oxoid, Basingstoke, UK) and an anaerobic indicator (Oxoid, Basingstoke, UK) were used (Ng *et al.,* 2008). A sterile loop was used to spread the bacteria from the tissue by touching the loop on the tissue and using the streak plate technique to obtain an even distribution of colonies. All the microbiological processing was carried out immediately on receipt of the tissue specimens to sustain the viability of fastidious anaerobic as well as aerobic organisms.

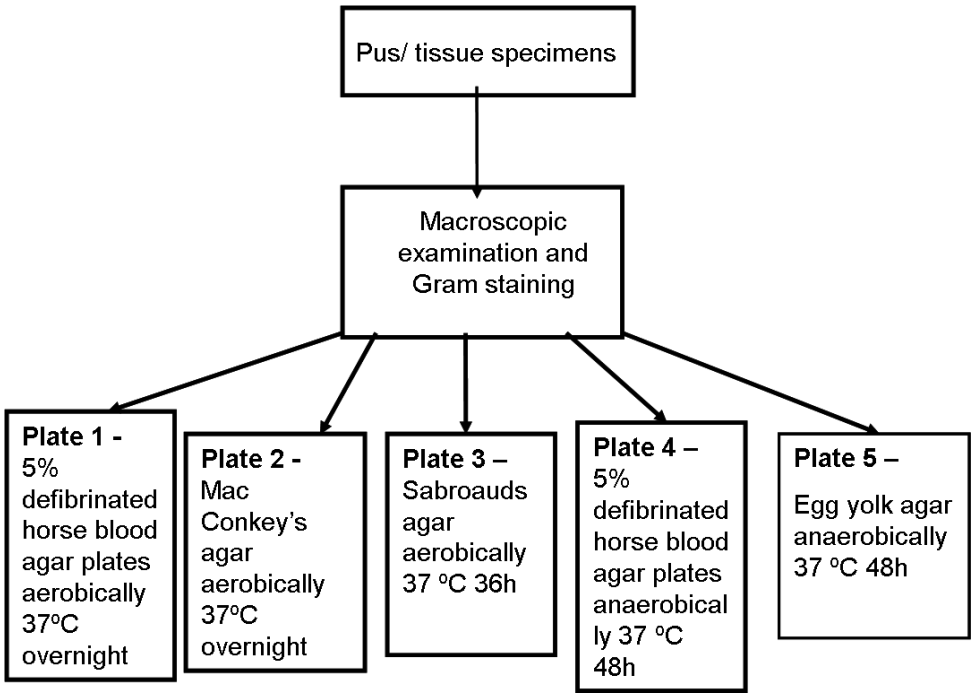


Figure 2.1 Selection of media agar plates for the microbiological processing of tissue and pus specimens

Plates 1 to 5 was used for the processing of clinical specimens obtained from the Indian and the UK diabetic foot patients and incubated at different temperatures, time and incubation conditions.

2.1.1.2 Clinical processing of pus specimens

For aspirated pus, a total of 27 pus samples were cultured to record their microbial aetiology. A sterile loop was used and a loop full of the pus was streaked on the selection of different media plates shown in figure 2.1 incubated under aerobic and anaerobic conditions. To obtain anaerobic conditions an anaerobic gas jar with anaerobic gas pack – Anaerogen sachet (Oxoid, Basingstoke, UK) and an anaerobic indicator (Oxoid, Basingstoke, UK) were used. All the microbiological processing was carried out immediately on receipt of the pus specimens to sustain the viability of fastidious anaerobic as well as aerobic organisms.

2.1.1.3 Identification of organisms to genus or species

After the respective incubation periods, shown in figure 2.1**,** the aerobes, anaerobes and fungi present were distinguished from each other on the basis of their morphological characteristics such as size, colour of colonies, texture, consistency and most importantly Gram staining . In the case of all the colony types obtained from the media plates that were incubated anaerobically an aero-tolerance test was performed such that examples of each colony type were sub cultured on aerobic and anaerobic blood agar

plates and left for overnight incubation. To rule out the presence of Gram – negative aerobes on the anaerobic plates the catalase, nitrate and spot indole tests were carried out on the different colony types. Traditional biochemical testing was carried out for aerobes. Techniques usually followed at the Microbiology laboratory at Raheja Hospital and the schemes of identification shown in the figure 2.2 were followed to identify the aerobes to genus level. Due to lack of expertise and lack of funding at Raheja Hospital's Microbiology department, anaerobic culturing was not a regular practice. For this pilot study a Rapid id 32A system (Biomerieux, France) was used for the identification of anaerobes, Hi Staph kit (Hi - media, Mumbai, India), Hi Strep kit (Hi – media, Mumbai, India) and Hi Candid (Hi – media, Mumbai, India) were used for identification of *Staphylococcus*, *Streptococcus* and *Candida* to species level. Although, due to import, logistical and financial constraints, the materials were sourced from different suppliers than those used in the UK study, they had undergone QA and QC and were good substitutes, though ideally the same kits should have been used in both studies.

Additionally Oxacillin discs (6 µg/ml) were used for the identification of methicillin resistant and methicillin sensitive *Staphylococcus* spp on Muller Hinton agar plates with a concentration of 4% of NaCl.

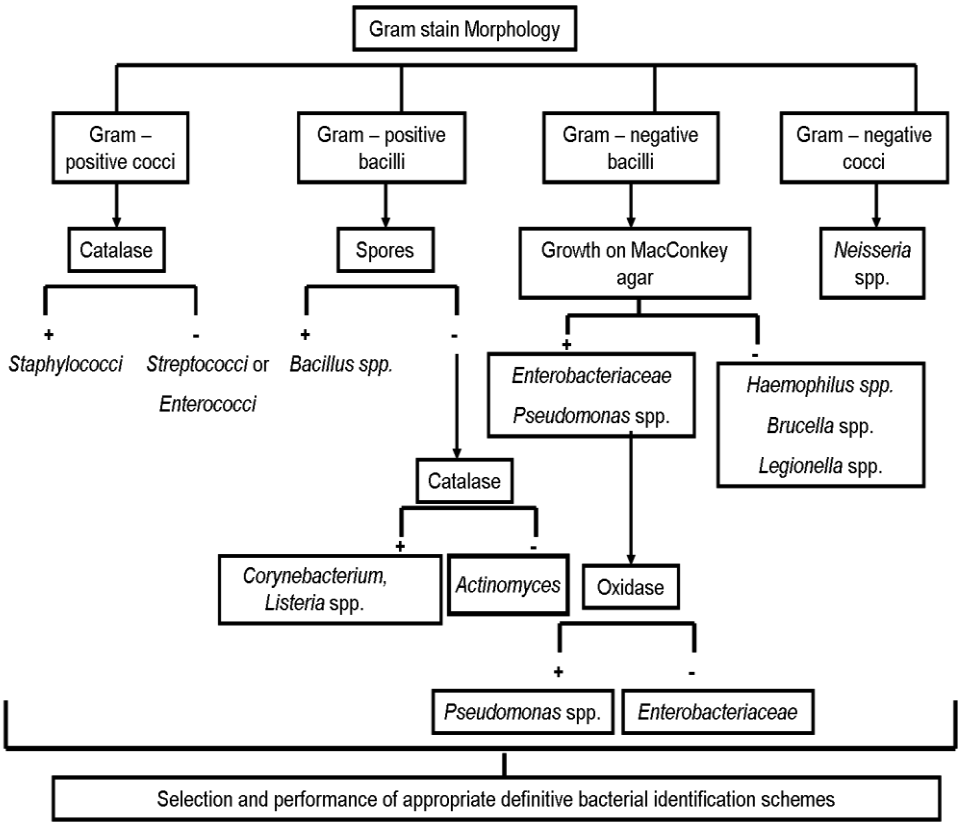


Figure 2.2 Scheme for the identification of aerobes to genus level using traditional biochemical tests (adapted using Bailey and Scotts, 2002).

The workflow shown in figure 2.2 was used in the identification of aerobes from the Indian DF patient's tissue and pus samples. The biochemical tests included for lactose fermentors included Indole, citrate, methyl red (MR), Voges-Proskauer (VP) and Triple sugar iron test (TSI) such that *E. coli* showed Indole (+ve), citrate (-ve), MR (+ve) and VP (-ve). *Klebsiella* spp. showed Indole (-ve), MR (-ve), VP (+ve) and citrate (+ve). *Enterobacteria* spp. showed Indole (-ve), MR (-ve), VP (+ve), citrate (+ve), TSI slant blackening. The biochemical tests for the identification of lactose non fermentors included Phenyl pyruvic acid test (PPA), urease, oxidase, nitrate, gelatin liquefaction such that *Proteus* spp. showed PPA (+ve), urease (+ve) and oxidase (-ve) while *Pseudomonas* spp. showed oxidase (+ve), urease (-ve), nitrate (+ve) and gelatin liquefaction. The biochemical tests were predicted on the organism's ability for substrate utilisation or their metabolic activities.

Streptococcus and *Enterococcus* spp. were distinguished using bile esculin test being (+ve) for *Enterococcus* spp. All the bio chemicals were prepared in the hospital and the materials were purchased from HiMedia, Mumbai – India.

2.1.2 Antimicrobial susceptibility testing

2.1.2.1 Antibiotics used in the Indian study

At Raheja hospital's Microbiology department the antibiotic susceptibility testing on aerobes was routinely carried out for the following antibiotics: Amikacin (Ak-30 µg), amoxicillin/clavulanic acid (Ac 20 by 10), ampicillin (A-10 µg), ampicillin/sulbactam (As-10 by 10), azithromycin (Az-15 µg), carbenicillin (Cb-100 µg), cefazolin (Cz 30 µg), cefepime (Cpm 30 µg), cefoperazome (Cs-75 µg), ceftazidime (Ca-30 µg), ceftizoxime (Ck-30 µg), cefuroxime (Cu-30 µg), chloromphenicol (C-30 µg), co-trimaxazole (Co-25 µg), gentamycin (G-10 µg), gatifloxacin (Gf-05 µg), imipenem (I-10 µg), lomefloxacin (Lo-10 µg), levofloxacin (Le-05 µg), meropenem (Mr-10 µg), nitrofurantoin (Nf-300 µg), penicillin (P-10 µg), piperacillin/tazobactam (Pt-100 by 10) and ticarcillin/clavulanic acid (Tc-75 by 10). All of the antibiotic discs used were obtained from Hi-Media (Mumbai, India). For the anaerobes isolated from the pus and tissue specimens however, MIC (minimal inhibitory concentrations) was obtained. This was an important aspect of this study as anaerobic culturing was not carried out on regular basis and this was the first time in many years that antibiotic sensitivity of anaerobes had been assessed. The antibiotics tested against the anaerobes included amikacin, ciprofloxacin, clindamycin, linezolid, levofloxacin, piperacillin/tazobactam and metronidazole. The MIC ranges for all the antibiotics were 0.01 – 256 µg/ml and all of these were obtained as E-comb (Hi – media, Mumbai, India). Briefly the E – comb is similar to the E- test (used in the UK) such that the different concentrations of antibiotics are impregnated on Hicomb filter paper discs of strips. The comb is directly placed on the respective media agar plate using a sterile forceps. On incubation, the resulting zone of inhibition appears as an eclipse that intersects the concentration marking. The results are expressed in terms of MIC of that particular antibiotic (Hi – media Manual). The E-comb test is validated as by the NCCLS (National community for clinical laboratory standards) standards and is very commonly used in Indian hospitals as a quick and convenient method for determining MIC.

2.1.2.2 Method used for antimicrobial susceptibility testing of aerobes and anaerobes

The Kirby Bauer's disc diffusion method was used routinely for the antibiotic susceptibility testing of aerobes at the Microbiological laboratory at Raheja hospital. NCCLS guidelines were followed for the selection of media, inoculum turbidity, and preparations of media plates along with the application of discs and the interpretations of zone of inhibition. For determining the MIC of anaerobes using the E-comb test, the agar diffusion method was used.

2.1.2.3 Selection of media and direct colony suspension for inoculum preparation

As recommended by the NCCLS, Muller Hinton agar plates with a concentration of 4% of NaCl were used for the susceptibility testing of aerobes and Brucella agar supplemented with 5% defibrinated horse blood, 5 mg/L haemin and 1 mg/L vitamin K_1 was used for the antibiotic susceptibility testing of anaerobes. The plates were prepared in- house in 150 mm diameter plates containing 60ml of agar media in case of anaerobic testing and 30 ml of agar media for aerobic testing. The different colony types were suspended in sterile saline in the case of aerobes and Brucella broth for anaerobes and prepared to a turbidity of 0.5 McFarland turbidity standards (using 0.5 ml of 0.048 mol/L of barium chloride to 99.5 ml of 0.18 mol/L of 0.36 N sulphuric acid with constant stirring to maintain a suspension). In total 4 antibiotic discs per media plate and 2 E-comb per media plate were placed using sterile forceps.

2.1.2.4 Control organisms used in the study

All the antibiotics were also tested on the control organisms such as *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 as suggested by the NCCLS during the susceptibility testing against aerobes. All the antibiotics were also tested on the control organisms such as *Bacteroides fragilis* ATCC 25285, *Bacteroides thetaiotaomicron* ATCC 29741 and *Eubacterium lentum* ATCC 43055 recommended by the NCCLS.

2.1.2.5 Interpretation of antibiotic susceptibility testing

The zone of inhibition around the tested antibiotics was measured in case of aerobes and interpretations were made using the breakpoints detailed in the

HiMedia manual validated according the NCCLS standards shown in Section 2.3.4 table 2.15. The breakpoints recommended by NCCLS in the Manual for the Methods for Antimicrobial Susceptibility Testing of Anaerobic Bacteria (M11-A7) were used for the MIC determination of anaerobes shown in Section 2.3.4.3 Table 2.17. An organism would be considered sensitive for a concentration of antibiotic and that implies that an infection due to the isolate may be appropriately treated with the dosage of an antimicrobial agent recommended for that type of infections unless other wise indicated. Intermediate levels of antibiotic susceptibility imply that the infection may be appropriately treated in body sites where the drugs are physiologically concentrated or when a high dosage of drug can be used. Resistant isolates are not inhibited by the usually achievable concentrations of the agent with normal dosage schedules and/or fail in the range where specific microbial resistance mechanisms are likely and clinical efficacy has not been reliable in treatment studies (NCCLS, M11 – A7).

2.2 Research design for the UK diabetic foot Cohort study

Unlike the pilot study carried out in India where the risk factors were noted the study carried out on the UK diabetic foot patients concentrated on determining the microbial aetiology of the diabetic foot ulcers and performing antibiotic susceptibility testing. This study was also carried out after obtaining ethics approval from the UK. To maintain patient's confidentiality there was no access to the patient's profile. Unlike the Indian study where tissue and pus samples were both used for microbiological studies , only 25 pus swabs were collected by the UCLH (University College London Hospitals) Clinical Microbiology laboratory and processed.

2.2.1 Identification of clinical isolates

2.2.1.1 Clinical processing of pus samples

For the clinical processing of the pus samples obtained from the UK, similar method were used as discussed in section 2.1.1.2.

2.2.1.3 Identification of organisms up to their genus or species

For the identification of aerobes, kits such as API 20E (bioMerieux, France) were used to identify the organisms belonging to the Enterobacteriaceae family. Pro -Staph (Pro – lab, Ontario,Canada) was used to identify the *Staphylococcus aureus* and *Staphylococcus epidermidis* . Pro – Strep (Pro

– lab, Ontario,Canada) was used for grouping of the Streptococcus species. For the identification of anaerobes up to species level Rapid id 32A system (bioMerieux, France) was used.

2.2.2 Antimicrobial susceptibility testing

2.2.1 Antibiotics used in the UK study

In the UK, since all the antibiotics were readily available, the MIC determination was carried out on the antibiotics generally prescribed for aerobic and anaerobic infections. E-test (bioMerieux, France) was used for determining the MIC of clinical isolates obtained in the UK study. The antibiotics used in this study were amoxicillin/clavulanic acid, clindamycin, daptomycin, erythromycin, imipenem, levofloxacin, linezolid, piperacillin/tazobactam and metronidazole both for aerobes and anaerobes. These MIC ranges for all the antibiotics were 0.01 – 256 µg/ml.

2.2.2 Method used for antibiotic susceptibility testing of aerobes and anaerobes

Similar to the Indian study discussed in section 2.1.2.2 – 2.1.2.6 the method, selection of media, turbidity, agar and plates, applications of E-test and control organisms used for the E-comb test were used for the E-test for the clinical isolates from the UK.

Although originally the aim had been to keep the usage of materials consistent, due to logistical reasons (importing and exporting rules) this was not facilitated, however similar antibiotic susceptibility testing was carried out on different isolates obtained from India and the UK.

2.3 Results and Discussion

All of the 89 patients included in the Indian cohort study had been diagnosed with type 2 diabetes mellitus of which 24 (26.96%) were females and 65 (73%) were males. The average age of females was 61 years and males were 57 years; both the sexes had an average 13 years of diabetes history and an average 10 days of hospitalisation. Of the total 89 patients included in this study 60 patients had undergone amputations of toe/toes although none had a leg amputed. After evaluating the questionnaire it was evident that the likely risk factors that could have led to amputations included peripheral vascular diseases, hypertension, ischaemic heart diseases, ischaemia, trauma and most significantly infection. Patients suffering with foot ulcers had other type 2 diabetes related complications affecting their kidneys, heart and liver. Due to the small numbers of each risk factor identified among Indian patients statistical analysis was not undertaken. Gangrene and infections dominated in all the 89 patients and hence a microbiological study identifying the organisms was imperative for empirical treatment.

2.3.1 Comparative analysis of clinical isolates from pus specimens from the Indian and the UK diabetic foot patients

The microbiological study was carried out on 27 pus samples along with 73 tissue biopsies from the Indian diabetic foot patients and 25 pus samples from the diabetic foot patients from the UK. This section (2.3.1) details the clinical isolates from pus specimens from India and the UK diabetic foot patients.

The diversity of both aerobic and anaerobic isolates was extensive for both the cohorts. From the 27 pus specimens from Indian diabetic foot patient's 30 aerobes and 20 anaerobes were cultured while from 25 pus specimens from the UK 35 were aerobes and only 5 anaerobes. Not surprisingly neither of the cohorts' pus samples showed infections due to fungi.

2.3.1.1 Distribution of aerobes from the Indian and UK diabetic foot pus samples

There were 12 and 13 Gram – positive aerobes and 18 and 7 Gram – negative aerobes isolated from the 27 and 25 pus samples from Indian and the UK diabetic foot patients respectively. The Gram – positive aerobes from the Indian diabetic foot pus samples mainly included the methicillin resistant *Staphylococcus* species, methicillin sensitive *Staphylococcus* species and *Streptococcus* species. With the use of Hi Staph kit the methicillin resistant species were identified and these included *S. aureus, S. arlettae, S. intermedius, S. kloosii, S. capitis, S. schleiferi* and *S. epidermidis*. *Streptococcus* species were mainly *Streptoccocus pyogenes*. The Gram – negative aerobes from the Indian diabetic foot pus samples mainly belonged to the Enterobacteriaceae family as shown in figure 2.3.

The Gram – positive aerobes from the diabetic foot patients from the UK mainly included the methicillin resistant *Staphylococcus* species, methicillin sensitive *Staphylococcus* species and the *Streptococcus* species. Pro – Strep kit identified the *Streptococcus* species to be from Group B, F, D and G and Pro – Staph kit identified the *Staphylococcus* species to be methicillin resistant *Staphylococcus aureus* (n = 6), methicillin sensitive *Staphylococcus aureus* (n = 2), methicillin resistant *Staphylococcus epidermidis* (n = 2) and methicillin sensitive *Staphylococcus epidermidis* (n = 1). The Gram – negative aerobes from the diabetic foot pus samples from the UK mainly belonged to the Enterobacteriaceae family as shown in figure 2.3.

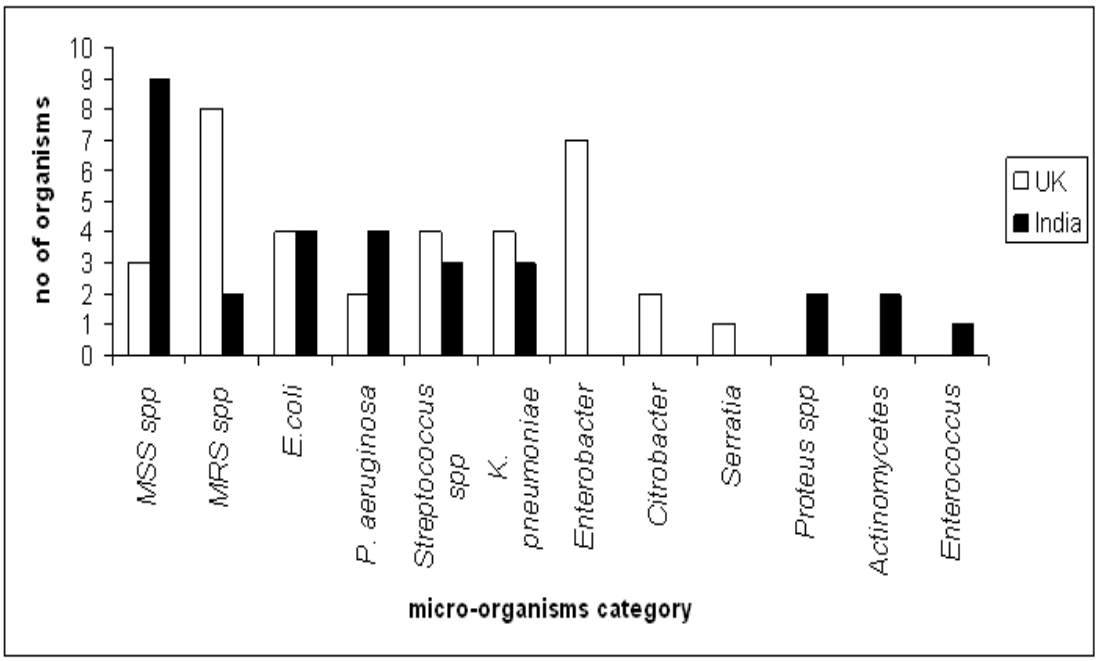


Figure 2.3 Distribution of aerobes from the diabetic foot pus samples from India and the UK

The total number of Gram – positive aerobes and Gram – negative aerobes from the Indian and UK diabetic foot patients. MSS spp – methicillin sensitive *Staphyloccocus* species, MRS spp – methicillin resistant *Staphylococcus* species, *E. coli – Escherichia coli*, *P. aeruginosa – Pseudomonas aeruginosa* and *K. pneumoniae – Klebsiella pneumoniae.* The aerobes from India were identified using traditionally used biochemical identification while Rapid id 20E was used for identifying Gram – negative aerobes from the Enterobacteriaceae family while Hi – Staph and Hi Strep was used to identify the *Staphylococcus* and *Streptococcus* species in the UK.

2.3.1.2 Distribution of anaerobes from the Indian and the UK diabetic foot pus samples

The figures 2.4 and 2.5 clearly show that in comparison to the UK cohorts the Indian diabetic foot pus samples showed high number of anaerobes suggesting the severity of infections. From the total 20 anaerobes from the 27 Indian pus samples there were 13 Gram – positive anaerobes mainly the *Clostridium* species and among the 7 Gram – negative anaerobes mainly included the *Bacteroides ureolyticus* (n = 5) with *Fusobacterium* (n = 1) and *Veillonella* species (n = 1). While from the total 5 anaerobes from the UK diabetic foot pus samples 3 of them were *Clostridium* species and others belonged to *Bacteroides ureolyticus* and *Veillonella* species.

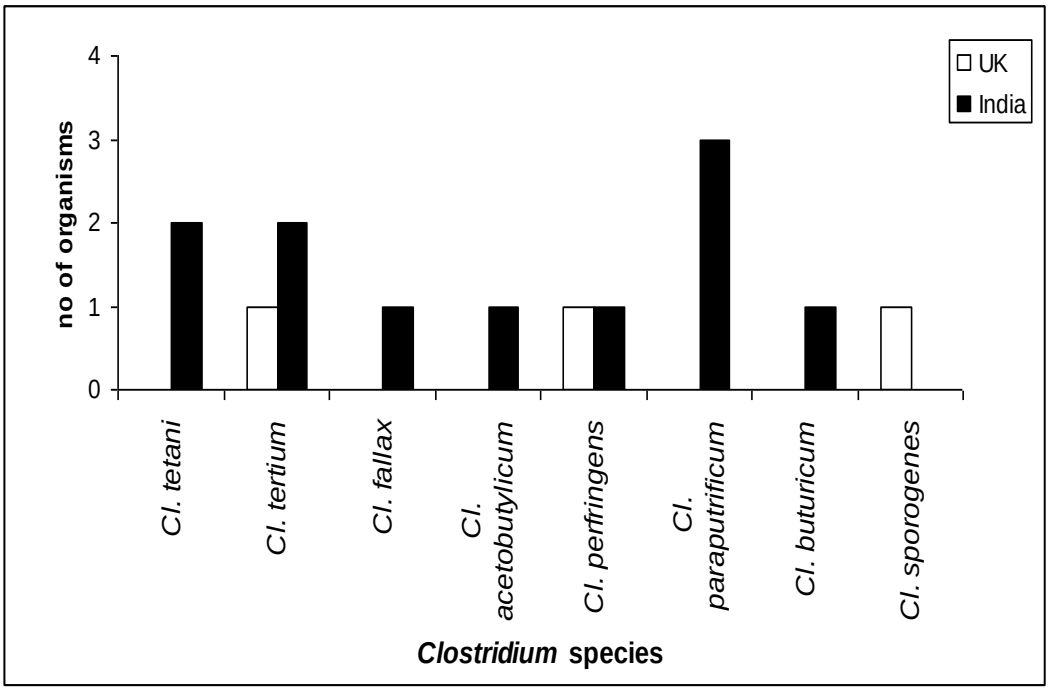


Figure 2.4 Distribution of *Clostridium* species from the diabetic foot pus samples from India and the UK

The number of *Clostridium* species recognised using Rapid id 32A for the Indian and the UK diabetic foot pus samples.

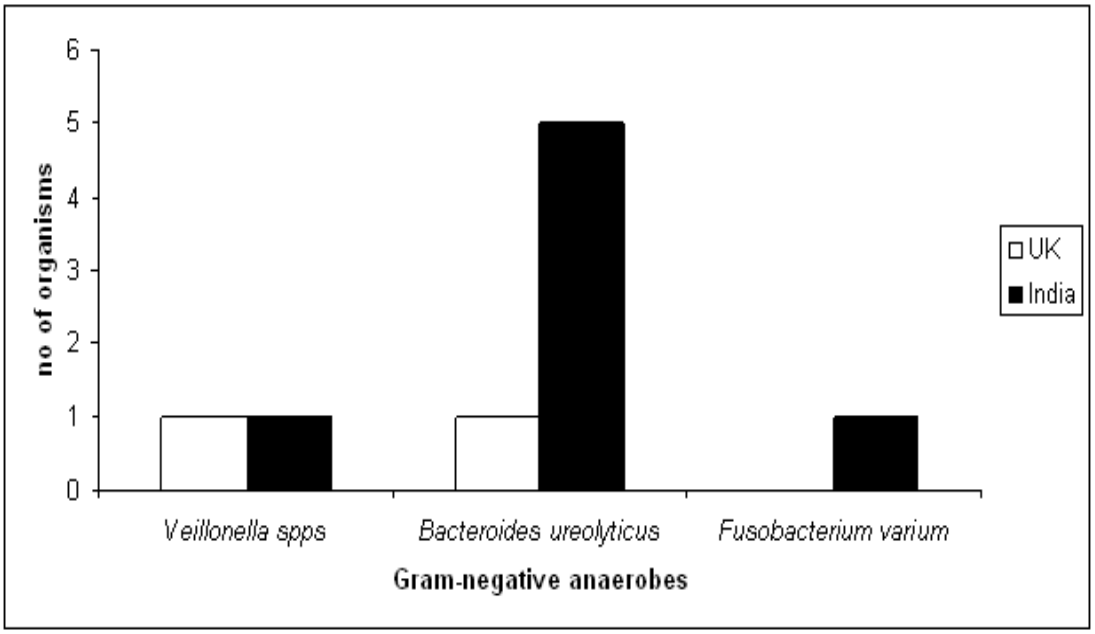


Figure 2.5 Distribution of Gram – negative anaerobes from the diabetic foot pus samples from India and the UK

The number of Gram - negative species recognised using Rapid id 32A for the Indian and the UK diabetic foot pus samples.

The number of pus samples only infected due to aerobes in the UK diabetic cohorts exceeds four fold that from India. While the number of pus samples with mixed infections from India was higher than that of UK as seen in table

2.10 Although it should be taken into consideration the table 2.10 only discusses the number of pus samples with aerobes or anaerobes or fungi or mixed infections. The numbers are reflected in figures 2.3 - 2.5 discussed earlier.

Table 2.10 Evaluation of aerobes, anaerobes and fungi from pus samples from the diabetic foot patients from India and UK

	Number of species grown	
Status	**India**	**UK**
Number of pus with aerobes only	7	31
Number of pus with anaerobes only	0	0
Number of pus with fungi only	0	0
Number of pus with mixed infection	16	6
Number of pus with no growth	4	1

The table 2.10 shows the evaluation of the microbial flora from the Indian and UK diabetic foot pus samples although statistically significant correlation cannot be made due to the small sample sizes.

2.3.2 Distribution of aerobes, anaerobes and fungi obtained from the tissue specimens from the Indian diabetic foot patients

Due to the severity of infections and amputations seen among the Indian diabetic foot patients, tissue biopsies were also sent to the Microbiological laboratory at Raheja Hospital – India for microbiological culturing. On microbiological processing of tissues samples, a wide variety of organisms were identified. Figure 2.6 shows the percentage of each aerobe cultured from tissue specimens with 34% belonging to methicillin sensitive *Staphylococcus* species and 13% methicillin resistant *Staphylococcus* species. Among the gram negative aerobes *E. coli* and *P. aeruginosa* were shown to comprise 10% of the total gram negative organisms. In total from 73 tissue samples 77 aerobes were cultured of which 42 were Gram – positive aerobes and 35 were Gram – negative aerobes.

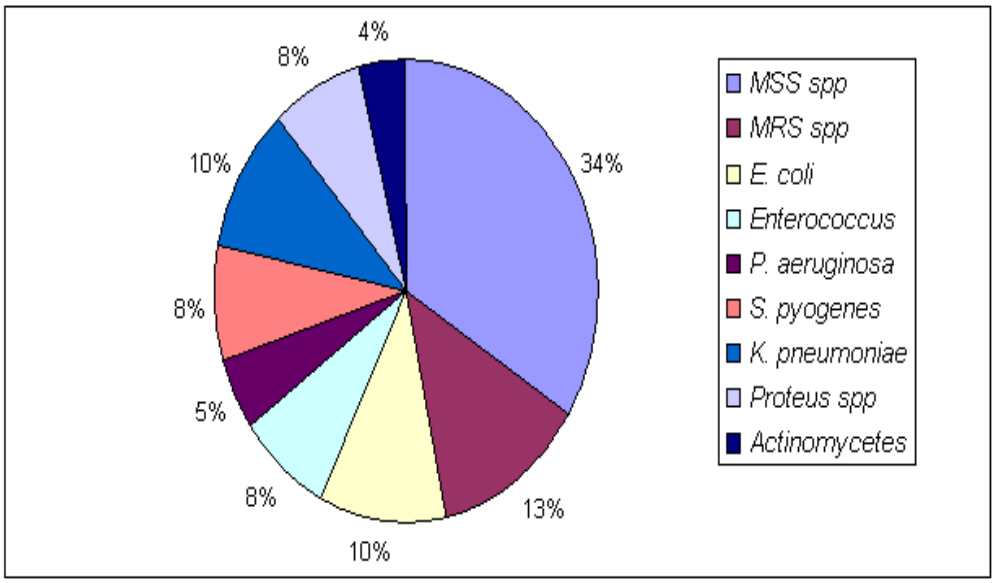


Figure 2.6 Distribution of aerobes from the tissue specimens from the Indian diabetic foot patients

The total number of Gram – positive aerobes and Gram – negative aerobes from the Indian diabetic foot patient's tissues. MSS spp – methicillin sensitive *Staphyloccocus* species (n = 26), MRS spp – methicillin resistant *Staphylococcus* species (n = 10), *E. coli* – *Escherichia coli* (n = 8), *P. aeruginosa* – *Pseudomonas aeruginosa* (n = 4) and *K. pneumoniae* – *Klebsiella pneumoniae* (n = 8). These were identified using traditionally used biochemical identification.

There were a total of 51 anaerobes cultured from the tissue specimens, 33 Gram – positive and 18 Gram – negative. The Gram – positive anaerobes were mainly from the *Clostridium* species other Gram – positive anaerobes (n = 13) included those from *Peptostreptococcus* species and *Eubacterium* and *Actinomycetes* as seen in Figure 2.7

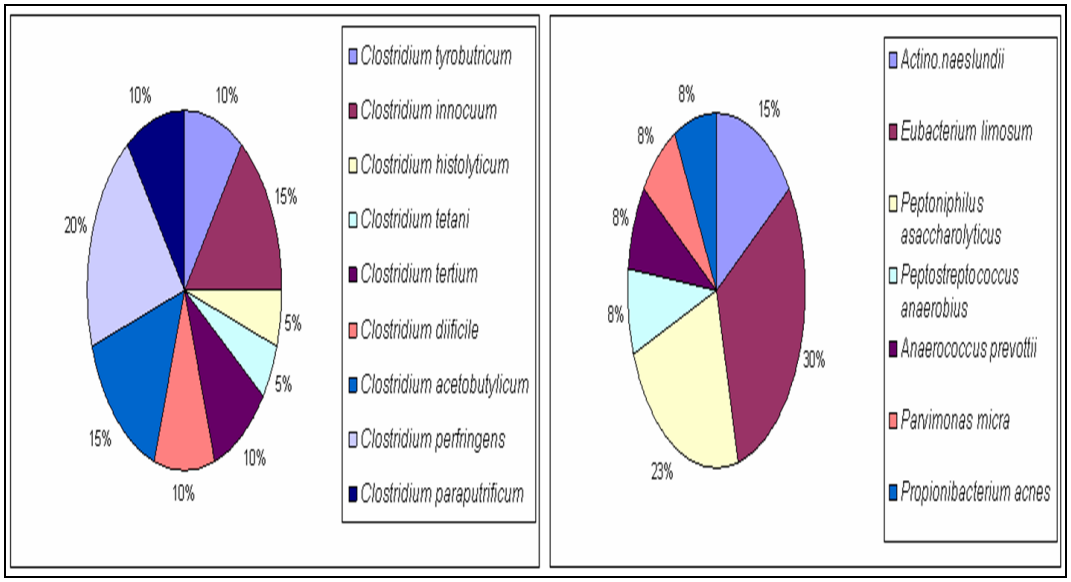


Figure 2.7 Distribution of Gram – positive anaerobes from the tissue specimens from the Indian diabetic foot patients

Clostridium species (n = 20) and other Gram – positive anaerobes (n = 13) cultured from the tissues specimens from the Indian diabetic foot patients. These were identified to their species level using Rapid ID 32A.

Among the 18 Gram – negative anaerobes 71% were identified as *Bacteroides ureolyticus Veillonella, Fusobacterium* and *Capnocytophaga* were also isolated as shown in figure 2.8.

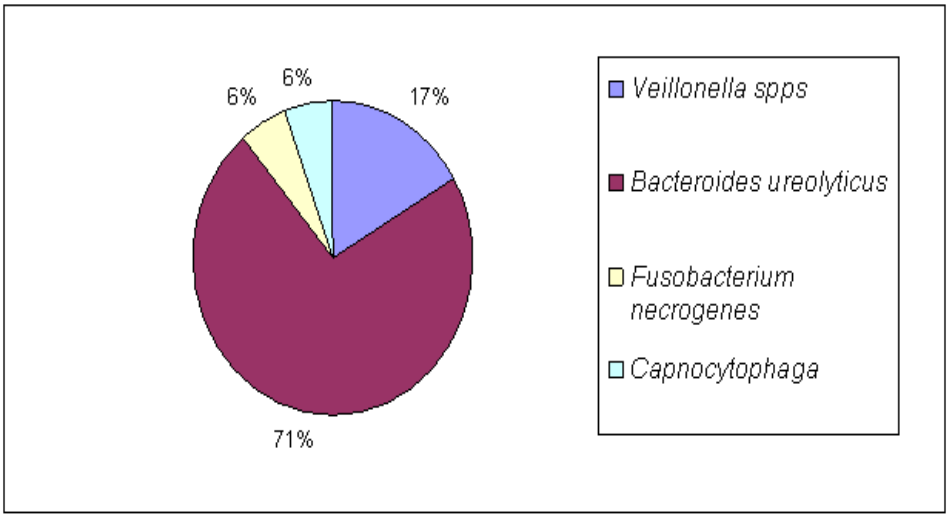


Figure 2.8 Distribution of Gram – negative anaerobes from the tissue specimens from the Indian diabetic foot patients
The identification of Gram – negative anaerobes up to their species level was facilitated with the use of Rapid Id 32A.

Fungi were only cultured from the tissue samples from the Indian diabetic foot patients and not from the pus samples from the diabetic foot patients from either India or the UK as shown in figure 2.9. The most common fungi were the *Candida* species with *Candida tropicalis* being the highest detected (n = 7) from the total 14 *Candida* species obtained from 73 tissues.

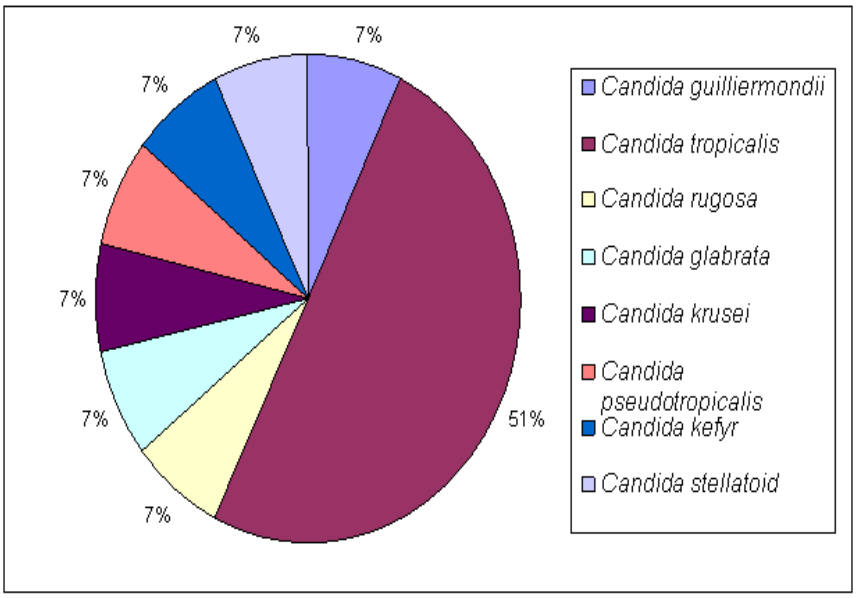


Figure 2.9 Distribution of fungi from the tissue specimens from the Indian diabetic foot patients
Candida species were identified using the Hi Candid kit available from Hi Media, Mumbai, India.

Of the total 73 tissues 22 tissues only had infection due to aerobes, while 5 tissues only had infections caused due to anaerobes as seen in table 2.11. 37 tissues showed mixed infections while none of the tissues showed infections caused solely due to *Candida* suggesting that fungi were from mixed cultures.

Table 2.11 Evaluation of aerobes, anaerobes and fungi from tissue samples from the diabetic foot patients from India

Status	Number of species cultured
Tissues with aerobes only	22
Tissues with anaerobes only	5
Tissues with fungi only	0
Tissues with mixed infection	37
Tissues with no growth	9

2.3.3 Distribution of aerobes, anaerobes and fungi from the Indian pus and tissue specimens according to their Wagner's grades

The diabetic foot patient's ulcers from the Indian study all were categorised according to their Wagner's grades (Appendix V). The categories of organisms obtained from these ulcers are grouped and shown in the table 2.12 .

Table 2.12 Spectrum of aerobic organisms from pus and tissue specimens from the Indian diabetic foot patients correlated to their Wagner's grades

Aerobes from pus and tissues	Wagner's Grades			
	I	II	III	IV
MSS spp (n = 35)	4	10	12	9
MRS spp (n = 12)	0	0	9	3
Enterococcus (n = 7)	0	3	3	1
E. coli (n = 12)	0	5	3	4
P. aeruginosa (n = 8)	0	1	3	4
S. pyogenes (n = 9)	2	3	2	2
K. pneumoniae (n = 11)	2	3	4	2
Proteus spp (n = 8)	0	0	4	4
Actinomycetes (n = 5)	0	0	2	3

The aerobes categorised in this table includes collections from both pus and tissue specimens from the Indian diabetic foot patients.

From the table 2.12 it is clear that MRS spps mainly were obtained from grades III and IV suggesting that the infections must have been deep

seated. MSS spps were isolated from all grades with higher numbers seen in grades II and III. Amongst the Gram – negative aerobes *P. aeruginosa*, *Actinomycetes* and *Proteus* species were isolated from higher grade infections, mainly grades III and IV.

Among the anaerobes from the tissue specimens it is evident from the table 2.13 shown below that they were more commonly obtained from grades III and IV again suggesting the severity of infections. This was expected as most of the tissues obtained were from an amputed toes or from patients who had high level of infection.

Table 2.13 Spectrum of anaerobic organisms from pus and tissue specimens from the Indian diabetic foot patients as per their Wagner's grades

Anaerobes from pus and tissues	Wagner's Grades			
	I	II	III	IV
Actino.naeslundii	0	0	1	1
Anaerococcus prevottii	0	0	0	1
Bacteroides ureolyticus	2	3	5	8
Capnocytophaga	0	1	0	0
Clostridium acetobutylicum	0	2	2	1
Clostridium buturicum	0	0	1	0
Clostridium diificile	0	0	1	1
Clostridium fallax	0	1	0	0
Clostridium histolyticum	0	0	1	0
Clostridium innocuum	0	0	2	1
Clostridium paraputrificum	0	2	2	1
Clostridium perfringens	0	2	1	2
Clostridium tertium	0	0	1	3
Clostridium tetani	0	0	2	3
Clostridium tyrobutricum	0	1	1	0
Eubacterium limosum	0	0	2	3
Fusobacterium necrogenes	0	0	0	1
Fusobacterium varium	0	0	0	1
Parvimonas micra	0	0	1	0
Peptoniphilus asaccharolyticus	0	0	2	1
Peptostreptococcus anaerobius	0	0	0	1
Propionibacterium acnes	0	0	1	0
Veillonella sps	0	1	2	1

The anaerobes categorised in the table 2.13 includes collections from both pus and tissue specimens from the Indian diabetic foot patients.

Similar to the anaerobes the *Candida* species were more commonly isolated from grades III and IV as shown in the table 2.14 below

Table 2.14 Spectrum of *Candida* species from the tissue specimens from the Indian diabetic foot patients as per their Wagner's grades

***Candida* species from tissues**	**Wagners Grades**			
	I	**II**	**III**	**IV**
Candida guilliermondii	0	0	0	1
Candida tropicalis	0	0	2	5
Candida rugosa	0	0	1	0
Candida glabrata	0	1	0	0
Candida krusei	0	0	1	0
Candida pseudotropicalis	0	1	0	0
Candida kefyr	0	0	1	0
Candida stellatoid	0	0	1	0

The *Candida* species categorised in the table 2.14 include collections from only the tissue specimens from the Indian diabetic foot patients as there were no fungi cultured from the pus samples.

Although there were high numbers of anaerobes and *Candida* species obtained from Grades III and IV as seen from tables, the figure below shows a steady increase in the number of anaerobes as the Wagner's grades increases from I - III. At grade IV the numbers of anaerobes and fungi were similar to those found at grade III. The number of aerobes also showed an increase up to grade III but at grade 4 there was a decrease in numbers.

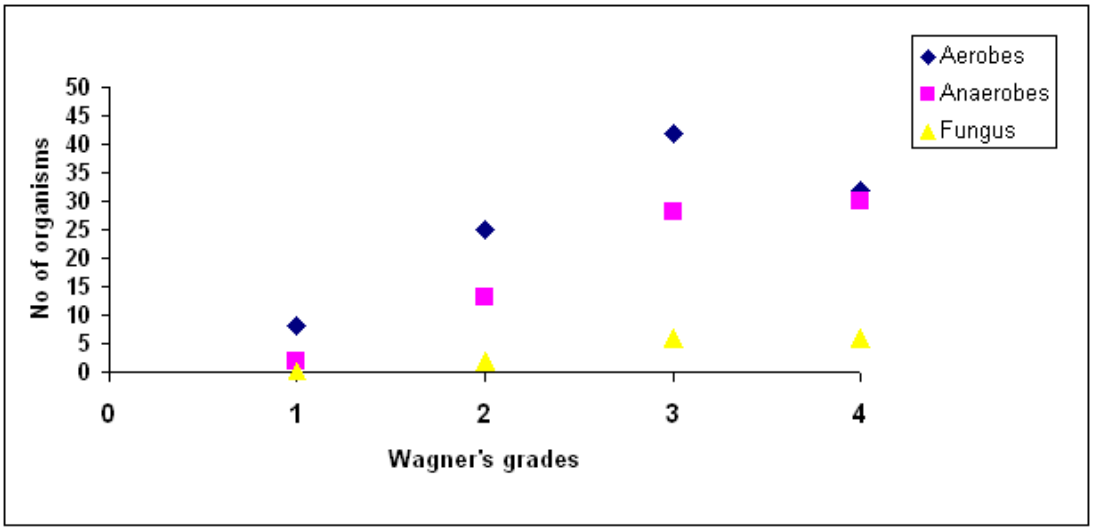


Figure 2.10 Average numbers of organisms per Wagner's grades isolated from the pus and tissue specimens from Indian diabetic foot patients

Total aerobes (n = 107), anaerobes (n = 71) and fungi (n= 14) combined from pus and tissues.

2.3.4 Antimicrobial susceptibility testing of aerobes and anaerobes from the diabetic foot patients from India and the UK

From the 27 pus and 73 tissue specimens from the Indian diabetic foot patients there were in total 107 aerobes and 71 anaerobes cultured. Antimicrobial sensitivity however was only carried out on 79 aerobes and 51 anaerobes. From the UK diabetic foot pus samples the total numbers of aerobes cultured were 35 and anaerobes 5, antimicrobial susceptibility testing however was only carried out on 23 aerobes and for all 5 cultured anaerobes.

As a routine practice antimicrobial testing by Kirby Bauer's disk diffusion method on aerobes is regularly carried out at Raheja Hospital in India. Therefore for antimicrobial susceptibility testing of aerobes antibiotic discs were used with the recommended NCCLS antibiotic potency. The antibiotic disc were purchased from Hi media (Mumbai India) and their manual was used to categorise the organisms antibiotic profiles (sensitive, intermediate and resistant) and these are extracted from the Hi media manual and represented in table 2.15.

Table 2.15 Zone-size interpretive charts for antibiotics used for testing aerobes from the Indian diabetic foot patient's pus and tissue specimens

Hi-media interpretation for the following antibiotics	Potency	Sensitive (mm or more)	Inter-mediate	Resistant (mm or less)
Amikacin	30 mcg	17	15-16	14
Amoxicillin/ Clavulanic acid	30 mcg for *Staphylococcus*	20	_	19
	30 mcg for enteric organism	18	14-17	13
Ampicillin	10 mcg for *Staphylococcus*	29	_	28
	10 mcg for *Strepto - coccus*	26	19-25	18
Ampicillin/Sulbactam	10 mcg	15	12-14	11
Azithromycin	15 mcg	18	14-17	13
Carbenicillin	100 mcg for enteric organism	23	20-22	19
	100 mcg for *Pseudomonas aerugi-nosa*	17	14-16	13
Cefazolin	30 mcg	18	15-17	14
Cefepime	30 mcg	18	15-17	14
Cefoperazone	75 mcg	21	16-20	15
Ceftazidime	30 mcg	18	15-17	14
Ceftizoxime	30 mcg	20	15-19	14
Cefuroxime	30 mcg	18	14-17	13
Chloramphenicol	30 mcg	21	18-20	17
Co-trimaxazole	23.75 mcg	19	16-18	15
Gentamicin	10 mcg	15	13-14	12
Gatifloxacin	5 mcg for *Staphylococcus*	23	20-23	19
	5 mcg for *Strepto-coccus*	21	18-20	19
Imipenem	10 mcg	16	14-15	13
	5 mcg *Staphylococcus*	19	16-18	15

Levofloxacin	5 mcg for enteric and *Strepto-coccus*	17	14-16	13
Lomefloxacin	10 mcg	22	19-21	18
Meropenem	10 mcg	16	14-15	13
Nitrofurantoin	300 mcg	17	15-16	14
Penicillin	10 units	29	-	28
Piperacillin/tazo-bactum	100/10 mcg	21	18-20	17
Ticacillin/Clavulanic acid	75/10 mcg for enteric and *Pseudom onas* spp	20	15-19	14
	75/10 mcg for *Staphyloc occus* spp	23	_	22

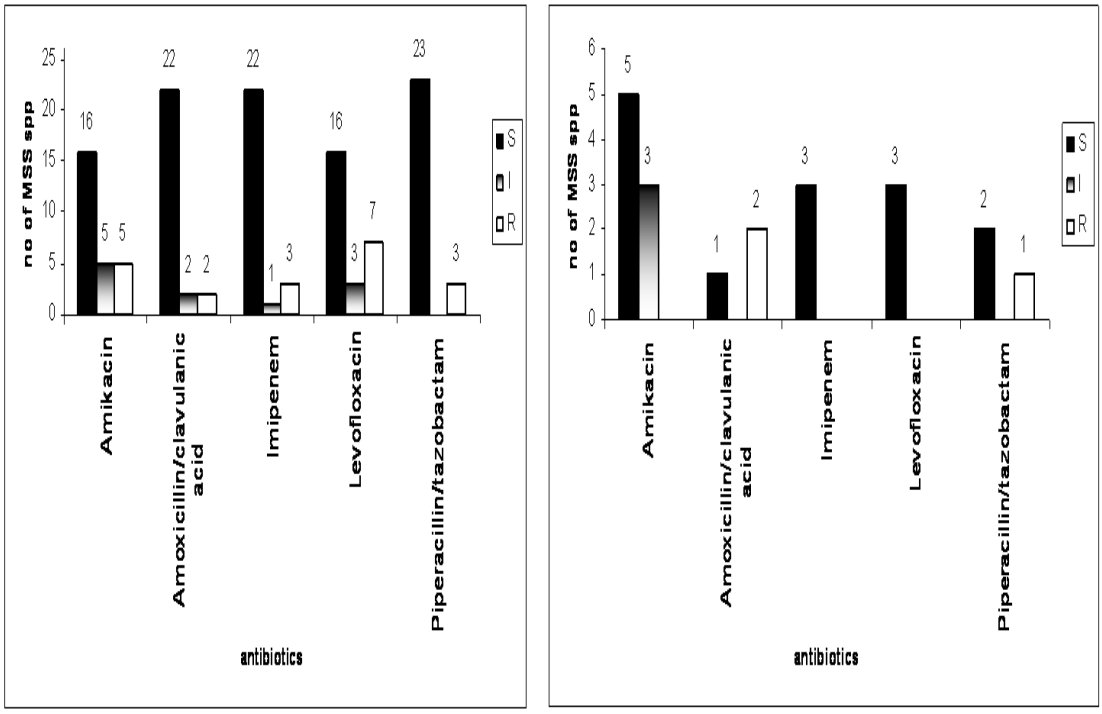
*****based on results obtained using Muller Hinton agar by disc – diffusion method

* Hi media chart used which is validated in accordance to the performance standards for antimicrobial disc tests by NCCLS.

2.3.4.1 Antimicrobial susceptibility testing of Gram – positive aerobes from the diabetic foot patients from India and the UK

There are some antibiotics which were commonly tested in the UK and India and therefore the data is represented in this manner. It is very important to note that there is no significant comparison as the antibiotic methods used in India and UK was different due to logistical issues of importing/exporting of antibiotics. In India the method followed was Kirby Bauer's disk diffusion using antibiotic discs (normally carried out at Raheja Hospital – India) as per NCCLS standards. While in the UK due to the availability of E-test, MIC was carried out on aerobes using the agar diffusion method as per NCCLS standards but represented not as MIC value but as sensitive, intermediate and resistant**.** The antimicrobial susceptibility testing was carried out on 45 Gram – positive aerobes cultured from the Indian DF patient's pus and tissue specimens while 14 Gram – positive aerobes cultured from the UK DF patient's pus specimens as shown in the figures below.

The antibiotics tested in India and the UK included amikacin, amoxicillin/clavulanic acid, imipenem, levofloxacin and piperacillin/tazobactam. The figure 2.11 below represents the MSS species susceptibility profiles and it is evident that few MSS spps showed resistance to the tested antibiotics in either the Indian and UK cohorts. Higher numbers in the range of 16-22 out of the 26 MSS spps from Indian DF patients showed susceptibility to all the tested antibiotics. Similar results were seen from the DF patients from the UK.



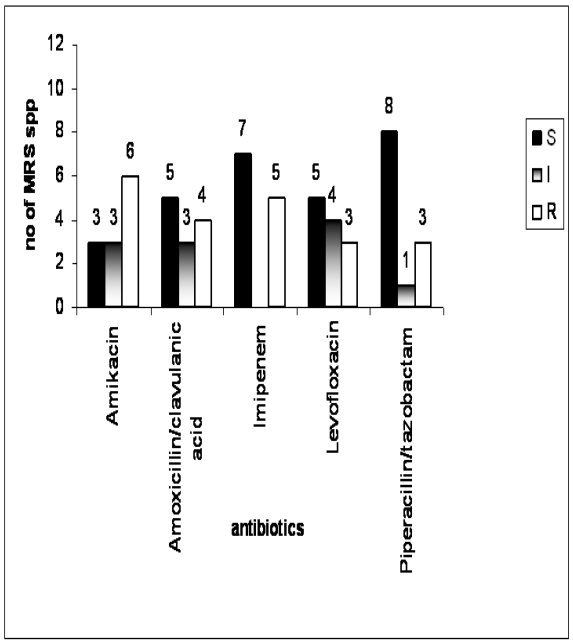
Activities of 5 antibiotics against 26 MSS spp from the Indian diabetic foot patients pus and tissue samples

Activities of 5 antibiotics against 3 MSS spp from the diabetic foot patients pus samples from the UK

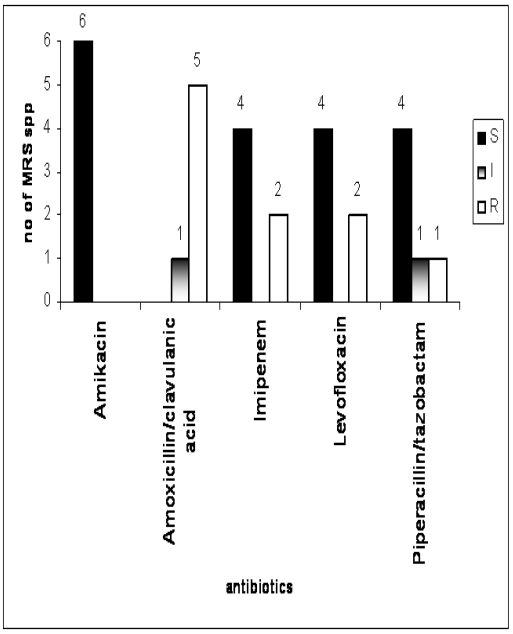
Figure 2.11 In vitro activities of antimicrobial agents against methicillin sensitive *Staphylococcus* species obtained from the Indian diabetic foot pus and tissue specimens and pus specimens from the UK

Antibiotics discs: Amikacin (30 µg), amoxicillin/clavulanic acid (20 by10), imipenem (10 µg), levofloxacin (05 µg) and piperacillin/tazobactam (100 by 10) used from MSS spps from Indian tissues and pus specimens. All the E-test strips ranged from 0.01 to 256 µg/ml used for the MSS species from the UK.

A different type of sensitivity to the tested antibiotics was seen in figure 2.12 for the MRS spp from the two cohorts. All the MRS spp from the UK DF patients showed sensitivity to amikacin, 5 out of 6 showed sensitivity to imipenem, levofloxacin and piperacillin/tazobactam and a complete resistance to amoxicillin/clavulanic acid. While in the range of 3 – 8 out of 12 of the MRS spp from the Indian DF patients showed sensitivity to the tested antibiotics worryingly there were a number of intermediate strains of MRS spp which could become resistant in the future.



Activities of 5 antibiotics against 12 MRS spp from the Indian diabetic foot patients pus and tissue samples

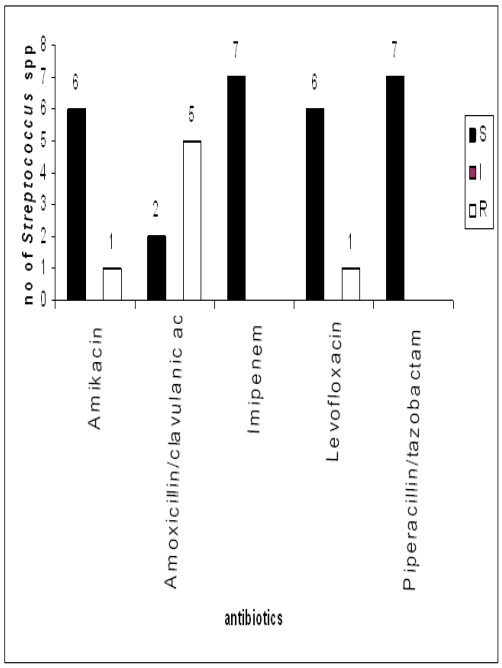


Activities of 5 antibiotics against 6 MRS spp from the diabetic foot pus samples from the UK

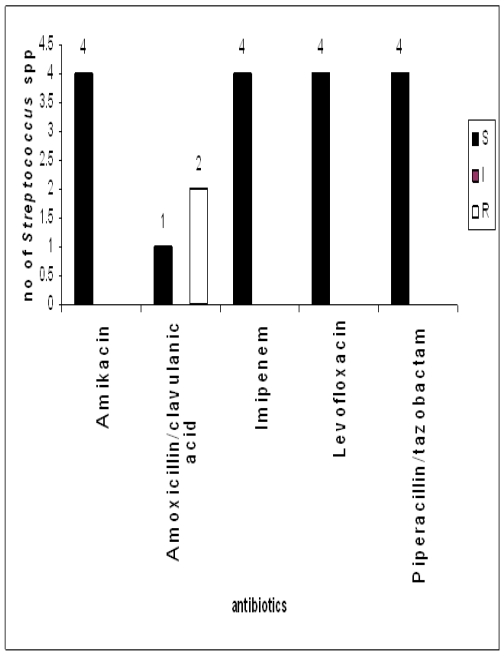
Figure 2.12 *In vitro* activities of antimicrobial agents against methicillin resistant *Staphylococcus* species obtained from the diabetic foot pus and tissue specimens from India and pus specimens from the UK

Antibiotics discs: Amikacin (30 µg), amoxicillin/clavulanic acid (20 by10), imipenem (10 µg), levofloxacin (05 µg) and piperacillin/tazobactam (100 by 10) used from MRS spps from Indian tissues and pus specimens. All the E-test strips ranged from 0.01 to 256 µg/ml used for the MRS species from the UK

As seen in the figure 2.13 all the *Streptococcus* strains from the Indian and the UK DF patient's specimens showed good sensitivity to the tested antibiotics with no intermediate levels seen. However, 5/7 tested from the Indian group and 2/3 of the UK group showed resistance to amoxicillin/clavulanic acid. In the Indian group 1/7 was resistant to amikacin and 1/7 to levofloxacin.



Activities of 5 antibiotics against 7 *Streptococcus* species from the Indian diabetic foot patients pus and tissue samples



Activities of 5 antibiotics against 4 *Streptococcus* species from the diabetic foot patients pus samples from the UK

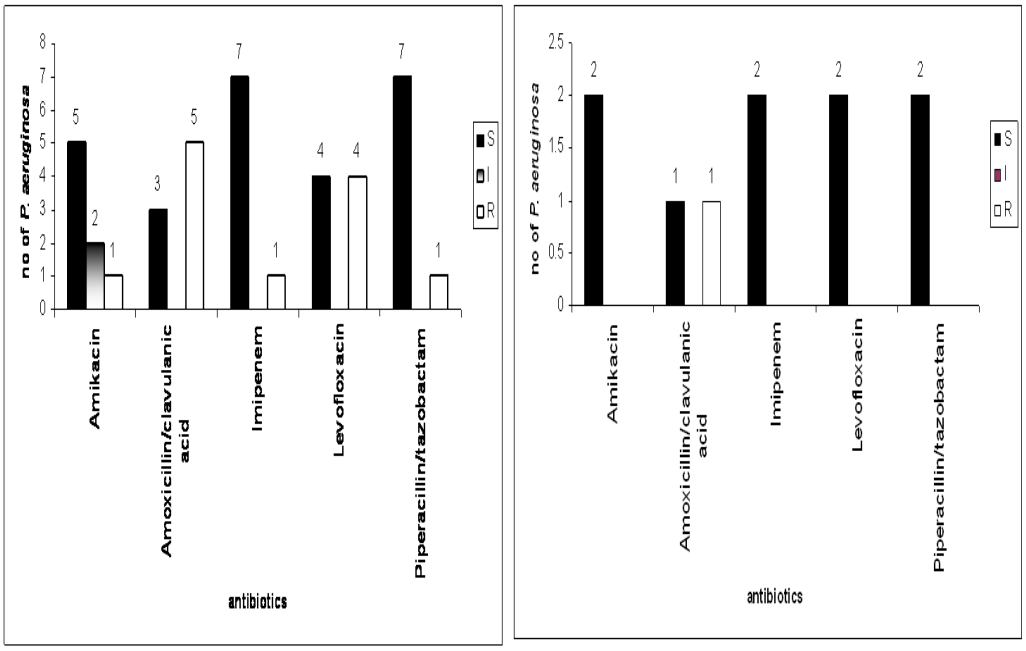
Figure 2.13 *In vitro* activities of antimicrobial agents against *Streptococcus* species from the Indian diabetic foot pus and tissue specimens and pus specimens from the UK

Antibiotics discs: Amikacin (30 µg), amoxicillin/clavulanic acid (20 by10), imipenem (10 µg), levofloxacin (05 µg) and piperacillin/tazobactam (100 by 10) used from *Streptococcus* spps from Indian tissues and pus specimens. All the E-test strips ranged from 0.01 to 256 µg/ml used for the *Streptoccocus* species from the UK.

2.3.4.2 Antimicrobial susceptibility testing of Gram – negative aerobes from the diabetic foot patients from India and the UK

The antimicrobial susceptibility testing was carried out on 34 Gram – negative aerobes from the Indian DF patient's pus and tissue specimens and 10 Gram – negative aerobes from the UK DF patient's pus specimens.

Good susceptibility was seen for the two *P. aeruginosa* strains from the UK DF patient's pus samples as seen in the Figure 2.14. Fifty percent of the isolates were resistant to amoxicillin/ clavulanic acid. Among the 8 tested *P. aeruginosa* strains from the Indian DF patient's pus samples 7 were sensitive to imipenem and piperacillin/tazobactam while 50% showed sensitivity and 50% resistance to levofloxacin. There were 5 strains (50%) showing resistance to amoxicillin/clavulanic acid.



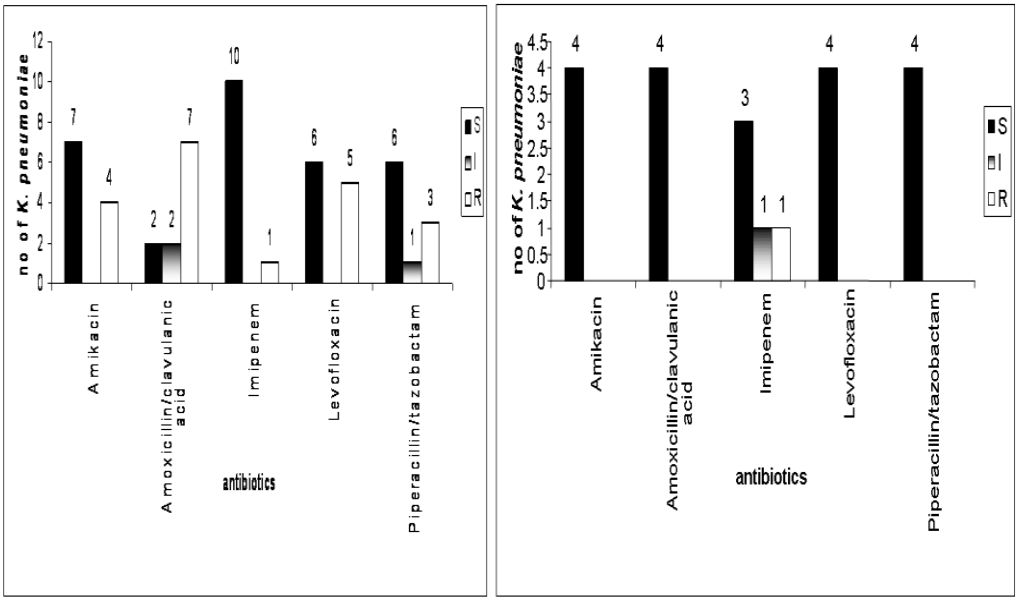
Activities of 5 antibiotics against 8 *P. aeruginosa* from the Indian diabetic foot patients pus and tissue samples

Activities of 5 antibiotics against 2 *P. aeruginosa* from the diabetic foot patients pus samples from the UK

Figure 2.14 *In vitro* activities of antimicrobial agents against *P. aeruginosa* from the Indian diabetic foot pus and tissue specimens and pus samples from the UK

Antibiotics discs: Amikacin (30 µg), amoxicillin/clavulanic acid (20 by10), imipenem (10 µg), levofloxacin (05 µg) and piperacillin/tazobactam (100 by 10) used from *P. aeruginosa* spps from Indian tissues and pus specimens. All the E-test strips ranged from 0.01 to 256 µg/ml used for the *P. aeruginosa* species from the UK.

In the figure 2.15 below, eleven *K. pneumoniae* strains from the Indian DF patients pus and tissue specimens showed good sensitivity to imipenem (10 out of 11) amikacin (7 out of 11) and levofloxacin and piperacillin/tazobactam (6 out of 11) although 5 strains did show resistance to levofloxacin. Among the isolates from UK DF patients *K. pneumoniae* showed good susceptibility to all the tested antibiotics with one isolate intermediate and one isolate resistant to imipenem.



Activity of 5 antibiotics against 11 *K. pneumoniae* from the Indian diabetic foot patients pus and the tissue samples

Activity of 5 antibiotics against 4 *K. pneumoniae* from the diabetic foot patients pus samples form the UK

Figure 2.15 *In vitro* activities of antimicrobial agents against *K. pneumoniae* from the Indian diabetic foot pus and tissue specimens and pus samples from the UK

Antibiotics discs: Amikacin (30 µg), amoxicillin/clavulanic acid (20 by10), imipenem (10 µg), levofloxacin (05 µg) and piperacillin/tazobactam (100 by 10) used from *K. pneumoniae* spps from Indian tissues and pus specimens. All the E-test strips ranged from 0.01 to 256 µg/ml used for the *K. pneumoniae* species from the UK

The antibiotic susceptibility testing was also carried out on *Citrobacter* species and *Serratia* species cultured from the diabetic foot pus samples from the UK but due to the very low numbers, data is not presented in this study.

On a routine basis in the Microbiology laboratory at Raheja Hospital in India the Kirby Bauer's disc diffusion method was performed using the antibiotics discussed in the materials and methods section. The table below (table 2.16) illustrates the number aerobes, from the pus and tissue specimens from Indian diabetic foot patients, tested that showed resistance against the 19 antibiotics used.

Table 2.16 *In vitro* activities of 19 antibiotic discs against aerobes from the Indian diabetic foot patient's pus and tissue specimens

Antibiotics	**No of resistant strains**						
	MRS spp (n = 12)	**MSS spp (n = 26)**	***Strepto-coccus* spp (n = 7)**	***P. aeru-ginosa* (n = 8)**	***K. pneu-moniae* (n = 11)**	***E. coli (n = 8)***	***Acineto-bacter (n = 7)***
Ampicillin (10 µg)	8	5	2	4	**8**	**6**	3
Ampicillin/sulbactam (10 by 10)	5	1	3	6	6	2	2
Azithromycin (15 µg)	5	7	2	3	**9**	1	2
Carbenicillin (100 µg)	5	7	1	5	4	4	3
Cefazolin (30 µg)	6	3	2	0	**7**	4	4
Cefepime (30 µg)	4	6	2	4	4	3	1
Cefoperazome (75 µg)	5	2	2	3	5	3	3
Ceftazidime (30 µg)	**7**	5	1	4	4	4	3
Ceftizoxime (30 µg)	**8**	6	1	4	4	4	3
Cefuroxime (30 µg)	**7**	4	1	0	4	4	3
Chloramphenicol (30 µg)	2	3	1	5	3	2	2
Co-trimaxazole (25 µg)	**7**	**14**	3	**6**	**7**	**6**	**5**
Gentamicin (10 µg)	6	3	1	3	4	2	1
Gatifloxacin (05 µg)	2	1	1	3	2	3	2
Lomefloxacin (10 µg)	**7**	**18**	2	4	5	**5**	2
Meropenem (10 µg)	6	1	1	2	1	1	3
Nitrofurantoin (300 µg)	0	1	2	4	3	2	4
Penicillin (10 µg)	**10**	2	2	4	5	2	**5**
Ticarcillin/clavulanic acid (75 by 10)	6	2	4	4	6	**5**	1

From the table 2.16 it is clear that there are some organisms showing high resistance to the antibiotics. Those organisms showing more than 50% resistant strains are highlighted in bold. More than 50% of the MRS spp

showed resistance to ceftazidime (30 µg), ceftizoxime (30 µg), cefuroxime (30 µg), co –trimaxazole (25 µg), lomefloxacin (10 µg) and penicillin (10 µg). While more than 50% of the MSS spp showed resistance to co – trimaxazole (25 µg) and lomefloxacin (10 µg). Gram – positive *Streptococcus* spp showed reasonably good sensitivity to all the tested antibiotics with very little resistance seen. In the Gram – negative aerobes more than 50% of *the K. pneumoniae* strains showed resistance to ampicillin (10 µg), azithromycin (15 µg), cefazolin (30 µg) and co – trimaxazole (25 µg), More than 50% of the *E. coli* strains showed resistance to ampicillin (10 µg), co – trimaxazole (25 µg) lomefloxacin (10 µg) and ticarcillin/clavulanic acid (75 by 10). *Acinetobacter* only showed resistance to co – trimaxazole (25 µg).

2.3.4.3 Antimicrobial susceptibility testing of anaerobes from the diabetic foot patients from India and the UK

As anaerobic culturing was not routine in Raheja hospital the antimicrobial testing therefore was also not common hence with the funds available from the Society for General Microbiology MIC testing was carried out on the anaerobes obtained from India and UK by using agar diffusion method. In India the MIC were obtained using E-comb purchased from Hi media (Mumbai, India) which is the most popularly used company for microbiological testing in the country. While in the UK the most popular and convenient MIC testing method using E-test that is used by many hospitals was utilised for anaerobic susceptibility testing. The interpretations were made using the NCCLS standards for each antibiotic and represented in the table 2.17.

Table 2.17 MIC interpretive chart for antibiotics used for testing aerobes and anaerobes from the UK diabetic foot patient's pus and anaerobes from the Indian diabetic foot patient's pus and tissue specimens

NCCLS interpretation for the following antibiotics	Sensitive	Intermediate	Resistant
Amikacin	≤16	32	≥ 64
Amoxicillin/ Clavulanic acid	≤ 4/2	8/4	≥16/8
Ciprofloxacin	≤ 8	16	≥ 32
Clindamycin	≤ 1	2	≥ 4
Daptomycin	≤ 1	-	-
Erythromycin	≤ 0.5	1-4	≥ 8
Imipenem	≤ 4	8	≥ 16
Levofloxacin	≤ 2	4	≥ 8
Linezolid	≤ 2	4	≥ 8
Piperacillin/tazobactam	≤ 32/4	64/4	≥ 128/4

All the results are based on results obtained using Brucella agar with supplements and using E-test from bioMerieux, France).

The tables 2.18 to 2.23 below are the MIC ranges using E-test (bioMerieux, France) and E-comb (HiMedia, Mumbai, India) for pus samples from the UK and pus and tissue samples from India respectively. Since most of the MIC ranged between intermediate to resistant for the anaerobes tested within the Indian diabetic foot cohorts; the data is represented in tables 2.18 to 2.23.

The tables 2.18 to 2.23 below are the comparison of antibiotics tested on the anaerobes cultured from India and UK. This data represents a pilot study and can be informative but cannot be considered statistically significant due to the low numbers of anaerobes isolated.

Among the Gram – positive anaerobes from the Indian DF patient's pus and tissue specimens *in vitro* antimicrobial testing was only carried out on 37 strains while from the UK only 3 strains were tested.

Table 2.18 Comparative *in vitro* activities of antimicrobial agents against *Clostridium* species from the pus and tissue specimens from Indian and pus samples from the UK diabetic foot patients

Organism	Country	Antibiotics	MIC distribution µg/L		
			0.016 – 4	**8 – 64**	**128 – 256**
Clostridium species (n = 28)	India	Amikacin	2	8	18
		Ciprofloxacin	1	2	25
		Clindamycin	1	3	23
		Linezolid	10	6	12
		Levofloxacin	3	3	21
		Piperacillin/ Tazobactum	1	7	20
		Metronidazole	20	0	0
Clostridium species (n = 3)	UK	Amikacin	0	1	2
		Amoxicillin/ Clavulanic acid	0	1	2
		Clindamycin	0	0	3
		Daptomycin	1	0	2
		Erythromycin	0	1	2
		Imipenem	3	0	0
		Levofloxacin	3	0	0
		Linezolid	0	2	1
		Piperacillin/ Tazobactam	2	1	0
		Metronidazole	3	0	0

The *Clostridium* species in the table 2.18 from the Indian diabetic foot patients specimens included the *Clostridium acetobutylicam, Clostridium butyricum, Clostridium difficile, Clostridium fallax, Clostridium histolyticum, Clostridium innocuum, Clostridium paraputrificum, Clostridium perfringens,Clostridium tetani, Clostridium tyrobutyricum* and *Clostridium tertium*.
The *Clostridium* species from the diabetic foot patient's specimens from the UK mainly included *Clostridium sporogens, Clostridium tertium* and *Clostridium perfringens.*

As seen in the table 2.18 there are very few *Clostridium* strains from the Indian DF patient's tissues and pus specimens showing complete sensitivity to the tested antibiotics. Of 28 *Clostridium* species isolated, 10 showed MIC in the range of 0.016 – 4 µg/L to linezolid and as per NCCLS the breakpoints are ≤ 2 µg/L not all the 10 strains would still be accepted as they were still sensitive to Linezolid. *Clostridium* spps from the UK DF pus specimens showed complete sensitivity to imipenem, levofloxacin and metronidazole (MIC for all antibiotics less than 2 µg/L). No significant

comparison can be made between the Indian and the UK *Clostridium* species as the numbers isolated were not comparable.

Among the Gram – negative species antimicrobial susceptibility testing was carried out on the *Bacteroides* and *Veillonella* species which were obtained from the Indian and the UK DF patient's clinical specimens. In total the *in vitro* antimicrobial testing was carried out on 14 Gram – negative anaerobes from the Indian DF patient's pus and tissue specimens and 2 from the DF patient pus samples from the UK.

Table 2.19 Comparative *in vitro* activities of antimicrobial agents against *Bacteroides* species from the pus and tissue specimens from the Indian and pus samples from the UK diabetic foot patients

Organism	Country	Antibiotics	MIC distribution µg/L		
			0.016 – 4	8 – 64	128 – 256
Bacteroi-des species (n = 8)	India	Amikacin	2	2	4
		Ciprofloxacin	2	2	4
		Clindamycin	1	3	4
		Linezolid	4	1	3
		Levofloxacin	3	0	5
		Piperacillin/ Tazobactum	2	2	4
		Metronidazole	4	0	4
Bacteroi-des species (n =1)	UK	Amikacin	0	0	1
		Amoxicillin/ Clavulanic acid	0	0	1
		Clindamycin	0	0	1
		Erythromycin	0	1	0
		Imipenem	1	0	0
		Levofloxacin	1	0	0
		Linezolid	1	0	0
		Piperacillin/ Tazobactam	1	0	0
		Metronidazole	1	0	0

The *Bacteroides* species from the Indian diabetic foot specimens mainly included the *Bacteroides ureolyticus* species while from the UK diabetic foot specimens were *Bacteroides fragilis.*

Bacteroides species isolated from the Indian DF patient's clinical specimens comprised high numbers of strains that were likely to show resistance when compared with the NCCLS breakpoints in table. Similar to the *Clostridium* strains shown in table from the Indian DF samples more than half of the *Bacteroides* spp showed MIC in the range of 0.016 – 4 µg/L however, we

cannot state that all the strains were sensitive as the breakpoints for linezolid according to NCCLS is ≤ 2 µg/L implying that the strains are either sensitive or intermediate. From the UK DF patient's pus specimens however the *Bacteroides* species showed sensitivity to imipenem, levofloxacin, linezolid, piperacillin/tazobactam and metronidazole (all MIC of less than 2 µg/L).

Table 2.20 Comparative *in vitro* activities of antimicrobial agents against *Veillonella* species from the pus and tissue specimens from the Indian and pus samples from the UK diabetic foot patients

All the 3 *Veillonella* species from the Indian DF patient's showed resistance to all the tested antibiotics but within the UK DF patient's samples *Veillonella* showed good sensitivity to levofloxacin, linezolid, piperacillin/tazobactam and metronidazole (all with the MIC of less than 2 µg/L).

Organism	Country	Antibiotics	MIC distribution µg/L		
			0.016 – 4	**8 – 64**	**128 - 256**
Veillonella species (n = 3)	India	Amikacin	1	1	1
		Ciprofloxacin	0	0	3
		Clindamycin	0	1	2
		Linezolid	0	1	2
		Levofloxacin	0	1	2
		Piperacillin/ Tazobactum	0	1	2
		Metronidazole	0	2	1
Veillonella species (n =1)	UK	Amikacin	0	0	1
		Amoxicillin/ Clavulanic acid	1	0	0
		Clindamycin	0	0	1
		Erythromycin	0	0	1
		Imipenem	0	1	0
		Levofloxacin	1	0	0
		Linezolid	1	0	0
		Piperacillin/ Tazobactam	1	0	0
		Metronidazole	1		

Table 2.21 *In vitro* activities of antimicrobial agents against *Peptostreptococcus* species from the pus and tissue specimens from the Indian diabetic foot patients

Among the *Peptostreptococcus* species from the Indian DF patient's clinical specimen's piperacillin/tazobactam had shown a MIC of less than 2 µg/L. This suggests that the strains were sensitive to the combination antibiotic.

Organisms	Antibiotics	MIC distribution µg/L		
		0.016 – 4	8 – 64	128 - 256
Peptostreptococcus species (n = 3)	Amikacin	0	2	1
	Ciprofloxacin	0	1	2
	Clindamycin	0	2	1
	Linezolid	1	0	2
	Levofloxacin	0	0	3
	Piperacillin/ Tazobactam	3	0	0
	Metronidazole	0	0	3

The *Peptostreptoccocus* species tested mainly included *Peptostreptococcus assacharolyticus, Anaerococcus prevottii* and *Parvimonas micra.*

Table 2.22 *In vitro* activities of antimicrobial agents against other Gram – positive anaerobes from the pus and tissue specimens from the Indian diabetic foot patients

Among the other Gram – positive anaerobes more than half showed a MIC range of 0.016 to 4 µg/L for linezolid and levofloxacin, but absolute sensitivity is dependent on the breakpoints suggested by NCCLS.

Organisms	Antibiotics	MIC µg/L		
		0.016 – 4	8 – 64	128 - 256
Other Gram - positive anaerobic species (n = 6)	Amikacin	0	6	0
	Ciprofloxacin	0	4	2
	Clindamycin	1	2	3
	Linezolid	3	1	2
	Levofloxacin	3	0	3
	Piperacillin/ Tazobactam	1	2	3
	Metronidazole	1	1	4

The other Gram – positive anaerobic species tested included the *Eubacterium lentum* (n = 3), *Actinomyces naeslundii* (n = 2) and *Propionibacterium acnes.*

Table 2.23 *In vitro* activities of antimicrobial agents against other Gram – negative anaerobes from the pus and tissue specimens from the Indian diabetic foot patients

Among the other tested Gram – negative anaerobes from the DF patients from India there were some strains showing resistance.

Organisms	Antibiotics	MIC distribution µg/L		
		0.016 – 4	8 – 64	128 - 256
Other Gram – negative anaerobes (n = 3)	Amikacin	0	1	2
	Ciprofloxacin	1	1	1
	Clindamycin	0	1	2
	Linezolid	0	0	3
	Levofloxacin	0	0	3
	Piperacillin/ Tazobactam	0	2	1
	Metrinidazole	0	2	1

The other Gram – negative anaerobic species tested included the *Fusobacterium varium, Fusobacterium necrogenes* and *Capnocytophaga.*

The antimicrobial susceptibility testing has indentified the presence of sensitive aerobes and multi-drug resistant anaerobes from the Indian DF patient's pus and tissue specimens and illustrated that there is good antibiotic susceptibility of aerobes and anaerobes from the UK DF patient's pus specimens. This study cannot be used for a strict comparison between the Indian and the UK DF patient's susceptibility testing due to the unequal sample size and low numbers of organisms isolated.

2.4 Discussion

The prevalence of diabetes is increasing in India faster than in any other country in the world. There are about 33 million diabetics mainly from the urban population (Viswanathan, 2005). Diabetes affects many organs of the body but our study was carried out only on the diabetic foot infections of affected patients in India. Ischaemic heart diseases and renal failure were very common in this cohort. This study was carried out on 89 diabetic foot patients of whom 60 had undergone amputation either of toe/toes. Although various information was recorded, statistically the numbers were not significant hence they cannot be predictive of the role of the risk factors to amputations among the Indian DF patients. Some of the most commonly seen causes of the formation of an ulcer seen in other studies (Viswanathan, 2005) were peripheral vascular disease and neuropathy, these were also seen in our study. Ischaemia and trauma are very common among the Indian DF patients as also reported by other studies (Kandemir *et al*., 2007; Bansal *et al*., 2008, Viswanathan, 2002 and Shankar *et al.,* 2005). These risk factors promote the development of diabetic foot ulcers with infections that can spread rapidly and lead to tissue destruction and subsequently amputation (Edmonds and Foster, 2004). In one of the studies carried out by Vijay, (2000) on 374 patients, 52% had infected foot ulcers. Infection thus plays a pivotal role in enhancing diabetic foot towards amputation.

The microbial flora of the normal skin is complex and diverse with a number of bacteria residing such as *Staphylococcus*, *Streptococcus*, *Propionibacterium* and *Corynebacteria*. It is also these primary bacteria that occur in the healthy skin that are also are cultured from the wound. Since the deep tissues are always surrounded by a number of commensal from the skin, recognition of the pathogen remains crucial. In the present study with 27 pus samples and 73 tissues samples from the Indian DF patient's there were total 107 aerobes, 71 anaerobes and 14 fungi cultured. From the 25 pus samples from the DF patient's from the UK there were total 35 aerobes and 5 anaerobes.

In the Indian DF patient's it was found that *Staphylococcus* species predominated in DF ulcers in concordance with other studies carried out in

India (Bansal *et al*., 2008 and Viswanathan *et al*., 2002). On sub-speciation *Staphylococcus schleiferi* was one of the species of *Staphylococcus* cultured from Indian diabetic foot patient's specimens. This organism is normally cultured from immunocompromised individuals (Kumar *et al*., 2007). Among the Gram – negative aerobes the most commonly encountered were the species belonging to the Enterobacteriaceae family such as *P. aeruginosa, K. pneumoniae* and *E. coli* in agreement with the other studies carried out in India (Gadepalli *et al.,* 2006 and Varaiya *et al.,* 2008). In the UK the common aerobes found included some similar to the ones from Indian DF specimens with *Serratia*, *Enterobacter* and *Citrobacter* species more commonly seen among the diabetic foot patients from the UK (Edmonds and Foster, 2004). *Candida tropicalis (51%)* was one of the most frequently isolated fungi from the DF patient's in India, these organisms were also found to be dominant in other studies carried out in India (Chincholikar and Pal, 2002 and Bansal *et al.,* 2008). Among the Indian DF patient's clinical specimens tissue and pus there were equal numbers of Gram – positive and Gram – negative aerobes cultured in disagreement with other studies where Gram – negative aerobes were dominantly cultured from the DF patient's specimens (Bansal *et al*., 2008; Shankar *et al*., 2005).

Most of the anaerobes cultured from the specimens from the Indian DF in our study were *Clostridium* species in agreement with the study carried out by Viswanathan, (2002). The culturing of high numbers of *Clostridium* species is not very surprising from the DF patient's clinical specimens from India as barefoot walking due to religious beliefs, the age of the patients, personal hygiene, outdoor occupation (e.g.farmers) all can favour the multiplication of the *Clostridium* species in the traumatised foot (Viswanathan, 2007; Jayasinghe *et al.,* 2007 and Pradeepa, Deepa and Mohan, 2002). Among the Gram – negative species the dominant species included the *Bacteroides fragilis* (n = 18) out of the total 25 Gram – negative anaerobes cultured from the tissues and pus specimens from Indian DF patients. *Bacteroides fragilis* was also one of the dominant species found in many other studies carried out in India (Chincholikar and Pal, 2002; Shankar *et al*., 2005 and Viswanathan *et al.,* 2002). *Bacteroides fragilis* has been reported to be one of the common Gram – negative anaerobe from DFI (De and Gogate, 2001 and Dogan and Baysal, 2010).

All of the ulcers were classified according to their Wagners grades and as the Wagner's grades increased subsequently the number of anaerobes also increased. This characteristic was also seen by Bansal and colleagues (2008) for aerobes although due to lack of resources they did not carry out testing for anaerobes. One of the peculiar characteristics of diabetic foot is the polymicrobial nature which was clear from this study in both the Indian and the UK DF cohorts as mixed infections were very common. In the UK there were comparatively less (n = 6 out of 25) mixed infected pus as compared to the Indian DF patient's with (n = 37 out of 73) mixed infected tissues and (n = 16 out of 27) mixed infected pus. The polymicrobial nature of DFI is raised in a number of studies all over the world (Citron *et al*., 2007, Bansal *et al*., 2008, Viswanthan, 2005, Gadepalli *et al.,* 2006 and Esposito *et al*., 2008). Nevertheless there were differences in the infected flora from the Indian DF patient's tissues and pus specimens and the DF patient's pus samples from the UK. It is evident from this study that, the tissue biopsies obtained from the Indian diabetic foot patients harboured a greater variety of aerobes, anaerobes and fungi than the pus. The main reasons for this would have been that the tissues sent for culturing often were obtained from the patients with gangrenous wounds or from an amputed toe with necrosis and although pus can favour the growth of number of organisms, with strict anaerobes exposure to air can cause cell death**.**

The *in vitro* antibiotic susceptibility testing carried out on the aerobes from India and the UK showed many aerobes that were sensitive to all the tested antibiotics such as amikacin, amoxicillin/clavulanic acid, imipenem, levofloxacin, linezolid, metronidazole and piperacillin/tazobactam but with high resistance seen among the anaerobes. Arguably it could imply that the aerobes when tested *in vitro* could show sensitivity but when present in a mixed culture could interact with other organisms and acquire resistance (Zayed *et al.,* 2009). There could also be a possibility that *in vivo* the antibiotics could have killed the aerobes with few resistant strains which would have survived and formed a layer in the wound environment thus hindering the entry of antibiotics prescribed to kill the anaerobes leading to multi-drug resistant anaerobes *in vitro*. The results of multi-drug resistant anaerobes seen in our study carried out at Raheja hospital in Mumbai, India were as predicted. One other study carried out in the same hospital in 2001

had documented multi-drug resistant organisms suggesting the use of antibiotic combination therapy in the management of patients with diabetic foot infections (Pathare and Sathe, 2001). Although anaerobes show susceptibility to metronidazole one of the studies carried out in the UK suggested reduced susceptibility of *C. difficile* isolates (n = 86) to metronidazole (24.4%) dissimilar to our study carried out in India where (28.57%) of *Clostridium* species showed resistance to metronidazole (Baines, O' Connor and Freeman, 2008). However higher resistance was seen among the *Clostridium* species from the Indian DFI in our study to Piperacillin/tazobactam. A study carried out by Esposito and his colleagues (2008) suggested that on administering combination antibiotics such as Piperacillin/tazobactam 93.4% of patients were either cured or there was improvement seen among them. In our study it was evident that amputation of the toe/toes among the total 60 out of the 89 DF patients included in the study would have occurred due to treatment failure as our results from the antimicrobial susceptibility results imply.

One of the major problems of carrying out antimicrobial susceptibility of anaerobes has been the non-compliance of standard procedures employed by the hospitals all over the world. A number of surveys carried out in the UK suggested that it was unclear to what extent the susceptibility testing of anaerobes was undertaken in the UK and what procedures were used for testing. Only 5% of laboratories used media specially designed for testing anaerobic organisms. Ninety-nine per cent of laboratories used a disc testing method and 15% used a combination of disc testing and a gradient method (E-test) of determining an MIC. The antibiotics most often tested, other than metronidazole, were penicillin, clindamycin, erythromycin and amoxicillin/clavulanic acid. Very few of the laboratories tested carbapenems (usually imipenem) and piperacillin/tazobactam, respectively (Andrew and Wise, 2002 and Smith *et al.,* 2010). In the developing world however there is a lack of investment, lack of access (non affordable) to drugs, lack of regulatory control, lack of quality standards for test evaluations and all these have an active role in the development of antibiotic resistance.

From the patterns of antimicrobial susceptibility to the tested antibiotics in India, a combination therapy can be used with the advantage of covering the gaps in empirical therapy but this could result in misuse and

inappropriate reuse of the antibiotics. Antibiotic failure can be due to wrong diagnosis, resistant pathogens, suboptimal dosing, an immuno - compromised host, delayed therapy, un-drained infection, presence of foreign body and medication non-compliance. There were limitations to our study mainly that the Indian diabetic foot study was only funded for 3 months and therefore the number of patients recruited in the study was too low and hence no statistical analysis could be carried out. Due to the number of patients recruited in the Indian study, there were low numbers of clinical specimens and subsequently of organisms belonging to the same genus therefore antibiotic susceptibility testing could not be statistically predictive with low numbers of organisms belonging to the same genus. The unmatched numbers samples that were microbiologically processed for the Indian and the UK DF cohorts did not allow a statistically meaningful comparison. However the qualitative information regarding the microbial ecology of the ulcer and the general antibiotic susceptibility patterns in India and UK were facilitated by our study. It would have been advantageous to carry out β-lactamases production testing but due to time constraints and resources, this was not carried out.

From the data obtained from this study on Indian diabetic foot patients, the foot problems such as ulceration, infection, gangrene, and amputations were common resulting in frequent and long-term admission to hospital. This is in agreement with the study carried out by Lavery *et al*. (2006) who concluded that for those patients who developed foot infections the risk of hospitalisation with 55.7 times greater than those who did not develop foot infections and the risk of amputation were 154.5 times higher. The economic and emotional consequences for the family of the patient can be significant (Aleem, 2003). One of the studies carried out in the southern parts of India found that patients without foot problems spent 9.3% of their total income, while patients with foot problems had spent 32.3% of their total income on treatment (Shobhana, 2000).

2.5 Conclusion

Although this is a pilot study it has highlighted the complexity of leg ulcers harbouring wide varieties of aerobes, anaerobes and fungi. The outcome of this study has been that it has facilitated routine culturing of anaerobes in Raheja hospital, Mumbai – India, where previously culturing of anaerobes from clinical specimens was not carried out and therefore none of the treatment options available to the patients were based on the nature of the organisms. Only broad spectrum antibiotics were only given; this has now been changed after the information provided by this study on the multi-drug resistant anaerobes.

CHAPTER – 3

GLYCAN-LECTIN INTERACTIONS

This chapter sets out the potential for glycan-lectin interactions in the rapid typing and identification of *Staphylococcus* species and anaerobes. Method development of the ELLA is detailed for whole cells, proteinase K treated cells and glycolipids for clinically significant organisms.

Study aims:
This study aimed to develop a rapid, easy and cost-effective typing and diagnostic method for clinically significant *Staphylococci* and anaerobes using biotinylated lectins in an adapted ELISA methodology (Afrough *et al.,* 2007).

3.1 Introduction

3.1.1 Gram - positive and Gram - negative cell walls

Bacteria possess cell walls which differ in Gram - positive and Gram - negative organisms with a thick and thin peptidoglycan layer respectively. The cell wall contains components that are unique to each bacterium with the peptidoglycan composed of alternating N-acetylglucosamine acid (NAGA) and N-acetylmuramic acid (NAMA) (Dziarski, 1991). This peptidoglycan layer protects the bacteria against protease enzymes and also from hydrolytic degradation by other organisms. The lipopolysaccharides have been shown to be medically important and are found in abundance in the Gram - negative outer membrane; the Gram - positive cell wall does not express lipopolysaccharide but expresses a relatively thick peptidoglycan layer.

3.1.2 Lectins as tools in glycoconjugate analysis

Lectins are ubiquitous in nature; they are the carbohydrate-binding proteins that each specifically recognise sugar structures and, in mammals mediate a variety of biological processes such as cell-cell and host-pathogen interactions, serum-glycoprotein turnover and innate immune responses (Drickamer, 1994; Powel and Varki, 1995). Lectins were first identified because of their property of agglutinating erythrocytes (Matsui *et al.*, 2001). In plants, lectins are widely distributed and hence are ingested daily in appreciable amounts by both humans and animals (Gallagher, 1984). Most lectins exist in the oligomeric form; as a result they bind to their ligands on several different cells and so agglutinate them. Various plant lectins that have been commercialised, for example *Dolichos biflorus* (anti-blood group A_1) (Falk *et al.*, 1991) and *Ulex europaeus* (anti-blood group H) (Matsui *et al.*, 2001) Mammals also possess lectins termed C-, S-, P- and I- type based on the structure of their carbohydrate–recognition domain (CRD) (Drickamer,1994; Powell and Varki ,1995)**,** while in some of the fungi there are carbohydrate binding adhesins which act as lectins (Ni. *and Tizard,* 1996). The CRD mediates the binding of lectins to the carbohydrates and is highly conserved for each lectin type. Bacterial lectins occur on fimbriae/pili, filamentous appendages that protrude from the surface of the cells and flagella, Lectins are valuable reagents for the clinical diagnostic laboratory because of their specificity and commercial availability. Lectins bind to defined glycan moieties in polysaccharides (Silfkin and Doyle, 1990).

3.1.3 Glycan-lectin interactions: scope for diagnostics

It is important for microbiological laboratories throughout the world to be able to identify pathogens efficiently and easily. In developing countries due to lack of resources they have relied on traditional culture characteristics. In developed countries there are many other ways to identify the pathogens such as typing with phage panel (Watson, 2003), the use of techniques such as PCR amplification (Kobayashi, 2006), mass spectrometry (Lay, 2001), MALDI-MS (Fenselau and Demirev, 2001), biotyping, serotyping, bacteriocin typing, protein typing, restriction endonuclease typing, gene probe typing, dot-blot (Stoitsova, Ivanova and Dimova, 2004) and ELISA. All require expensive reagents and have been employed with the aim of typing

clinically significant micro-organisms (Merlino *et al.*, 2003)**.** Although there is a range of methods for identifying pathogens it is important to realise the practicalities of their use in routine situations in hospitals. Typing is significant as it may inform the epidemiological investigations at different levels ranging from local to regional to national population data which can assist in the control of infection, identify the carriers and establish the prevalence of individual strains. Common reasons for microbial typing are to identify the common sources of infection, to discriminate between mixed strain infections and distinguish relapse or re-infection. Lectins discussed in section 3.1.2 have been explored diagnostically for typing clinically significant organisms (Munoz *et al.,* 2003; Munoz *et al*., 1999 and Annuk *et al*., 2001). An enzyme linked assay using biotinylated lectins termed as ELLA (enzyme linked lectin sorbent assay) is one of the easiest and cost – efficient methods which can be used for typing and identification of clinically significant microbes.

Anaerobes are the most over-looked pathogens due to difficult isolation procedures, requiring competent staff and traditionally used laborious culturing and expensive biochemical identification. Species within the genus are often hard to differentiate because they tend to be largely negative in traditional biochemical tests. Classification of anaerobes has long been regarded as unsatisfactory and until today is very difficult and constantly changing with addition of new species. Following genomic investigations, species have been renamed for example *Peptostreptoccocus magnus* has been renamed *Finegoldia magna, Peptostreptococcus prevottii to Anaerococcus prevottii* and *Peptostreptococcus micros* is now known as *Parvimonas micra* (Song *et al.,* 2007a and 2007b).

Identification has relied on characterisation of volatile fatty acids using gas liquid chromatography which involved lengthy procedures. In laboratories where time is the key factor and rapid identification is crucial for doctors to prescribe antibiotics it remains a major issue that needs to be addressed (Murdoch and Mitchelmore, 1991). Traditional antibiotic resistance patterns, once adopted as a method to type some anaerobes up to their species level, need to be re-considered especially with the emerging antibiotic resistance among anaerobes. In contrast the current molecular methods utilised in identification of microbes to their species and genus level can be

useful in a research setting but not in clinical laboratories with limited resources (Nagy *et al.,* 2006). More significantly, an initial rapid identification of the presence of anaerobes can guide the clinicians to prescribe antimicrobial therapy or surgical interventions. There have been many other rapid and reproducible methods of identification of aerobes described, for example the pre-formed enzyme identification kits that generate profile numbers that are interpreted using computers available from several manufacturers (Bascomb and Manafi, 1998; Huletsky *et al.,* 2005 and Horstkotte *et al.,* 2001) and the use of chromogenic media recently designed for the rapid identification of MRSA strains (Louie, *et al.,* 2006). Although these kits are reliable, rapid and reproducible they are often very expensive and limited to the identification of strains from their databases. There is an urgency to introduce such methods to identify and discriminate pathogens such as methicillin resistant *Staphylococcus aureus,* methicillin sensitive *Staphylococcus epidermidis, and* methicillin resistant *Staphylococcus epidermidis* as well as in the identification of the clinically significant anaerobes. These pathogens have been isolated repeatedly from diabetic foot ulcers and there is interaction between them leading to the formation of biofilms (Dowd *et al.,* 2008).

3.2 Materials and Methods

3.2.1 Culturing and sub culturing of microbes

Initial research was carried out on the ATCC (American Type Culture Collection), NCTC (National Collection of Type Cultures) and AC strains (clinical strains obtained from UCLH) as shown in table 3.1. The strains obtained were checked for their purity using commercially available kits.

Table 3.1: ATCC/NCTC strains and growth requirement

Type of strains	Isolation agar and Culture media	Incubation period
Methicillin sensitive *Staphylococcus aureus* (NCTC 6571)	5% defibrinated sheep blood agar cultured in Tryptone soy broth	Incubated at 37ºC overnight aerobically
Methicillin resistant *Staphylococcus aureus* (NCTC 33591)	5% defibrinated sheep blood agar cultured in Tryptone soy broth	Incubated at 37ºC overnight aerobically
Methicillin sensitive *Staphylococcus epidermidis* (AC strain)	5% defibrinated sheep blood agar cultured in Tryptone soy broth	Incubated at 37ºC overnight aerobically
Methicillin resistant *Staphylococcus epidermidis* (AC strain)	5% defibrinated sheep blood agar cultured in Tryptone soy broth	Incubated at 37ºC overnight aerobically
Streptococcus pyogenes (AC strain)	5% defibrinated sheep blood agar cultured in Tryptone soy broth	Incubated at 37ºC overnight aerobically
Other aerobes from Enterobacteriaceae family (AC strains)	Mac Conkey's agar in LB broth	Incubated at 37ºC overnight aerobically
Finegoldia magna (ATCC 29328)	5% sheep blood agar cultured in brain heart infusion broth with 0.01% tween 80	Incubated at 37ºC for 48h anaerobically
Other anaerobes (AC strains)	5% sheep blood agar cultured in brain heart infusion broth with 0.01% tween 80	Incubated at 37ºC for 48h anaerobically

Table 3.1 shows all the organisms used in this work with their growth requirements. All media was obtained from Oxoid (Basingstoke, UK).

MRSA, MSSA, MRSE and MSSE were analysed for their resistance or sensitivity by molecular methods for the presence or absence of the *mec A* gene and by biochemical tests for coagulase activity at UCLH.

3.2.2 Biotinylated lectins

The biotinylated lectins used in Table 3.2 were selected to cover a wide range of sugar specificities. All the lectins were purchased from Vector labs UK in the form of Lectin kits I, II and III. These were provided as 1 mg powders and dissolved as per instructions in recommended solvents. The final concentration used in the assay was 0.6 µg/ml.

Table 3.2: Panel of 22 biotinylated lectins, its origin and glycans specificity

LECTIN	GENERAL GLYCAN SPECIFICITY	ORIGIN
Concanavalin A (CON A)	α- mannose. α-glucose	Jack bean
Pisum Sativum agglutinin (PS)	α –mannose	Garden pea
Lens culinaris agglutinin (LC)	α-mannose with core α-fucose. α-glucose	Lentil seeds
Phaseolus vulgaris erythroagglutinin (PVE)	complex structures of mannose.	Red kidney bean
Phaseolus vulgaris Leucoagglutinin (PVL)	complex structures of mannosegalβ1,4GcNAcβ1,6 man	Red kidney bean
Ulex europaeus agglutinin (UEA)	α-fucose, fucα1,2gal	Furze seed
Datura stramonium (DS)	β1,4-N-acetylglucosamine oligomersgalβ1,4glcNAcβ1,4 man	Thorn apple
Lycopersicon esculentum (LE)	β-N-acetylglucosamine (GlcNAc)3, GlcNAc 1,4 oligomers, chitobiose, chitotriose	Tomato fruit
Griffonia simplicifolia lectin II (GSII)	Terminal α (β)- N-acetylglucosamine	*Griffonia* seeds
Solanum tuberosum (ST)	β-N-acetylglucosamine, (Glcβ1,4Nac)n, chitotriose	Tomato fruit
Succinylated wheat germ agglutinin (SWGA)	β-N-acetylglucosamine but not sialylated	Wheat germ
Erythrina cristagalli (EC)	β-galactose, galβ1,4glcNAc	*Erythrina cristagalli* seeds
Wheat germ agglutinin *(WGA)*	β-N-acetylglucosamine, sialic acid glcNAcβ1,4glcNAcβ1,4glcNAc, chitotriose	Wheat germ
Jacalin (JAC)	β-galactose ,galβ1,3galNAcα1-Ser/Thr in O-glycans, Terminal α-gal	Jackfruit seeds
Soybean agglutinin (SBA)	α and β N-acetylgalactosamine, galactose (minor), galNAcα1,3gal	Soybean
Peanut agglutinin (PNA)	Galactose, galβ1,3galNAcα1-Ser/Thr	Peanuts
Ricinus communis agglutinin I (RCI)	β-gGalactose, N-acetylgalactosamine	Castor bean
Griffonia simplicifolia lectin I (GS I)	α-galactose, α-N-acetylgalactosamine galα1,3gal	*Griffonia* seeds
Vicia villosa lectin (VV)	α and β terminal N-acetylgalactosamine galNAcα1-O-ser	Hairy vetch seed
Sophora japonica (SJA)	β-acetylgalactosamine, β-galactose	Japanese pagoda tree
Dolichos biflorus (DBA)	Terminal α-N-acetylgalactosamine	Horse gram Seeds
Sambucus nigra (SN)	α – 2,6 sialic acid gal	Elder bark

The sugar specificity of the lectins and their abbreviations has been extracted from (Afrough *et al.,* 2007 and Lis and Sharon1998).

3.2.3 Buffer solutions used in ELLA analysis

The buffers used in the ELLA analysis for whole cells, proteinase K treated whole cells and glycolipids is Phosphate buffered saline (PBS) with the composition of NaCl 5.85 g, Na_2HPO_4 4.72 g, NaH_2PO_4 2.64 g dissolved in 1 litre of distilled water (pH 7.2) and the Tris-HCl buffer with composition of 12.1 g tris base was dissolved in 100 ml of distilled water and pH was adjusted to 8.8 with HCl, Sigma Ltd, UK). For coating the anaerobes on the 96 well ELISA plate carbonate buffer pH 9.5 was used. The composition of carbonate buffer (100mM) was 3.03 g Na2CO3, 6.0 g NaHCO3 in 1 litre of distilled water (pH 9.5). All of the chemicals and buffers were obtained from Acros Organics (USA).

3.2.4 Analysis of glycans on whole cell

Method development was carried out using *Staphylococcus aureus* strains, as the glycan - lectin profile of this organism was already reported (Silfkin and Doyle, 1990).Previous research on microbial glycans has focussed on agglutination assays (Flemming and Wigender, 2001) and the use of confocal microscope or fluorescence microscopy for the detection of glycan-lectin interactions (Strathmann, Wigender and Flemming, 2002, Leriche, Sibille and Carpentier, 2000 and Thomas *et al*., 1997). This research aimed to develop a rapid, easy, reliable, reproducible and cost effective method for the rapid identification of the glycan–lectin interactions. An enzyme linked lectin assay was developed using biotinylated lectins termed as ELLA. Different types of tubes, 96 well Nunc polystyrene plates and tissue culture plates were explored for organism immobilisation. With the aim to develop an easy method a tube-based approach was investigated using simple whole cells. Initial assays were carried out with *Staphylococcus aureus*. Some troubleshooting was required during the method optimisation as discussed in section 3.2.4 a, b and c.

3.2.4 (a) Method developments for ELLA for glycans analysis

In the method development of ELLA there were a series of different factors that were explored as discussed below:

- Concentration at which the cells could adhere optimally to the 96 well plates.
- Volume of culture suspension to be used to coat onto 96 well plates
- Optimum temperature and time for incubation for cells to firmly adhere/coat to the plates.
- Use of appropriate buffer, blocking agents for the assay.
- Blocking time to avoid non-specific binding.
- Coating of colonies directly onto 96 well plates in case of anaerobes.
- Use of formal saline as fixative to avoid cell loss in the case of anaerobes.
- Use of washed cells as well as cells in broth in the case of aerobes and anaerobes.
- Use of freeze dried cells in coating the plates in the case of anaerobes.
- Incubating the above plates aerobically and anaerobically at 37°C and at 4°C.
- Use of higher OD in the case of anaerobes in washed cells.
- Increasing the time of incubation for coating of anaerobes.
- Measuring the OD to determine cell loss before blocking and after each washing during the assay.
- Use of washed anaerobic cells and coating with different buffers at different pH.
- Use of washed anaerobic cells and incubating them aerobically at 37°C.

These troubleshooting and optimisation steps were required for the attachment of the organism to the 96 well ELISA plates as well as for the exposure of the glycans for accurate recording of the glycan-lectin activity. Some of the significant steps in the optimisation and the method used throughout the study are described in detail below.

3.2.4 (b) Optimisation of a tube-based method for glycan analysis of whole cells

Immobilisation of microbes was facilitated by using Zirconium (Sigma) a heavy metal acting as a non soluble support as suggested by Ibrahim *et al*., (1985)**.** *Staphylococcus aureus* of known cell density was washed with distilled water and zirconium chloride was then added to the tube. 0.05 % Tween 20 was used as a detergent to avoid any non specific binding and the tube was filled to the brim. This was then left for 2 h at room temperature and then 60 µl of 0.6 µg/ml of biotinylated lectins were added and allowed to

interact with bacterial glycans for 2 h and then washed with washing buffer phosphate buffer saline (PBS - pH 7.2). Streptavidin alkaline phosphatase (100 µl of dilution recommended by the manufacturer) (Vector laboratories) was added and incubated for 1h at room temperature. The mixture was then washed with PBS (pH 7.2) three times and 100 µl of 1 mg/ml paranitrophenol-phosphate substrate added. The reaction was allowed to proceed for 30 min at room temperature. The reaction was then quenched with 50 µl of 3 M sodium carbonate. Absorbance was measured at 405 nm. All these experiments were carried out with fresh working solutions and with controls.

3.2.4 (c) Optimisation of ELLA method for glycan analysis of whole cells

An ELISA based method had been employed by Strathmann *et al*., (2002) for the detection of exopolysaccharides in biofilms using peroxidase labeled lectins. In their study they used 96 well microtitre plates for the immobilisation of bacterial exopolysaccharides. With modifications to their method the current method has been developed as an in-house rapid method of detection of glycans utilising biotinylated lectins. A panel of 22 biotinylated lectins (0.6 µg/ml) have been used in this assay. A number of different experiments were carried out for the optimisation and for the successful running of the ELLA method. A 96 well polystyrene ELISA plate (Nunc Ltd, Germany) was used in this assay. Method development for this assay was also carried out using *Staphylococcus aureus* which is known to interact with Con A (Silfkin and Doyle, 1990).

Sample organisms of known density (absorbance 0.3-0.5 at 600 nm) were washed with distilled water and in the case of aerobes PBS (pH 7.2) was used as the coating buffer. Wells were coated with 50 µl of sample overnight at 37 ºC and washed with PBS and 0.05 % of Tween 20 was used to prevent non-specific binding for 2 h. Biotinylated lectins, 60 µl of 0.6 µg/ml solution, were added to each well and gently mixed on shaker for 2 h. Washing was carried out to remove any unbound lectins using PBS pH 7.2 and streptavidin alkaline phosphatase (100 µl) was added and incubated at room temperature for 1 h. Washing was carried out 3X with PBS buffer pH 7.2 and 1mg/ml of paranitrophenol-phosphate (100 µl) substrate was added. Plates were kept in dark for 30mins at room temperature and then 50 µl of

3 M sodium carbonate was added to stop the reaction and absorbance was read at 405 nm. Changes were made to the above protocol, for the glycan analysis for anaerobes, in which, 2 % gelatin was used as the blocking buffer and carbonate buffer (10.6 g/l of 0.1M sodium carbonate (anhydrous), 8.4 g/l of 0.1 M sodium bicarbonate mixed in 200 ml of distilled water and the pH was adjusted to 9.5) instead of using the PBS pH 7.2 buffer for coating the anaerobes on the 96 well polystyrene microtitre plate.

3.2.5 Glycan analysis by proteolytic treatment of whole cells

Bacterial cultures were grown and the cell densities were adjusted to 0.3-0.5 at 600 nm with distilled water, these were used for the ELLA with whole cells. For the proteolytic treatment the cells were washed once with PBS (pH 7.2) and centrifuged at 3000 rpm for 10 min. The pellet was re suspended in PBS (pH 7.2) and then heated at 100 ºC for 15 min to remove the cell-surface proteins (Annuk *et al*., 2001). After washing with PBS (pH 7.2) at 3000 rpm for 10 min, the pellet was treated with 6 µl of Proteinase K (Sigma) (10 mg/ml). The suspension was then incubated for 1h at 60 ºC and then 5 min at 100 ºC to inactivate the Proteinase K. The suspension was then centrifuged at 3000 rpm for 10 min. The pellet was then re-suspended in distilled water to give and OD 0.3 at 600 nm before analysing with biotinylated lectins.

3.2.6 Analysis of glycans of glycolipids

With the aim to obtain a defined glycan profile for different aerobes and anaerobes, glycolipids and lipopolysaccharide from the Gram - positive and Gram -negative organisms were extracted respectively.

3.2.6(a) Glycolipid extraction

Lipopolysaccharide (LPS) remains unique to different bacteria and is a determinant of their pathogenicity (Boneca *et al*., 2000). Research on lipopolysaccharides is vast, but with the limitations of low yields during extraction, they can be difficult to extract (Kay *et al.,* 2006) Thus a lipopolysaccharide extraction kit (Intron Bio Molecular solutions) was purchased that yielded 30 µg of LPS from a 5 ml culture suspension. The company protocol was followed and good yields of glycolipids from Gram – positive organisms and LPS from Gram – negative organisms were extracted in 1h.

LPS extraction methods can be used for the extraction of the peptidoglycan layer from the Gram - positive micro-organisms (Dziarski, 1991 and Wang et al., 2003, De Jonge, *et al.,* 1992) to further confirm that the material obtained was glycolipids; fatty acid composition was determined by mass spectrometry for one organism as shown figure 4.3 in the results of chapter 4.3.4. The carbohydrate and protein content for all the glycolipids and lipopolysaccharide were also determined discussed in chapter 4.3.2 in figure 4.1.

3.2.6b ELLA method for detection of glycans from glycolipids of aerobes and anaerobes

The extracted glycolipids and lipopolysaccharide were dissolved in 10 mM Tris-HCl buffer with pH adjusted to 8.8 with HCl, Sigma Ltd, UK. 50 µl of the dissolved glycolipids and lipopolysaccharide were coated onto 96 well ELISA plates (Nunc, Germany) and incubated for 4 h at 37 ºC. Further assay conditions were adopted from the in-house developed ELLA method described in section 3.2.4c.

The table 3.3 shown below shows the organisms whose glycan-lectin interactions were analysed in this study along with their respective sample nature.

Table 3.3 List of organisms and nature of their Glycan-lectin interactions studied in this study

Organisms	Whole cells	Proteinase K treated whole cells	Glycolipids
Enterobacter faecalis	√		
Escherichia coli (EC)	√		
Methicillin resistant Staphylococcus aureus (MRSA)	√	√	√
Methicillin resistant Staphylococcus epidermidis (MRSE)	√	√	√
Methicillin sensitive Staphylococcus aureus (SA)	√	V	V
Methicillin sensitive Staphylococcus epidermidis (MSSE)	√	√	√
Proteus mirabilis (PM)	√		
Pseudomonas aeruginosa (PA)	√		
Streptococcus pyogenes (SP)	√		
Bacteroides fragilis	√		√
Finegoldia magna	√		√
Fusobacterium nucleatum	√		√
Peptostreptococcus anaerobius	√		√
Peptoniphilus asaccharolyticus	√		√
Parvimonas micra	√		√
Prevotella bivia	√		√
Anaerococcus prevottii	√		√

The glycan – lectin interactions were only carried out on the organisms shown in table 3.3. ELLA was carried out on whole cells, Proteinase K treated whole cells and the extracted glycolipids in case of Gram – positive organisms and LPS in case of Gram – negative organisms.

3.2.7 Inhibition assay

Inhibition assays were carried out to confirm the specificity of two of the biotinylated lectin-glycan interactions. The assay was performed using different concentrations of D (+) mannose and D (+) galactose. The sugars were preincubated in lectin solutions at final concentration of 0 mM, 10 mM, 20 mM, 50 mM, 100 mM, 200 mM, 400 mM and 600 mM. These were incubated for 2 hours before performing the ELLA assay. The inhibition assay was only carried out for the glycolipids of *Finegoldia magna* that were scored greater than (3+) in the original ELLA assay. The lectins used were *Pisum sativum* specific for mannose and *Soya bean agglutinin* specific for galactose.

3.3 Results

3.3.1 Method Optimisation

To optimise the method for the analysis of glycans on microbial cells, a series of different procedures have been explored with the objective to develop a rapid, easy and reliable method. *Staphylococcus aureus* has been shown to have glycans containing glucose and reacts with Con A lectin (Silfkin and Doyle, 1990). Therefore biotinylated Con A was utilized as the lectin of choice with *Staphylococcus aureus* (NCTC 6571) as the microbe for method optimization. There are a number of aspects which had to be optimized one of the most important being the immobilization of microbes onto an adherent surface, buffer systems, blocking buffer and cell density of the organism.

3.3.1A Glycan analysis by tube method

Glycan analysis in tubes was performed on simple whole cells of *Staphylococcus aureus* (NCTC 6571) with Con A. A range of tubes such as eppendorf tubes, polystyrene tubes, 50 ml polycarbonate tubes were used for the assay. Immobilization of organism onto the tubes was difficult and hence, zirconium chloride was used which is a non soluble support to facilitate immobilisation. For aerobes like *Staphylococcus* (NCTC 6571) *aureus* (Figure 3.1) the tube method with zirconium chloride gave promising and consistent results, but for anaerobes immobilization was not facilitated and the cells were not attached. The cells usually washed away during the washing step with PBS. So the tube method would not be a choice for the rapid identification of glycans of anaerobes.

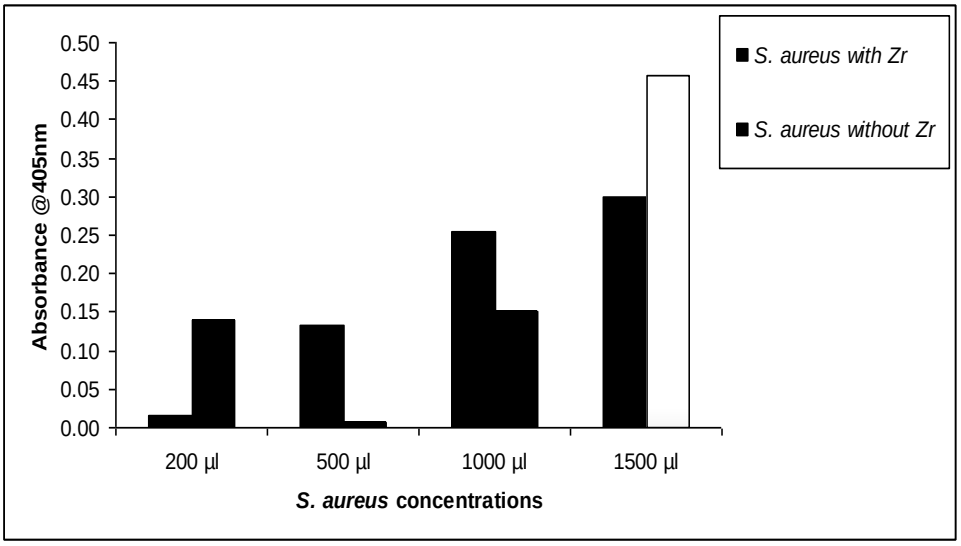


Figure 3.1: Glycan analysis of *Staphylococcus aureus* (*Sa*) (NCTC 6571) whole cells with biotinylated Con A lectin with and without zirconium by tube method.

In the absence of zirconium (Figure 3.1), there was no correlation between signal and organism concentration, whereas a dose dependent relationship was seen in the presence of zirconium. It is concluded from this set of experiment that zirconium chloride is the choice for successful immobilisation of aerobes.

3.3.1B Glycan analysis by ELLA method using 96 well polystyrene microtitre plates

The tube method was not cost-efficient because, for the identification of glycans on the microbial cells, there were a panel of 22 biotinylated lectins to be utilised with different glycan specificity. An ELISA based solid phase lectin assay was developed using the 96 well polystyrene plates. Enzyme linked lectin sorbent assay (ELLA) also worked well for the aerobes but for anaerobes, trouble shooting was required mainly for the immobilisation of anaerobes onto the ELISA plates.

The table 3.4 below represents the optimised buffers to be used and the concentration of microbial cells and type of blocking buffer which would facilitate the immobilisation of organisms on the 96 well polystyrene microtitre plate.

Table 3.4: ELLA method optimisation for aerobes and anaerobes

Microbes used	Washing buffer	Coating buffer	Blocking agent	OD of microbial cell @600nm
Staphylococcus aureus (aerobe)	PBS pH 7.2	PBS pH 7.2	0.5% Tween 20	0.3
Pseudomonas aeruginosa (aerobe)	PBS pH 7.2	PBS pH 7.2	0.5% Tween 20	0.3
Finegoldia magna (anaerobe)	PBS pH 7.2	Carbonate buffer pH 9.5	2% Gelatin	0.8

Immobilisation of aerobes and anaerobes as shown in table 3.4 were facilitated due to the buffers with specific pH and blocking the non specific sites on the 96 well polystyrene plate facilitated by the specific buffers shown in table 3.4. All the glycan – lectin interactions data obtained in this study are based on the buffers, blocking agents, pH and cell density shown in table 3.4

From these series of optimisation trials it was concluded that PBS (pH 7.2) was the better buffer to immobilise aerobes for glycan analysis and immobilisation onto 96 well ELISA plate while carbonate buffer (pH 9.5) remained the buffer of choice for the subsequent immobilisation of anaerobes (Table 3.4) in agreement with other studies where carbonate buffer had been employed to adhere LPS and live anaerobes on the ELISA plates (Bull, Matthews and Rhodes, 1986 and Bantroch, Buhler and Lam, 1994). In case of the anaerobes a higher cell density was required than aerobes for successful ELLA.

In all experiments from optimisation of tube method to the fully developed ELLA method for aerobes as well as anaerobes, negative controls used were (no lectin), (no streptavidin) and (no organism) to rule out false-positive results.

To further analyse the effectiveness of glycan - lectin interaction at different phases of bacterial growth and to confirm the results obtained in the previous optimisation trials a growth curve of *Staphylococcus aureus* (NCTC 6571) was constructed and at each phase aliquots were taken and an ELLA was performed with biotinylated lectin Con A (figure 3.2).

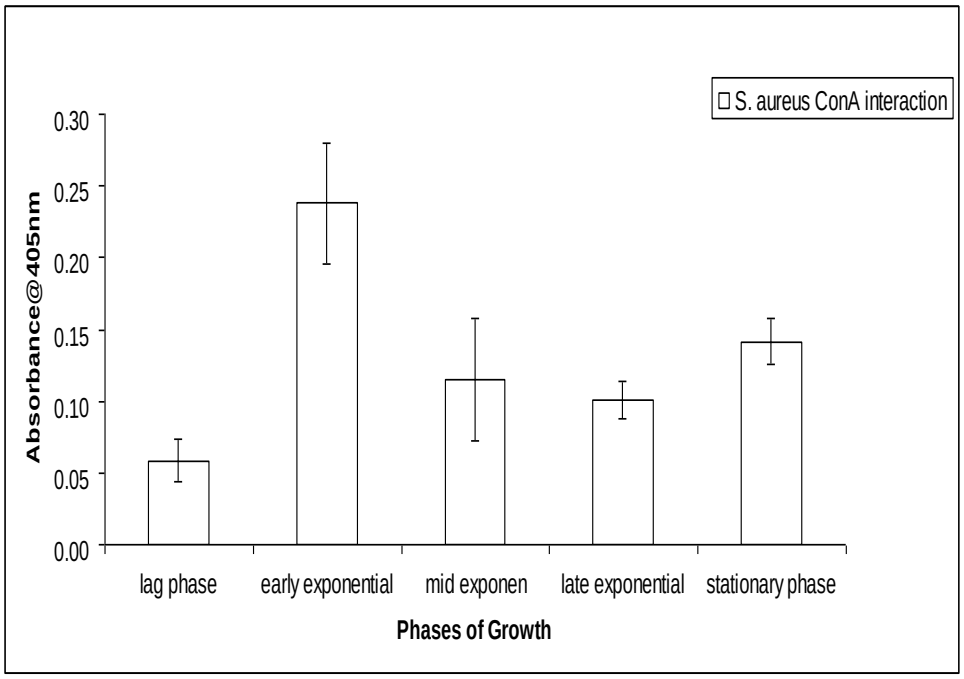


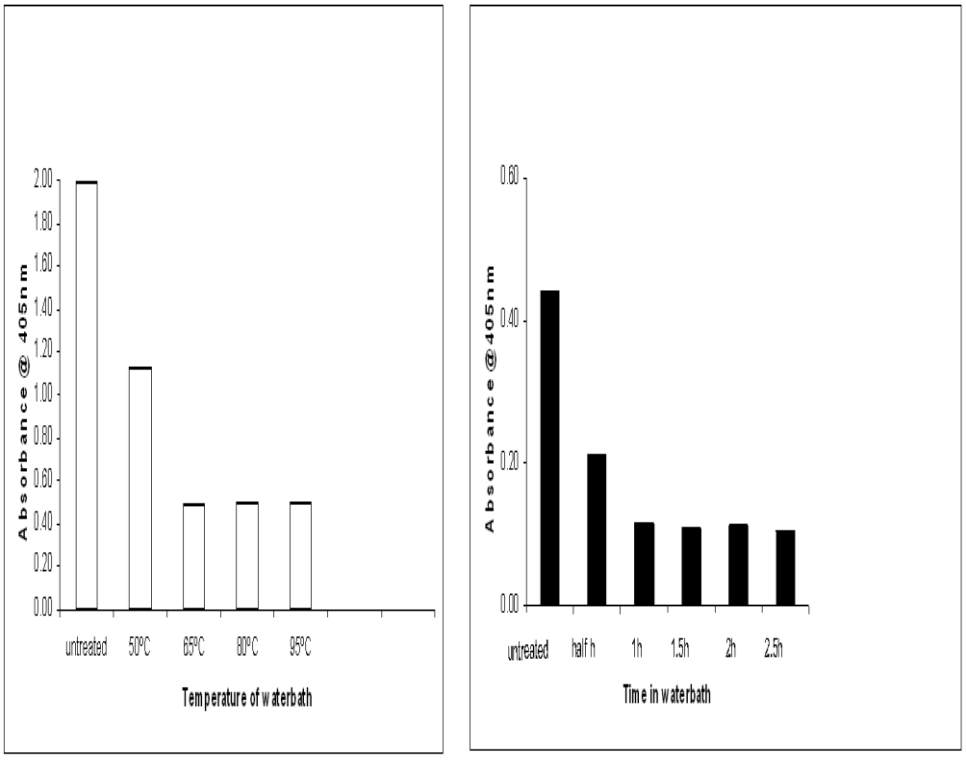
Figure 3.2: Glycans expressed at different phases of bacterial growth detected with Con A

Controls used were no biotinylated lectin, no streptavidin and no organisms and the data represented in this figure is the ELLA method of analysis of glycan – lectin interactions of *Staphylococcus aureus* whole cells with Con A lectin at different phases of growth. All work was carried out in triplicates and the data represented in this figure is after taking away the background.

From this experiment it was confirmed that for aerobes an early exponential phase at approximate OD of 0.3-0.5 at 600 nm was the choice for further ELLA analysis (Figure 3.2).

Many aerobes have been shown to have phosphatase activity which would interfere with the ELLA detection using phosphatase-streptavidin conjugates (Satta *et al*, 1988). A pilot assay carried out on *Staphylococcus aureus* (NCTC 6571) revealed the presence of phosphatase activity which was overcome by denaturation of the enzyme by heating at a series of different temperatures and times (figure 3.4). *MRSA* (NCTC 33591), *Escherichia coli, MRSE, MSSE, Pseudomonas aeruginosa, Enterococcus faecalis, Proteus mirabilis, Streptococcus group B and Streptococcus pyogenes* were all

checked for the phosphatase activity prior to being tested with biotinylated lectins (Data not shown).



S. aureus whole cells exposure at different temperatures *S. aureus* whole cells exposure at different time

Figure 3.3 Denaturation time and temperature: Phosphatase activity of *Staphylococcus aureus* (NCTC 6571).

The figure 3.3 represents the ELLA analysis of *S. aureus* with Con A after series of temperature treatments and different exposure time to denature the phosphatase activity.

The above figure 3.3 suggests that denaturation of the phosphatase activity could be facilitated by heating the sample up to 50ºC for 1h. For all aerobes denaturation was performed before further ELLA analysis.

The scoring system shown below in table 3.5 was followed to record the glycan-lectin activity for aerobes and anaerobes.

Table 3.5: Levels of glycan-lectin binding

Absorbance @405nm	Level of activity score
0-0.05	+/-
0.05-0.1	+
0.1-0.2	++
0.2-0.5	+++
0.5 and above	++++

Table 3.5 represents the scoring system which would be followed to record the glycan-lectin activity for all the aerobes and anaerobes listed in table 3.1. These scores represent the glycan – lectin activity identified after carrying out ELLA analysis and taken away the background from controls such as no lectin, no streptavidin and no organisms.

3.3.2 Glycan analysis by ELLA of whole cells of aerobes and anaerobes:

During the initial optimisation the glycan lectin interactions of the whole cells of a range of aerobes and anaerobes were also carried out to determine whether there are any lectins whose binding were unique to particular organisms and if those lectins could be explored diagnostically.

The table 3.6A shows the glycan-lectin interactions of whole cells of some aerobes growing in diabetic foot ulcers (raw data shown in the appendix VI to X).

Table 3.6A: Glycan-lectin interactions of whole cells of some aerobes growing in diabetic foot ulcers (raw data shown in the appendices XA to XE)

AEROBES	BIOTINYLATED LECTINS																				
	JAC	ST	SBA	PNA	DB	SJA	RC I	GII	WGA	SWGA	PVE	LC	EC	PS	PVL	VV	LE	GI	UEA	DS	CONA	SN
E. coli whole cells	+/-	+/-	+/-	++	+/-	+/-	+/-	-	+	+/-	+	+/-	-	-	-	+	+/-	+/-	+/-	+	+	+/-
P. aeruginosa whole cells	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+/-	+	-
S. pyogenes whole cells	+	+/-	++	+/-	+	-	+++	-	+	+/-	-	-	+/-	+	+	-	+/-	+/-	+	+	+++	++
E. faecalis whole cells	-	-	-	-	-	-	-	-	+++	+/-	-	-	-	+	+/-	+	+	-	+	+	++	+
P. mirabilis whole cells	-	-	-	-	-	-	-	-	-	+/-	++	+	+	++	+	+	-	-	+	+	++	+/-

Table 3.6A represents the ELLA glycan analysis of whole cells of some of the pre-dominant aerobes in DF ulcers. The scores are based on the scoring system shown in table 3.5 and after taking away the background from the controls no lectin, no streptavidin and no organisms. The unique lectins are scored in green colour while the higher (2 or >2) glycan-lectin binding affinities are shown in blue.

ELLA analysis on whole cells of clinically significant aerobes are presented in table 3.6A. The lectins that were unique to *S. pyogenes* whole cells were *Jacalin*, *Soybean* agglutinin, *Dolicus biflorus*, and *Ricinus communisis I*. Among all the other aerobes whole cells, *P. mirabilis* showed distinctive interactions with *Lens culinaris* and *Erythrina cristagalli*. From the total 22 biotinylated lectins *E. faecalis* showed only *Lycopersicon esculentum* lectin to be uniquely bound. Similarly *P. aeruginosa* showed only Wheat germ agglutinin to be uniquely bound. However there were other lectins with higher binding activity which are shown in blue in table 3.6A. ELLA analysis on whole cells showed many other biotinylated lectins which were bound weakly (shown with symbol +/-). The table 3.6B below shows the lectins that bound to aerobes and the binding glycans found in diabetic foot ulcers.

Table 3.6B: Glycan specificity of whole cells of aerobes in diabetic foot ulcers

Biotinylated lectins	*E. coli*	*P. aeruginosa*	*S. pyogenes*	*E. faecalis*	*P. mirabilis*	GLYCAN SPECIFICITY
Concanavalin A (CON A)	√	√	√	√	√	α- mannose. α-glucose
Pisum Sativum agglutinin (PS)	X	X	√	V	√	α –mannose
Lens culinaris agglutinin (LC)	X	X	X	X	√	α-mannose with core α-fucose. α-glucose
Phaseolus vulgaris erythroagglutinin (PVE)	√	X	X	X	√	complex structures of mannose.
Phaseolus vulgaris Leucoagglutinin (PVL)	X	X	√	X	√	complex structures of mannosegalβ1,4GcNAcβ1,6 man
Ulex europaeus agglutinin (UEA)	X	X	√	√	√	α-fucose, fucα1,2gal
Datura stramonium (DS)	√	X	√	√	√	β1,4-N-acetylglucosamine oligomersgalβ1,4glcNAcβ1,4 man
Lycopersicon esculentum (LE)	X	X	X	√	X	β-N-acetylglucosamine (GlcNAc)3, GlcNAc 1,4 oligomers, chitobiose, chitotriose
Succinylated wheat germ agglutinin (SWGA)	X	√	X	X	X	β-N-acetylglucosamine but not sialylated
Erythrina cristagalli (EC)	X	X	X	X	√	β-galactose, galβ1,4glcNAc
Wheat germ agglutinin *(WGA)*	√	√	X	√	X	β-N-acetylglucosamine, sialic acid glcNAcβ1,4glcNAcβ1,4glcNAc, chitotriose
Jacalin (JAC)	X	X	√	X	X	β-galactose ,galβ1,3galNAcα1-Ser/Thr in O-glycans, Terminal α-gal
Soybean agglutinin (SBA)	X	X	√	X	X	α and β N-acetylgalactosamine, galactose (minor), galNAcα1,3gal
Peanut agglutinin (PNA)	√	X	X	X	X	Galactose, galβ1,3galNAcα1-Ser/Thr
Ricinus communis agglutinin I (RCI)	X	X	√	X	X	β-gGalactose, N-acetylgalactosamine
Vicia villosa lectin (VV)	√	X	X	√	X	α and β terminal N-acetylgalactosamine galNAcα1-O-ser
Dolichos biflorus (DBA)	X	X	√	X	X	Terminal α-N-acetylgalactosamine
Sambucus nigra (SN)	X	X	√	√	X	α – 2,6 sialic acid gal

Microbes*: E. coli – Eshcherichia coli, P. aeruginosa – Pseudomonas aeruginosa, P. mirabilis – Proteus mirabilis, S. pyogenes – Streptococcus pyogenes, E. faecalis – Enterococcus faecalis.*

Table 3.6B shows the presence of α glucose, α and β terminal N-acetylglucosamine in *E. coli* as well as α and β-N-acetylgalactosamine, sialic acid, β1,4-N-acetylglucosamine oligomers and the complex structures of mannose. While *P. mirabilis* expresses β galactose, α and β 1,4 - acetylgalactosamine, α mannose with core α fucose, α glucose and α fucose. *S. pyogenes* possesses mannose structures including the tri and the tetra antennary, N-acetyl glucosamine, α 2, 6 sialic acid along with α and β N-acetylgalactosamine. *E. faecalis* expresses galactose, α-N-acetylgalactosamine, α mannose, α glucose, terminal α-N acetylgalactosamine, GalNAcα1,3 [Fucα1,2]Gal, α-fucose, Fucα1,2Gal, β-N-acetylglucosamine, sialic acid and α – 2,6 sialic acid-linked to galactose. It should be noted that the specificities of these lectins is based largely on work carried out on eukaryotic systems and the specificities should therefore be analysed with some caution.

The table 3.7A shows the glycan-lectin interactions of whole cells of some anaerobes present in diabetic foot ulcers (raw data shown in appendices XIIA, XIIIA, XIVA and XVA).

Table 3.7A: Glycan-lectin interactions of whole cells of some anaerobes present in diabetic foot ulcers (raw data shown in appendices XIIA, XIIIA, XIVA and XVA).

ANAEROBES	BIOTINYLATED LECTINS																				
	JAC	ST	SBA	PNA	DB	SJA	RC I	GII	WGA	SWGA	PVE	LC	EC	PS	PVL	VV	LE	GI	UEA	DS	CONA	SN
P. bivia whole cells	++	+	+/-	+/-	-	+/-	+/-	-	+	+	+/-	-	-	+	-	+/-	+	+/-	+/-	+/-	+/-	+/-
A. prevotti whole cells	+	-	+/-	-	-	+/-	+/-	+/-	-	-	-	+/-	-	-	+/-	-	-	+/-	-	+/-	-	+/-
P. micra whole cells	-	-	-	+/-	-	-	-	-	+++	++	++	++	++	+++	+++	++	-	-	-	-	-	-
P.acne whole cells	-	+/-	+/-	+/-	+/-	+/-	+/-	+	-	-	-	+/-	+	-	+	-	-	+/-	+/-	+/-	+/-	-

Table 3.7A represents the ELLA glycan analysis of whole cells of some of the pre-dominant anaerobes in DF ulcers. The scores are based on the scoring system shown in table 3.5 and after taking away the background from the controls no lectin, no streptavidin and no organisms. The unique lectins are scored in green colour and the higher (2 or >2) glycan-lectin binding affinities are shown in blue.

ELLA analysis on whole cells of clinically significant anaerobes as seen in table 3.7A the lectin binding that was unique to *P. micra* whole cells included *Phaseolus vulgaris esculentum*, *Lens culinaris* and *Vicia villosa* lectins. Among all the other anaerobes *P. bivia* whole cells showed distinctive interactions with *Lycopersicon esculentum* lectin and *Solanum tuberosum* lectin. From the total 22 biotinylated lectins *P. acne* whole cells bound only *Griffonia simplicifolia II* lectin, while *A. prevottii* whole cells bound many biotinylated lectins weakly shown with symbol (+/-). However there were other lectins with higher binding activity which are shown in blue in table 3.7A. The table 3.7B below lists the biotinylated lectins binding to the above anaerobes found in diabetic foot ulcers.

Table 3.7B: Glycan specificity of whole cells of anaerobes in diabetic foot ulcers

Biotinylated lectins	*P. bivia*	*A. prevotti*	*P. micra*	*P. acne*	GLYCAN SPECIFICITY
Pisum Sativum agglutinin (PS)	√	X	√	X	α –mannose
Lens culinaris agglutinin (LC)	X	X	√	X	α-mannose with core α-fucose. α-glucose
Phaseolus vulgaris erythroagglutinin (PVE)	X	X	√	X	complex structures of mannose.
Phaseolus vulgaris Leucoagglutinin (PVL)	X	X	√	√	complex structures of mannosegalβ1,4GcNAcβ1,6 man
Lycopersicon esculentum (LE)	√	X	X	X	β-N-acetylglucosamine (GlcNAc)3, GlcNAc 1,4 oligomers, chitobiose, chitotriose
Succinylated Wheat germ agglutinin (SWGA)	√	X	√	X	β-N-acetylglucosamine but not sialylated
Erythrina cristagalli (EC)	X	X	√	√	β-galactose, galβ1,4glcNAc
Wheat germ agglutinin *(WGA)*	√	X	√	X	β-N-acetylglucosamine, sialic acid glcNAcβ1,4glcNAcβ1,4glcNAc, chitotriose
Jacalin (JAC)	√	√	X	X	β-galactose ,galβ1,3galNAcα1-Ser/Thr in O-glycans, Terminal α-gal
Vicia villosa lectin (VV)	X	X	√	X	α and β terminal N-acetylgalactosamine galNAcα1-O-ser
Solanum tuberosum (ST)	√	X	X	X	β-N-acetylglucosamine, (Glcβ1,4Nac)n, chitotriose
Griffonia simplicifolia II (GII)	X	X	X	√	Terminal α (β)- N-acetylglucosamine

Microbes*: P.bivia – Prevotella bivia, A. prevotti- Anaerococcus prevottii, P. micra – Parvimonas micra, P. acnes – Propionibacterium acnes.*

There were differences in the glycan profiles among the anaerobes tested (Table 3.7B) for example *P. bivia* and *P. acne* express β-galactose ,Galβ1,3GalNAcα1-Ser/Thr in O-glycans, terminal α-galactose and β-N-acetylglucosamine $(GlcNAc)_3$, GlcNAc1,4 oligomers including chitobiose and chitotriose and complex structures of mannose. *Parvimonas micra* expresses β-galactose,

Galβ1,4 GlcNAc, α-mannose in bi and triantennary complex type N glycans with core α-fucose, β-N-acetylglucosamine (not sialylated), and the complex structures of mannose including the tri and tetra antennary. *A. prevotti* however only showed interaction with Jacalin and thus expresses β galactose and terminal α galactose.

3.3.3 Comparative glycan analysis by ELLA of whole cells and Proteinase K treated whole cells of *Staphylococci* species

In order to obtain a homogenous profile of glycan-lectin interactions on whole cells Proteinase K was used to treat the whole cells of *Staphylococci* only. This study was part of method development to determine the glycan – lectin profile shown after the treatment with Proteinase K. The table 3.8A below shows the comparative glycan-lectin interactions of the whole cells and the Proteinase K treated whole cells of *Staphylococci* species (raw data shown in appendix VI A, B to IX A and B).

Table 3.8A: Comparative Glycan-lectin interactions of whole cells and Proteinase K treated whole cells of *Staphylococci* species (Raw data shown in appendices VI A, B to IX A and B).

AEROBES	BIOTINYLATED LECTINS																				
	JAC	ST	SBA	PNA	DB	SJA	RC I	GII	WGA	SWGA	PVE	LC	EC	PS	PVL	VV	LE	GI	UEA	DS	CONA	SN
MSSA whole cells	+	+/-	-	+/-	+	-	-	-	+	-	-	++	-	-	-	-	+	+	-	-	++	+
MSSA Proteinase K treated whole cells	-	-	+/-	-	++	-	-	-	+++	-	++	-	-	-	-	-	+	-	+/-	-	-	+/-
MRSA whole cells	+++ +	+/-	++	++	+/-	+	++	++ +	++++	++++	+/-	+	++	+/-	++	+	++	++	++	+	++	+++
MRSA Proteinase K treated whole cells	+	++	+++	+/-	+/-	-	-	-	+++	+/-	-	-	+/-	+/-	+/-	+/-	+	+/-	+	+/-	+/-	++
MRSE whole cells	+/-	++	+/-	+++	+/-	+/-	-	++ +	+++	+++	+/-	_	_	+	-	+	++ +	+/-	_	_	+/-	+
MRSE Proteinase K treated whole cells	+/-	+++	-	+/-	+/-	-	+/-	+/-	+	++	+	+/-	_	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-	+/-
MSSE whole cells	+/-	-	-	+++ +	+/-	-	-	-	+	+	+/-	+/-	+/-	+/-	+/-	+/-	+	+/-	+/-	+/-	+/-	+
MSSE Proteinase K treated whole cells	+	+	+++	+	++	+/-	+/-	-	+/-	-	-	-	-	-	+/-	+/-	-	-	+/-	+	+/-	-

Table 3.8A represents the comparative ELLA glycan analysis of whole cells and Proteinase K treated whole cells of *Staphylococcus* species The scores are based on the scoring system shown in table 3.5 and after taking away the background from the controls no lectin, no streptavidin and no organisms. The unique lectins are scored in green colour while the higher (2 or >2) glycan-lectin binding affinities are shown in blue.

As seen in table 3.8A the comparative glycan – lectin profiles obtained by ELLA analysis demonstrated that some carbohydrate residues were exposed and thus were available for binding while other residues were destroyed on treatment with Proteinase K and hence there were difference in the lectin binding of the whole cells and Proteinase K treated whole cells of *Staphylococcus* species. The lectins that were uniquely binding to MSSA whole cells were *Jacalin* and *Griffonia simplicifolia I* lectin while MRSA whole cells bound *Sophora japonica* lectin and *Lens culinaris* lectin. *Pisum sativum* was the only lectin that was uniquely bound by MRSE whole cells in comparison to whole cells of all other *Staphylococcus* species as seen in table 3.8A. MSSE on the other hand did not show uniquely bind any lectin but showed weak binding to a number of lectins. There was other stronger glycan - lectin binding seen which is shown in the table 3.8A in blue.

Proteinase K treatment was only carried out on the *Staphylococcus* species. Among the Proteinase K treated cells there were many weaker interactions seen from MSSA, MRSA and MRSE with higher interactions seen with MSSE Proteinase K treated cells. Table 3.8B and table 3.8C illustrate the glycans expressed by the *Staphylococcus* species whole cells and Proteinase K treated cells respectively.

Table 3.8B: Glycan-lectin profiles for the whole cells of *Staphylococcus* species

Biotinylated lectins	MSSA whole cells	MRSA whole cells	MRSE whole cells	MSSE whole cells	GLYCAN SPECIFICITY
Concanavalin A (CON A)	√	√	X	X	α- mannose. α-glucose
Pisum Sativum agglutinin (PS)	X	X	√	X	α –mannose
Lens culinaris agglutinin (LC)	√	√	X	X	α-mannose with core α-fucose. α-glucose
Phaseolus vulgaris Leucoagglutinin (PVL)	X	√	X	X	complex structures of mannosegalβ1,4GcNAcβ1,6 man
Ulex europaeus agglutinin (UEA)	X	√	X	X	α-fucose, fucα1,2gal
Datura stramonium (DS)	X	√	X	X	β1,4-N-acetylglucosamine oligomersgalβ1,4glcNAcβ1,4 man
Lycopersicon esculentum (LE)	X	√	√	√	β-N-acetylglucosamine (GlcNAc)3, GlcNAc 1,4 oligomers, chitobiose, chitotriose
Succinylated wheat germ agglutinin (SWGA)	X	√	√	X	β-N-acetylglucosamine but not sialylated
Erythrina cristagalli (EC)	X	√	X	√	β-galactose, galβ1,4glcNAc
Wheat germ agglutinin *(WGA)*	√	√	√	X	β-N-acetylglucosamine, sialic acid glcNAcβ1,4glcNAcβ1,4glcNAc, chitotriose
Jacalin (JAC)	√	√	X	X	β-galactose ,galβ1,3galNAcα1-Ser/Thr in O-glycans, Terminal α-gal
Soybean agglutinin (SBA)	X	√	X	X	α and β N-acetylgalactosamine, galactose (minor), galNAcα1,3gal
Peanut agglutinin (PNA)	X	X	√	√	Galactose, galβ1,3galNAcα1-Ser/Thr
Ricinus communis agglutinin I (RCI)	X	√	X	X	β-gGalactose, N-acetylgalactosamine
Vicia villosa lectin (VV)	X	√	√	X	α and β terminal N-acetylgalactosamine galNAcα1-O-ser
Dolichos biflorus (DBA)	√	X	X	X	Terminal α-N-acetylgalactosamine
Sambucus nigra (SN)	√	√	√	√	α – 2,6 sialic acid gal
Saphora japonica (SJA)	X	√	X	X	β-acetylgalactosamine, β-galactose
Griffonia simplicifolia I (GI)	√	√	X	X	α-galactose, α-N-acetylgalactosamine galα1,3gal
Griffonia simplicifolia (GII)	X	X	√	X	Terminal α (β)- N-acetylglucosamine
Solanum tuberosum (ST)	X	X	√	X	β-N-acetylglucosamine, (Glcβ1,4Nac)n, chitotriose

MSSA and MRSA whole cells expressed similar glycans as seen in table 3.8B. The glycans expressed were α glucose, α mannose with core α fucose, α and β terminal N-acetylglucosamine with and without sialic acid as well as N-acetylgalactosamine, β1,4-N-acetylglucosamine oligomers, α-2,6 sialic acid linked to galactose and the complex structures of mannose by MSSA and MRSA whole cells. While MRSE whole cells expresses terminal α and β N - acetylglucosamine, α galactose, α mannose and α-2,6 sialic acid. MSSE whole cells express N-acetylglucosamine, α 2, 6 sialic acid along with β galactose. The table 3.8C shows the glycans expressed by Proteinase K treated *Staphylococcus* species.

Table 3.8C Glycan-lectin profiles for the Proteinase K treated *Staphylococcus* species

Biotinylated Lectins	MSSA Proteinase K treated cells	MRSA Proteinase K treated cells	MRSE Proteinase K treated cells	MSSE Proteinase K treated cells	GLYCAN SPECIFICITY
Phaseolus vulgaris erythroagglutinin (PVE)	√	X	X	X	complex structures of mannose.
Ulex europaeus agglutinin (UEA)	X	√	X	X	α-fucose, fucα1,2gal
Datura stramonium (DS)	X	X	X	√	β1,4-NAcetylglucosamine oligomersgalβ1,4glcNA β1,4 man
Lycopersicon esculentum (LE)	√	√	√	X	β-N-acetylglucosamine (GlcNAc)3, GlcNAc 1,4 oligomers, chitobiose, chitotriose
Succinylated wheat germ agglutinin (SWGA)	X	X	√	X	β-N-acetylglucosamine but not sialylated
Wheat germ agglutinin *(WGA)*	√	√	√	X	β-N-acetylglucosamine, sialic acid glcNAcβ1,4glcNAcβ1,4glNAc, chitotriose
Jacalin (JAC)	X	√	X	X	β-galactose galβ1,3galNAcα1 Ser/Thr in O-glycans, Terminal α-gal
Soybean agglutinin (SBA)	X	√	X	√	α and β N acetylgalactosamine, galactose (minor), galNAcα1,3gal
Peanut agglutinin (PNA)	X	X	X	√	Galactose, galβ1,3galNAcα1-Ser/Thr
Dolichos biflorus (DBA)	√	X	X	V	Terminal α-N Acetylgalactosamine
Sambucus nigra (SN)	X	√	X	X	α – 2,6 sialic acid gal
Solanum tuberosum (ST)	X	√	√	√	β-N-acetylglucosamine, (Glcβ1,4Nac)n, Chitotriose

From the table 3.8C, the Proteinase K treated whole cells showed comparable interactions to those of the whole cells of *Staphylococcus* species in agreement to the study carried out by Annuk *et al.*, 2001 where the proteolytic treatment greatly reduced the auto-agglutination for majority of the species apart from *L. acidophilus* species. All the 4 Proteinase K treated *Staphylococcus* species showed interactions with wheat germ

agglutinin, suggesting the presence of GlcNAc/sialic acid. Proteinase K treated MRSA showed interactions with *Jacalin, Solanum tuberosum, Soy bean agglutinin, Succinylated* wheat germ agglutinin, *Ulex europaeus I agglutinin, Datura stramonium* and *Sambucus nigra* lectin expressing β- N –acetylglucosamine, β- galactose, α and β N – galactosamine and α 2,6 sialic acid linked to galactose along with α fucose and fucose linked α 1,2 galactose. Proteinase K treated cells of MSSA however showed the interactions with *Dolicus biflorus* and *Phaseolus vulgaris erythroagglutinin*. Proteinase K treated MRSE and MSSE showed interactions with *Solanum tuberosum, Soybean agglutinin* and *Peanut agglutinin* while MRSE alone showed interactions with *Griffonia simplicifolia II, Succinylated* wheat germ agglutinin*, Phaseolus vulgaris leucoagglutinin* and *Lycopersicon esculentum* lectin. Proteinase K treated MSSE cells however showed interactions with *Solanium tuberosum*, *Peanut agglutinin*, *Soy bean agglutinin, Dolicus biflorus* and *Datura stramonium* expressing β 1,4 – N acetylglucosamine, α and β N –acetylgalactosamine , galactose and Gal β 1,3 GalNAc.

3.3.4 Glycans-lectin interactions potential in microbial diagnostics

3.3.4.1 In the rapid typing of *Staphylococci* species

An in house developed ELLA method was explored on the extracted glycolipids of some of the clinically significant aerobes and anaerobes pre dominantly found in DFI.

The ELLA method on the glycolipids of *Staphylococcus* species showed the following glycan-lectin profiles (table 3.9 - raw data shown in appendices VIC, VIIC, VIIIC and IX C).

Table 3.9: Glycan-lectin profiles of glycolipids of *Staphylococcus spp.* (Raw data shown in appendices VIC, VIIC, VIIIC and IXC).

Biotinylated Lectins	Glycolipids of *Staphylococcus spp.*			
	MRSA NCTC 33591	MSSA NCTC 6571	MRSE	MSSE
JAC	-	-	-	-
ST	-	-	+/-	-
SBA	+/-	-	-	+/-
PNA	-	-	++++	+/-
DB	+/-	-	-	+/-
SJA	-	-	-	-
RC	+/-	+/-	-	+
GII	+/-	+	-	+/-
WGA	++	++++	++	+/-
SWGA	+/-	+++	++++	-
PVE	+/-	+/-	+	-
LC	-	+	+/-	+/-
EC	+	+/-	+/-	-
PS	+/-	+/-	+/-	-
PVL	+/-	+/-	+/-	-
VV	+	+/-	+/-	-
LE	+/-	+/-	+/-	+/-
GI	+/-	+++	+/-	-
UEA	+/-	+/-	+/-	-
DS	+/-	+/-	+/-	-
CON A	+/-	+/-	+/-	-
SNA	-	+/-	+/-	+/-

Table 3.9 represents the ELLA glycan analysis of the extracted glycolipids of clinically significant *Staphylococcus* species. The scores are based on the scoring system shown in table 3.5 and after taking away the background from the controls no lectin, no streptavidin and no organisms. The unique lectins are scored in green colour while the higher (2 or >2) glycan-lectin binding affinities are shown in blue.

***Lectins**: Jacalin (JAC), Solanum tuberosum (SOL), Soybean agglutinin (SBA), Peanut agglutinin (PNA), Dolichos biflorus (DB), Sophora japonica(SJA), Ricinus communis agglutinin I (RC), Griffonia simplicifolia lectin II (GSII),* Wheat germ agglutinin (WGA), Succinylated wheat germ agglutinin (SWGA), *Phaseolus vulgaris erythro agglutinin (PVE), Lens culinaris agglutinin (LC), Erythrina cristagalli (EC), Pisum Sativum agglutinin (PS), Phaseolus vulgaris leucoagglutinin (PVL), Vicia villosa lectin(VV), Lycopersicon esculentum(LE), Griffonia simplicifolia lectin I (GS I), Ulex europaeus agglutinin (UEA), Datura stramonium (DS),* Concanavalin A (CONA), *Sambucus nigra (SN).*

Microbes: methicillin resistant *Staphylococcus aureus (MRSA),* methicillin sensitive *Staphylococcus aureus (MSSA),* methicillin resistant *Staphylococcus epidermidis (MRSE) and* methicillin sensitive *Staphylococcus epidermidis (MSSE).*

MRSA, MSSA, MRSE and MSSE were confirmed by the presence of *mec A* gene by molecular typing at UCLH.

The lectins that bound specifically to MRSA and MSSA were *Lens culinaris, Erythrina cristagalli, Vicia villosa, Lycopersicon esculentum, Griffonia simplicifolia I* as seen in Table 3.9. The lectins shown to be specific to MRSE and MSSE were *Peanut agglutinin*, *Phaseolus vulgaris erythroagglutinin* and *Ricinus communis I.* MRSA showed activity with *Erythrina cristagalli* and *Vicia villosa* binding to the glycans α and β N-acetylgalactosamine, β-N-acetylglucosamine, GalNAcα1, 3 Gal and β-galactose. While MSSA expressed α-mannose with core α-fucose, α-glucose and α-N-acetylglucosamine. MRSE showed interactions with *Peanut agglutinin* and *Phaseolus vulgaris erythroagglutinin* expressing glycans β galactose with GalNAcα1, 3 Gal and complex structures of mannose. MSSE however showed uniquely interactions only with *Ricinus communis I* expressing β galactose and α and β N-acetylgalactosamine. There were other lectins that were also expressed which are shown in blue in table 3.9 along with the other weaker expressed lectins shown with symbol (+/-).

3.3.4*.2 ELLA* in the rapid identification of anaerobes

ELLA has been employed to identify many aerobes (Munoz *et al.,* 1999 and Annuk *et al*., 2004). The assay carried out in this study has successfully obtained the glycan - lectin profiles for a number of glycolipids from the Gram – positive anaerobes and lipopolysaccharide from the Gram - negative anaerobes pre dominantly found in DFI. The following table (Table 3.10A and 3.10B) shows the glycan-lectin profiles of the extracted lipopolysaccharide and glycolipids of Gram - negative and Gram - positive anaerobes respectively (raw data shown in appendices XIC, XIIB, XIIIB, XIVB, XVB, XVI A,B and C).

3.3.4.2a: Glycan-lectin profiles for Gram - negative anaerobes

Table 3.10A: Glycan-lectin profiles of glycolipids of Gram - negative anaerobes (Raw data shown in Appendices XVIA, B and C).

Biotinylated lectins	Gram negative anaerobes lipopolysaccharides		
	F. nucleatum	*B. fragilis*	*P. bivia*
JAC	-	+++	++
ST	-	+/-	-
SBA	+/-	-	+/-
PNA	+/-	+	++
DB	+/-	+/-	-
SJA	-	-	-
RC	+/-	-	-
GII	+/-	-	++
WGA	++	+/-	++
SWGA	+	+/-	-
PVE	-	+/-	-
LC	+/-	-	-
EC	-	+/-	-
PS	+	-	-
PVL	+	+/-	-
VV	-	++	-
LE	+++	+/-	++
GI	+	+/-	-
UEA	+/-	+/-	+/-
DS	++	++	-
CON A	++	+	+
SNA	++	+/-	-

Table 3.10A represents the ELLA glycan analysis of the extracted glycolipids of some of the clinically significant Gram – negative anaerobes from the DFI. The scores are based on the scoring system shown in table 3.5 and after taking away the background from the controls no lectin, no streptavidin and no organisms. The unique lectins are scored in green colour while the higher (2 or >2) glycan-lectin binding affinities are shown in blue.
***Lectins**: Jacalin (JAC), Solanum tuberosum (SOL), Soybean agglutinin (SBA), Peanut agglutinin (PNA), Dolichos biflorus (DB), Sophora japonica(SJA), Ricinus communis agglutinin I (RC), Griffonia simplicifolia lectin II (GSII),* Wheat germ agglutinin (WGA), Succinylated wheat germ agglutinin (SWGA), *Phaseolus vulgaris erythro-agglutinin (PVE), Lens culinaris agglutinin (LC), Erythrina cristagalli (EC), Pisum Sativum agglutinin (PS), Phaseolus vulgaris leucoagglutinin (PVL), Vicia villosa lectin(VV), Lycopersicon esculentum(LE), Griffonia simplicifolia lectin I (GS I), Ulex europaeus agglutinin (UEA), Datura stramonium (DS),* Concanavalin A (CONA), *Sambucus nigra (SN).*
Microbes: *Fusobacterium nucleatum (F. nucleatum), Bacteroides fragilis (B. fragilis), Prevotella bivia (P. bivia)*

The results (Table 3.10A) suggest that lectins could be further explored diagnostically for the rapid identification of the clinically important Gram - negative anaerobes *F. nucleatum, B. fragilis* and *P. bivia*. The lectins *Succinylated* wheat germ agglutinin, *Pisum sativum, Phaseolus vulgaris leucoagglutinin, Griffonia simplicifolia I* and *Sambucus nigra* all showed interactions with *F. nucleatum* but not with *B. fragilis* and *P. bivia*. However, *P. bivia* showed binding to *Griffonia simplicifolia II*, whereas this lectin has no or less affinity for *B. fragilis* and *F. nucleatum* respectively. Similarly *B. fragilis* showed binding with *Vicia villosa* lectin that had no affinity for *F. nucelatum* and *P. bivia.*

3.3.4.2b: Glycan-lectin profiles of glycolipids of Gram - positive anaerobes

Table 3.10B: Glycan - lectin profiles of glycolipids of Gram - positive anaerobes (Raw data shown in Appendices XIC, XIIB, XIIIB, XIVB, and XVB).

Biotinylated Lectins	Gram positive anaerobes glycolipids					
	F. magna	*Anaercoccus prevotti*	*P. anaerobius*	*Peptnniphilus asaccharolyticus*	*P. acnes*	*Parvimonas micra*
JAC	+	-	+	++	++	-
ST	-	-	-	+/-	+/-	-
SBA	+++	-	+/-	+/-	-	-
PNA	++++	+	+++	++	++	-
DB	+	++	+/-	+/-	++	-
SJA	+	-	-	+/-	++	-
RC I	+++	-	-	+/-	++	-
GII	+	-	++	+/-	++	-
WGA	+	++	+++	++	+++	++++
SWGA	+++	+	+/-	+	+	-
PVE	+	-	-	+/-	++	-
LC	++++	-	+/-	-	-	-
EC	+/-	+/-	+/-	-	+/-	-
PS	++++	+/-	+/-	-	+/-	-
PVL	+++	-	+/-	-	+	-
VV	+/-	+/-	+/-	+/-	+	-
LE	-	+	+	+	++	++
GI	-	+++	++	+++	+/-	-
UEA	-	+/-	+/-	-	+/-	-
DS	+/-	++	+/-	-	+/-	-
CON A	+/-	+	+	++	-	-
SN	+++	++	-	+	+/-	-

Table 3.10b represents the ELLA glycan analysis of the extracted glycolipids of some of the clinically significant *Peptostreptococcus* species most commonly cultured from a DF ulcer. The scores are based on the scoring system shown in table 3.5 and after taking away the background from the controls no lectin, no streptavidin and no organisms. The unique lectins are scored in green colour while the higher (2 or >2) glycan-lectin binding affinities are shown in blue.

Lectins: *Jacalin (JAC), Solanum tuberosum (SOL), Soybean agglutinin (SBA), Peanut agglutinin (PNA), Dolichos biflorus (DB), Sophora japonica (SJA), Ricinus communis agglutinin I (RC), Griffonia simplicifolia lectin II (GSII),* Wheat germ agglutinin (WGA), Succinylated wheat germ agglutinin (SWGA), *Phaseolus vulgaris erythro-agglutinin (PVE), Lens culinaris agglutinin (LC), Erythrina cristagalli (EC), Pisum Sativum agglutinin (PS), Phaseolus vulgaris leucoagglutinin (PVL), Vicia villosa lectin(VV), Lycopersicon esculentum (LE), Griffonia simplicifolia lectin I (GS I), Ulex europaeus agglutinin (UEA), Datura stramonium (DS),* Concanavalin A (CONA), *Sambucus nigra (SN).*

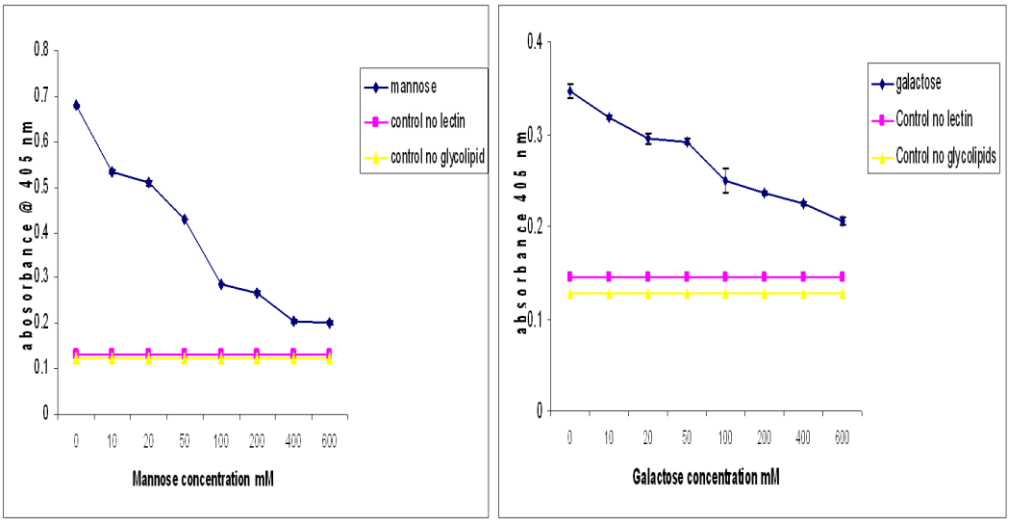
Microbes: *Peptostreptococcus anaerobius (P. anaerobius), Parvimonas micra (Parvi. micra), Finegoldia magna (F.magna), Peptoniphilus asaccharolyticus (Pepton.asaccharolyticus) Anaerococcus prevottii (Anaer.prevottii) and Propionibacter acnes (P.acnes).*

The glycan-lectin activity profiles for clinically significant Gram - positive anaerobes are shown in Table 3.10B. Some of the lectins are either unique in their binding to particular anaerobes or have varied levels of binding that could be explored as the basis of a diagnostic tool. The lectin whose binding was unique to *F. magna* was *Soybean* agglutinin while all the others organisms tested showed no or lesser affinity for this agglutinin. *Parvi. micra* showed no affinity for the lectins *Succinylated wheat germ agglutinin*, *Lens culinaris* and *Pisum sativum* agglutinin while *F. magna* did bind tothem. This could also be used in the diagnostic panel to type *Parvi. micra* and *F. magna* belonging to the *Peptostreptococcus* family*. A. prevottii and Parvi. micra* glycolipids shows no affinity to *Soybean agglutinin*, *Phaseolus vulgaris leucoagglutinin* and *Lens culinaris* while *F. magna* glycolipids does show stronger affinity. *A. prevotti* alone also shows affinity with *Dolicus biflorus* agglutinin. Thus *Dolicus biflorus, Soybean agglutinin, Phaseolus vulgaris leucoagglutinin* and *Lens culinaris* could be employed in the diagnostic panel for typing of the clinically significant *Peptostreptococcus* species. *P. anaerobius* and *Peptoniphilus asaccharolyticus* glycolipids did not show any unique lectin binding. For the typing of these species levels of glycan lectin binding could also be explored. *P. acne* is a Gram - positive anaerobe that belongs to the *Propionibacterium* genus and is a clinically significant skin pathogen. It shows unique interactions with the *Vicia villosa* lectin while all other organisms tested showed no affinity to this lectin. Thus *Vicia villosa* lectin could be included in the diagnostic panel to discriminate *P. acnes* from the other species of the *Peptostreptococcus* family.

3.3.5 Inhibition assay

To further confirm that the biotinylated lectins used in the ELLA glycan analysis for all the aerobes and anaerobes discussed in this study were binding to the glycan on the organisms an inhibition assay was carried out only on one anaerobe *F. magna* glycolipids that were scored greater than (3+) in the original ELLA assay. The lectins used were *Pisum sativum*

specific for mannose and *Soy bean agglutinin* specific for galactose as shown in the figure 3.4.



The effect of monosaccharide mannose pre-incubation with *Pisum sativum* agglutinin on *Finegoldia magna* glycolipids

The effect of the monosaccharide galactose pre-incubation with *soybean agglutinin* lectin on *Finegoldia magna* glycolipids

Figure 3.4. Inhibition of lectin binding with monosaccharides
F. magna glycolipids were coated on to 96 well polystyrene microtitre plate and blocked with 2% Gelatin. 0.6 µg/ml of biotinylated lectin *Pisum sativum* agglutinin and *Soybean agglutinin* were preincubated with their respective monosaccharide mannose and galactose respectively were added to the microtitre plate and ELLA analysis was carried out discussed in section 3.2.6.

Figure 3.4 shows that with the increasing concentrations of monosaccharides mannose and galactose there is a decrease in the binding as measured using ELLA suggesting that the lectins were bound specifically to the glycans from the glycolipids of *Finegoldia magna*. No inhibition was noted for assay performed on wells with no glycolipids and no sugar.

3.4 Discussion

Considering the data obtained by ELLA on whole cells, proteinase K treated whole cells and extracted glycolipids of clinically significant aerobes and anaerobes predominantly found in DFI, an attempt has been made to explore the use of lectin binding as a diagnostic tool. The in house developed ELLA method has been used in this study is user friendly and economical for the analysis of microbial glycans using biotinylated lectins. In the process of development of the ELLA method various troubleshooting and optimisation steps were required such as denaturation conditions, attachment of the organisms to the 96 well Nunclon^{Tm} plates with problems of adhering anaerobes to the plate and loss of cells during washing steps with PBS (discussed in section 3.2.4a) and exploration of the use of different buffers with different pHs (Table 3.3). One of the characteristics shown by aerobes and anaerobes interpreted from table 3.3 is that different buffers at different pH facilitated the attachment of these organisms on the polystyrene microtitre plate. This has been due to the fact that at pH 7.2 phosphate buffered saline was best for their attachment of aerobes on the 96 well microtitre plate. For the attachment of anaerobes however carbonate buffer at pH 9.5 was selected. Sodium carbonate buffer was used as oppose to the calcium carbonate buffer as there have been studies suggesting the interference of calcium with lectin binding. Different buffers along with their respective pH helped to change the charges on the organisms to facilitate secured attachment. Although there have been various ELISA assays using bovine serum albumin as the blocking buffer in this study however tween 20 and gelatin was used to block the non-specific binding for aerobes and anaerobes respectively. Bovine serum albumin itself contained sugars so the use of it as the blocking buffer would incur false-positive interpretation of the glycan-lectin interactions. Many aerobes have been shown to have phosphatase activity which would interfere with the ELLA detection using phosphatase-streptavidin conjugates (Satta *et al*., 1988; Porschen and Spaulding, 1974). *S. aureus* showed phosphatase activity that interfered

during the assay and gave false-positive results. Therefore a denaturation step was carried out by heating at 55ºC for 1 h before performing the ELLA (figure 3.3). Similar denaturation steps were required in case of other aerobes such as methicillin resistant *Staphylococcus aureus*, *Enterobacter* species, *Escherichia coli* and the anaerobes *Parvi. micra*, *Peptinophilus asaccharolyticus* and *B. fragilis***.** During the optimisation whole cells gave a heterogenous glycan-lectin profiles therefore proteinase K treatment was offered to the whole cells with the aim to cleave open the peptidoglycan layer to produce a more homogenous profile. It is also well noted from figure 3.2 that the glycans where expressed in the early exponential phase and that there was decreased activities in the other phases. This could be because of varied specificity the organism used such that with Staphylococcus aureus with Con A biotinylated lectin it could be that in the early exponential phase in the growing state there must be Con A bound to glucose and then to mannose. While in the mid exponential and other phases there must be Con A bound to just glucose then to other sugars and hence the chain did not incorporate mannose therefore showing no activity. Therefore to obtain a consistent and avoid any false-positive interpretation all the cells were picked from the early exponential phase except for glycolipids.

There are number of biomarkers which have been investigated for the rapid identification or typing of micro-organisms. The presence of the biomarker sialidase has been utilised for the identification of anaerobes such as black-pigmented *Bacteroides* and *Capnocytophaga* species by performing a simple spot sialidase test (Moncla *et al.,* 1990). MALDI -TOF mass spectrometry has been coupled with internet-based proteome database search algorithms in an approach for direct micro-organism identification. This approach had been applied to characterize intact *H. pylori* a Gram-negative bacterium (Demirev *et al*., 2001). Similarly there are many other molecular methods used such as 16s RNA RT PCR, and mass spectrometry. RT-PCR (reverse transcriptase) was employed in the differentiation of *Staphylococcus aureus* from *Staphylococcus epidermidis*

in a case study from a culture negative osteomyelitis (Kobayashi *et al*., 2006). An identification technique needs to be simple, rapid, reproducible and economical for it to be employed on a regular basis in the routine laboratory. Analysis of surface carbohydrates by lectin binding in an ELLA format does fulfil the criteria and there have been many studies trying to use lectins for diagnostics. Munoz and his colleagues (1999) tried to use lectins for the rapid identification of clinically important MRSA, not surprisingly he used only cell extracts and simply performed slide agglutination test trying to develop a rapid and easy method of identification. His study however highlighted that lectin typing can be a good first line approach to the epidemiology of MRSA for its rapid performance in laboratories but it did not show reproducibility. Our study needs to be developed further to analyse many clinical isolates from different countries grown on different culture medium as (Munoz *et al*.,1999) also highlights that culture medium used for growth plays an active role in sugar expression.

Lectin typing has also been employed for many other organisms such as *Lactobacillus*, *Candida* and also Archebacteria, but all have used simple agglutination methods performed on whole cells. Annuk *et al*., (2001) performed lectin typing on proteolytic treated whole cells. However these agglutination tests are limited for some organisms and may not facilitate agglutination of all clinical isolates (Kuusela *et al*., 1994). In one of the studies carried out by Masarova and his colleagues (2004) an optical based biosensor was used as a tool in bacterial identification. Here they employed lectins attached on Biacore ^{TM} sensor chips and whole cells or endotoxins were injected onto the surface. Originally antibodies were attached to the Biacore ^{TM} for the detection of micro-organisms because of the strong and specific interactions but this involved an expensive procedure for antibody isolation and purification and as some of these monoclonal antibodies were specific to antigens usage was limited. Our study has taken further steps towards improving lectin typing by employing a simple-user friendly ELLA as the technique, performing lectin typing on

whole cells and Proteinase K treated as well as glycolipids and lipopolysaccharides extracted from the organisms. This study has successfully used panel of 22 biotinylated lectins with various glycan specificity giving broad overview of glycan-lectin interactions and their potential in microbial diagnostics.

In this study we noted that glycans, due to their conformational and structural differences show varied interactions with lectins. The glycan specificities of the lectins were derived from the results as shown in Table 3.2. It was noted that although LC and Con A lectin are specific for α mannose and to a lesser extent α glucose, LC has a preference for bi and tri anntennary complexes with a core α fucose and α glucose. Con A on the other hand recognises unsubstituted α mannose on bi and hexa antennary chains. Similarly LE lectin binds to β-N-acetylglucosamine especially in oligomers such as $(GlcNAc)_{3,}$ GlcNAc 1, 4 oligomers, chitobiose and chitotriose in contrast to the GII lectin which has specificity for terminal α β-N-acetylglucosamine. With these conformational and structural preferences, it is clear that lectins can be employed in microbial diagnostics where results would be scored as degree of binding and hence affinity towards glycans and the uniqueness of the lectins that were expressed by aerobes and anaerobes. Some of the lectins bound only to specific organisms whereas others were more promiscuous multimeric proteins such that they bind monosaccharides of different specificity groups in the same combining site while few others possess more than one combining site for different monosaccharides in each subunit or can recognise oligosaccharides only. Usually the monosaccharides bind to the primary combining site of the lectins. The glycan–lectin binding specificity is based on the research data available from eukaryotes and therefore the results interpretations therefore need to be made with caution. The binding patterns and uniqueness can also be explored to distinguish different species in their respective genus.

Previous studies on lectin typing of methicillin resistant *Staphylococcus aureus* have utilised agglutination assays of 77 clinical isolates with 32

commercially available lectins have results which differed to our study (Kuusela *et al*., 1994) that the lectins agglutinating the MRSA isolates were Con A, *Helix pomatia* specific for D-GalNAc (and to a lesser extent GlcNAc)and *Lens culinaris*. They reported auto-agglutination in their study and have claimed to have obtained varied glycan-lectin profiles. Among the 77 clinical isolates about 30 agglutination patterns were observed. Some lectins that were not included in their study proved to be significant in our study .They also did not pre-treat the cell suspensions with Proteinase K. In our study the whole cells and Proteinase K treated whole cells of *Staphylococcus* species showed different lectins that were expressed in coherence with the proposition made by Munoz and colleagues (1999) suggesting that proteolytic enzymes can partially hydrolyse the proteoglycans, such that some carbohydrate residues can be exposed and thus may become available for binding while other residues can even become destroyed or modified. With whole cells the ELLA results showed many weakly positive interactions with many lectins implying that all the glycans on the microbial cells such as peptidoglycan, glycolipids, and glycoproteins were expressed thus producing more heterogeneous profiles. The results obtained in this study are in agreement with other studies (Munoz, Alvarez and Alonso, 1999; Masarova, Dey and Danielsson, 2004 and Stoitsova, Ivanova and Dimova, 2004) carried out that relied on agglutination assays, or the use of Biosensor devices, or dot-blot analysis to identify the glycans. In our study however a panel of 22 biotinylated lectins, with a broader range of glycan specificities were employed.

Although anaerobes are involved in number of infections they are often overlooked and have undergone numerous changes in nomenclature and classification with new species being added. API 32A which is the most regularly used identification system in the microbiological laboratories do not include these new species in the table of identification or the computer analysis software (Kitch and Appelbaum, 1989). Some other identification systems used in clinical laboratories include the BBL™ crystal anaerobe

identification system (Pape, Wadlin and Nachamkin, 2006; Cavallaro, Wiggs and Miller, 1997) do not include the new species but most importantly these systems are very expensive. Due to costs, regular monitoring of anaerobic cultures in laboratories is limited, affecting the prescription of more empirical antibiotics and further leading to antibiotic resistance. Typing data should be in a format that is early assimilated into a national picture. Different bacterial species often exhibit different populations or structures. Some species are characterized by highly diverse populations at one extreme and closely similar member at the other. The ideal diagnostic tool would be "ASSURED" affordable, sensitive, specific, user-friendly (simple to perform, minimal training), rapid-robust, equipment – free and deliverable to those who need it. The method in this study uses simple ELLA and biotinylated lectins which are inexpensive and moreover satisfies most of the characteristics required for a good typing and identification method. From the data obtained identifying the glycan – lectin interactions for aerobes whole cells for the identification of *E. coli, P. aeruginosa, S. pyogenes, E. faecalis* and *P. mirabilis* whole cells the lectins that could be employed in a diagnostic panel (kit) could be WGA, JAC, SBA, DF, RCI, LC, EC and LE (table no 3.6A). Similarly for the identification of anaerobes whole cells the lectins that could be used in a diagnostic panel (kit) would be PVE,LC,VV, LE, ST and GII (table 3.7A) which would help identification *of P.bivia, A. prevotti, Parvi. micra* and *Prop. acne*.

ELLA results obtained in this study could also be used for typing of *Staphylococcus* species such that a panel of biotinylated lectins could be designed using Jac, GI, SJA, LC and PS for whole cells of *Staphylococcus* species and LC, EC, VV, LE, GI, PNA, PVE and RC I (table 3.9) lectins for the typing of glycolipids of *Staphylococcus* species. Similarly ELLA results could use a panel of these biotinylated lectins such as SBA, DB, SWGA, LC, PS, SBA, PVE and LC (table 3.10B) with either unique binding or no binding for typing of Gram- positive anaerobes of the *Peptostreptococcus* species. VV lectin could be included to identify

additionally *Propionibacterium acne* species among the tested Gram positive anaerobes (table 3.10B). Among the Gram negative anaerobes the diagnostic panel (kit) could include SWGA, PS, PVL, GI, SN and GSII (table 3.10A) lectins to discriminate the *F. nucleatum, B. fragilis* and *P. bivia* species. Thus this study although has identified the glycan- lectin profiles for number of aerobes, anaerobes exploring different type of glycans from the whole cells, treated whole cells and glycolipids to further explore its role in microbial diagnostics ELLA analysis needs to be repeated on higher numbers of clinical strains to confirm utility in microbial diagnostics and to be statistically significant.

3.5 Conclusion

This study has utilised lectins and has developed a cost effective, easy to use and practical rapid method for the typing of various clinically significant aerobes and anaerobes. Further research is required to determine the robustness of this method in the clinical arena.

CHAPTER – 4

CHARACTERISATION OF GLYCOLIPIDS OF FINEGOLDIA MAGNA

This chapter discusses the various pathogenic characteristics and the mechanisms used by micro-organisms in causing infections. It reports the identification of sialic acid and the utilisation of GC-MS in the characterisation of the glycans of an anaerobe *Finegoldia magna*.

Study rationale:
Recognition of the pathogenic qualities of the various anaerobic organisms can assist in their prompt identification and in the initiation of appropriate therapy.

4.1 Introduction

4.1.1 Different modes of pathogenesis

The ability of an organism to cause disease in a host is referred to as the pathogenicity. There are a number of virulence factors that make an organism a pathogen and initiate an infection. There are sequences of events that occur after a pathogen enters the host from attachment, to invasion by overcoming the natural defence barrier. After entry into the host, the pathogens multiply and spread until the infection becomes persistent and untreatable (Wilson and Devine, 2003).

Many Gram - negative pathogens have functionally diverse adhesive pili which are fibrous organelles on their surface and mediate attachment to the host cells (Sauer, *et al.,* 2000). Minor pili like structures have also been seen among the Gram – positive organisms which have shown to play a major role in host cell colonisation (Proft and Baker, 2008). In other cases, adhesion could be directly associated with the microbial surface. Some Gram - negative organisms such as *Campylobacter coli* and *Campylobacter jejuni* have fimbriae, flagella which help them in their first

step towards infection facilitating attachment to the host (Doig *et al.,* 1996). *Klebsiella* species and some of the mycobacterium species (Stokes, *et al.,* 2004) have capsules that are glycan rich and studies have shown that they play a role in virulence. In some instances the bacteria take advantage of the rigidity of the branched oligosaccharide to improve the binding. (Imberty, Mitchell and Wimmerova, 2005) while others have proteins which are heavily glycosylated as seen in many of the Gram - negative species such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Neisseria* and *Campylobacter* species.

Virulence factors such as bacterial toxins, exotoxins and endotoxins are some of the other ways by which bacteria are capable of imparting toxicity to the cells. Most toxins produced are active even in nano molar concentrations. Gram - negative organisms have LPS as a component and there have been number of studies suggesting the toxicity is due to the lipid A moiety of the LPS (Sperandeo, Deho and Polissi, 2009). Some of the uropathogenic and enteropathogenic *Escherichia coli* have pathogenicity islands which are large elements that are integrated into the chromosome of bacteria which carry the virulence determinants. for instance some strains of *E. coli* have *Pai I* which encodes for haemolysin, *Pai II* encoding for P fimbriae as well as haemolysin, *Pai III* is a type II secretion system that mediates the infection of proteins directly into the cytoplasm of the target host cell. *Pai IV* encodes for hemolysin adhesion associated pili and *Pai V* encodes for hemolysin and cytotoxic necrotizing factor (Hentschel, and Hacker 2001). One of the classic examples of bacteria whose virulence is determined by the presence of enterotoxins is *Clostridium difficle* a significant anaerobe known to cause many fatal infections (Boquet *et al*., 2000).

There are other ways by which bacteria have evolved by making cell surface molecules that mediate bacterial attachment, these cell surface carbohydrates and proteins help in protecting bacteria as is seen among the Gram - positive organism *Staphylococcus aureus.* It produces a collagen binding protein (Deivanayagam *et al*., 2000) while *Streptococcus*

pyogenes shows the presence of M proteins which are classic virulence determinants interacting with the host molecules such as fibrinogen, albumin, plasminogen and IgG (Cunningham, 2000).

4.1.2 Role of glycans in pathogenesis

Glycans interact with lectins and this is the molecular basis of a number of interactions between bacteria and their environment. Thus the binding of the surface carbohydrates of one bacterial species by surface adhesins of another causes bacterial co-aggregation (Rickard *et al*., 2003 and Kolenbrander *et al.,* 1993), and this plays a role in the formation of biofilms.

Many bacteria are also protected by capsules, which are often glycosylated and play a role in attachment to the host as discussed earlier. One of the most significant contributions to understanding pathogenesis has been made with the advent of lectins as it has now become possible to identify the glycan-lectin interactions adopted by various bacteria with their host in initiating an infection. There have been many occasions where lectins from the bacterial cell wall have been utilised as the first mode of attachment to the host glycans. For example the AB5 - type proteins of *Vibrio cholera,* enterotoxic *E.coli* and *Bordella pertussis* have one toxin and five lectin subunits which enables them to bind to the gangliosides of gut or lung walls (Merrit and Hol, 1995). Similarly *Clostridium* species causing botulism have a lectin domain enabling them to bind to the ganglioside GM1 on neurons (Lalli *et al.,* 2003). The opportunistic human pathogen *Pseudomonas aeruginosa* produces two soluble lectins PA-1L (gene *lec A*) and PA-IIL (gene *lec B*) which bind to galactose and fucose/mannose containing glycoconjugates of the host. These glycans present on the host cells act as specific targets for the pathogen binding and are involved in the establishment of infections (Lloyd *et al*., 2007). Conversely there are glycans present on micro-organisms such as *E.coli* which use host mannose specific receptors to bind to bladder epithelium causing cystitis, and galactobiose specific

receptors to invade the upper urinary tract causing pyelonephritis (Imberty *et al.,* 2004).

4.1.3 Role of sialic acid in pathogenesis

Sialic acid is a collective term for a family of 9-carbon monosaccharide which are often found as terminal sugar residues on the glycans of glycoproteins and glycolipids. These are found both on eukaryotes as well as prokaryotes. Although N-acetylneuraminic acid is one of the most abundant of the sialic acids there are other neuraminic acid derivatives which occur in a variety of natural sources. The naturally occurring sialic acids are from the families of N-acetyl, N-glycolyl, O-phosphate, O-sulphate, O-methyl and O-lactyl (Schauer, 2000). Sialic is saliva in Greek, and neuraminic means brain and amine (Angata and Varki, 2002).

Studies have shown that sialic acid has played an active role in enabling some microbes to colonize, persist and cause disease in mammalian species. Sialic acids are present on many pathogens, for example *Escherichia coli* K1, *Haemophilus influenzae*, *Pasteurella multocida, Neisseria* species, *Campylobacter jejuni* and *Streptococcus agalactiae* (Severi *et al.,* 2007). Bacteria can also utilize sialic acid as a carbon and nitrogen source by scavenging it from the surrounding environment. The sialic acid molecule is extensively found in mucus rich environments such as the gut and lungs where many pathogens thrive (Severi *et al.,* 2005 and Chang *et al*., 2004). This kind of sialic acid catabolism has been demonstrated in five species**:** *Clostridium perfringens**,** Escherichia coli**,** P. multocida**,** H. influenzae**,*** and *Bacteroides fragilis* (Nees, Schauer, Mayer, 1976 and Brigham *et al*., 2009). Chang *et al*., *(2004)* showed in *E. coli* that the ability to degrade sialic acid was important for the colonization of the mouse colon. This finding suggests that the ability to utilize sialic acid as a carbon source may be important for bacteria to colonize this niche (Almagro-Moreno and Boyd, 2009). Sialic acids help in stabilizing glycoconjugates and cell membranes, and also act as chemical messengers within the host (Schauer, 2000).

There are many other ways by which the presence of sialic acid can prove to be harmful to the host. Current studies have shown that several bacterial pathogens such as enterohaemorrhagic *Escherichia coli, Haemophilus influenzae, H. ducreyi, Pasteurella multocida, Neisseria gonorrhoeae, N. meningitidis, Campylobacter jejuni,* and *Streptococcus agalactiae* can attach sialic acid residues on to their outer surfaces (sialylate) masking them from the host immune system. These sialic acids can help in evading the host defence by inhibiting the direct activation of the alternative pathway of complement (Edwards *et al.,* 1982). This is seen in the anaerobe *Porphyromonas endodontalis* a black pigmented obligate anaerobe frequently responsible for causing mixed microbiota of infected root canal systems and peripheral abscesses (Collazo *et al.,* 2003). Interestingly, these pathogens have developed different mechanisms for obtaining sialic acid that include *de novo* biosynthesis of sialic acid in *E. coli and N. meningitidis*, sialic acid scavenging as seen in *N. gonorrhoeae*, and precursor scavenging as recognised in *H. influenzae* (Almagro-Moreno and Boyd, 2009). Owing to their negative charge sialic acids have been involved in the binding and the transport of positively charged molecules as well as in the attraction and repulsion of cells and molecules.

4.1.4 *Finegoldia magna,* sites of infections and pathogenic factors

Finegoldia magna, belonging to the group *Peptostreptococcus spp* is one of the Gram - positive anaerobes most commonly isolated from diabetic foot ulcers. All the *Peptostreptococcus* species are part of the normal skin flora of the mucosal surface in the oropharynx, and in the gastrointestinal and genitourinary tracts (Murdoch, 1998; Stephens *et al.*, 2003). In association with other pathogens they can become opportunistic pathogens. Among all the Gram - positive anaerobes, *F. magna* mainly causes skin infections and is often recovered from osteoarticular surgery (Felten, *et al*., 1998) and is among the most frequently isolated bacterial species from chronic leg ulcers (Wall *et al.*, 2002 and Stephens *et al*.,

2003). The *F. magna* recovered from infectious sites is almost always found in association with other pathogens. Recently there have been many advances in identifying the pathogenic factors of *F. magna*. It has Protein PAB (DeChâteau *et al*., 1996) suggesting colonisation and ability to produce various enzymes. The Protein PAB has been seen to be responsible for binding to Human serum albumin to their surface (Lejon *et al.,* 2004). The enzymes produced by *F.magna* which could contribute to pathogenesis include hippurate hydrolase, serine dehydratase, threonine dehydratase, gelatinase, alkaline phosphatase, esterase C4, catalase and collagenase (Krepel *et al.,* 1992). Other factors include encapsulation (Brook and Walker, 1985) and increased oxygen tolerance of clinical isolates. Most strains also have shown to express subtilisin-like enzymes such as Suf A that have proteolytic activity against many antimicrobial molecules (Karlsson *et al.,* 2007).

About 10% of *F.magna* strains have Protein L which has high affinity for immunoglobulin light chains (Bjorck, 1988). A study has also shown that Protein L has contributed to colonisation of *F.magna*. Another protein termed as FAF protein (*F. magna* adhesion factor) has also been involved in the aggregation and the colonisation of *F.magna* strains (Frick *et al*., 2008). This study suggested that when the *F.magna* strains with the FAF protein were treated with proteases the cells showed no adherence to any other cells. Molecular techniques were also used to confirm the presence of *FAF* gene in some strains of *F. magna*. The study by Frick and colleagues (2008) also highlighted that those strains which did not show the FAF protein could use other methods in colonisation. Another study has suggested that in the growth medium *in vitro* many of the *F. magna* isolates inhibited the proliferation of fibroblasts and keratinocytes (Stephens *et al*., 2003). Many strains of *F. magna* have also been shown to express the *ABC transporter* gene which is associated with the efflux of antibiotics out of the cells leading to antibiotic resistance and multi drug resistance pumps (Jauhangeer, 2004). All of the above discussed features

are utilised by *F.magna* in colonising, host cell attachment or embracing adaptations to avoid host defence and imparting infections.

4.1.5 Tools in the characterisation of glycolipids

Glycoproteins and glycolipids are also the major classes of glycoconjugates found on human epithelial and endothelial tissue (Hakomori, 1973 and Ruddiger and Gabius, 2001). These glycoconjugates play a wide role in many biological processes and many utilise carbohydrate-lectin binding to facilitate cell-cell and small molecule – cell recognition (Severi *et al.,* 2007). Therefore it becomes necessary to acknowledge all the different types of tools that can be explored to identify these important components of cells. Qualitative and quantitative measurements of the monosaccharide residues on glycoconjugates can easily be made by combinations of approaches including colorometric assays, paper or thin chromatography (Carlson, 1968) or using more sophisticated but expensive tools such as gas chromatography-mass spectrometry (GC-MS) , high pH anion exchange chromatography (HPAEC) (Townsend and Hardy, 1991) and nuclear magnetic resonance (NMR) (Hounsell, 1994) to help fully characterise the glycolipids and glycoproteins. Structural analysis of glycans requires chemical (reduction in alkaline or hydrolysis) or enzymatic treatment (endo-exoglycosidases) of the glycans. After derivatisation the sample can be analysed by mass spectrometry which is a valuable tool to rapidly characterise small amounts of materials. It can also be used in determining the chemical structures of important peptides and chemical compounds. It relies on the principle of ionizing the chemical compounds and generating charged molecules or molecule fragments and finally measuring their mass-to-charge ratios.

4.2 Materials and Method

4.2.1 Micro-organisms, culture conditions and glycolipids extraction

ATCC strain 29328 of *Finegoldia magna* was provided by the Anaerobic Reference Laboratory of London (UCLH). *Finegoldia magna* ATCC 29328 was grown under strict anaerobic conditions at 37ºC on 5% defibrinated horse blood agar and then cultured in brain heart infusion broth (Oxoid) with 0.01% Tween 80.

The method and materials used for the extraction of glycolipids of *Finegoldia magna* and biotinylated lectins was as outlined in chapter 3.2.5A and 3.2.2 respectively.

4.2.2 Proteins and Carbohydrate assay:

Bradford assays were performed for the detection and the quantification of proteins. Bradford reagent was obtained from Sigma and the protocol provided by them was followed (Bradford, 1976). Phenol-sulphuric assay was the method of choice for the detection and quantification of complex carbohydrates (Dubois *et al.*, 1951).

4.2.3 Warren assay for sialic acid

Sialic acid analysis was carried by the Warren method (Warren *et al.,* 1959). Fetuin (0 to 15 µg), known to contain sialic acid, was used as the control. Both the controls and *Finegoldia magna* glycolipids were acid hydrolysed with 0.1M sulphuric acid for 2 h at 80 ºC and then the tube was cooled for 20 min at room temperature. In brief 20 µl of periodate reagent (0.2M sodium periodate in 9M phosphoric acid) was added to 100 µl of fetuin and to the hydrolysed glycolipids. This mixture was incubated for 20 min at room temperature. To this 100 µl of 10% sodium arsenite in 0.05 M sulphuric acid with 0.5 M sodium sulphate was added slowly until the brownish yellow colour appeared and disappeared entirely. Thiobarbituric acid, 250 µl of 0.6 M, in 0.5 M sodium sulphate was added and the reactions were kept in boiling water for 20 min and then cooled at RT. The

mixture was then added to another eppendorf tube and 750 µl of cyclohexane was added and vortex rigorously centrifuged for 7 min at 2000rpm for phase separation. The upper phase was then measured at 549nm in a quartz 0.5 ml cuvette.

4.2.4 Preparation of glycans for high pH anion exchange chromatography (HPAEC):

The calibration standards of a mix of monosaccharides of concentration 0.5 nmole/10 µl, 1.0 nmole/ 10 µl, 2.0 nmole/10 µl mix were hydrolysed using 400 µl of 2 N trifluoroacetic in 200 mM sodium hydroxide buffer at 100 ºC for 4 hours. While 400 µl of 2 M acetic acid in 100 mM of sodium hydroxide buffer was added to hydrolyse the sialic acid standards (N-acetyl-neuraminic acid and N-glycol-neuraminic acid) and *F.magna* glycolipids at 80 ºC for 4 hours. After hydrolysis the hydrolysates were frozen on dry ice and lyophilised. The lyophilised hydrolysates were resuspended in degassed nanopure water and sonicated in cold water for 7 min. Standards and hydrolysed *Finegoldia magna* glycolipids (10 µl) were injected in the DIONEX machine.

4.2.5(A) Composition and linkage analysis by Gas Chromatography - Mass spectrometry

Composition analysis: To analyse the composition of glycolipids of *Finegoldia magna* the following chemical derivatisation were carried out before injecting the sample.

Sample preparation: A sample of 800 µg of glycolipids of *F. magna* was weighed and 20 µg of inositol as an internal standard control was added. An aliquot of 200 µl of 2 M trifluoroacetic acid (TFA) was added to the sample and incubated for 2 h at 121 ºC. The sample was allowed to cool at room temperature and dried with nitrogen. Isopropanol (100 µl) was then added and the suspension was dried with nitrogen. This was repeated twice.

Reduction: Freshly prepared solution of 10 µl of 10 mg/ml of sodium borodeuteride in 1 M ammonium hydroxide was added to hydrolysed sample and incubated at RT for 2 hour. The above solution was

neutralised with glacial acetic acid and dried down with nitrogen. 15 µl of 9:1 methanol: acetic acid was added and dried with nitrogen. Again this was repeated twice. To further remove the borates 15 µl of methanol was added and dried with nitrogen, the procedure was repeated until a crusty white residue was seen on the side of the tube.

O-acetylation: Acetic anhydride (250 µl) of and 230 µl concentrated TFA was added to the above sample and incubated at 50 ºC for 10 minutes. After cooling 1 ml of isopropanol was added and the suspension was dried in a stream of nitrogen. To neutralise the sample 0.2 M of sodium carbonate was added and mixed gently. A 1 ml aliquot of dichloromethane was added and centrifuged briefly. The aqueous layer was discarded and 1 ml of water was added and centrifuged. The organic layer was taken to another tube and concentrated prior to injection onto GC-MS (SP2330 fused silica column, Supelco).

4.2.5(B) Composition of lipids – by gas chromatography mass spectrometry

A preliminary study was carried out to check the composition of the lipid portion of the samples. The dried glycolipid extracts (0.5 mg) were suspended in chloroform-methanol-water (65: 25:4, v/v) to remove the non lipid material. From the sample (10 µg) of the above lipid fraction was hydrolyzed in 0.5 ml 1 M HCl for 4 hrs at 100 ºC in sealed tubes. Following evaporation of HCl, samples were reduced and peracetylated according to the method discussed earlier in section 4.2.5(A) Internal standard C-17 heptadecanoic acid was added as a control.

4.2.6 Linkage analysis by gas chromatography – mass spectrometry:

Permethylation, depolymerisation, reduction and acetylation of glycolipids of *Finegoldia magna* were carried out and the resultant partially methylated alditol acetates (PMAAs) analysed by gas chromatography-mass spectrometry. Initially, aliquots of 0.5 mg/ml of glycolipids in 200 µl DMSO were prepared and placed on a magnetic stirrer for 4-8 hours for complete dissolution and then freezing of the sample. This reaction and all

subsequent reactions were performed in disposable glass tubes with Teflon-lined screw cap fittings.

Permethylation of the samples was carried out by using the Hakomori methylation. (Hakomori, 1964) The permethylation of the material was carried out by adding methyl iodide (500 -700 µl) with the head space purged with nitrogen gas and carefully stirred for 3 hrs. To quench the reaction I ml of nano pure water was added and mixed. Methyl iodide was then bubbled off completely with low flow of air leaving a clear solution. Reverse phase chromatography was performed manually using Sep-Pak C18 cartridges. The sample was then extracted with acetonitrile (1 ml). Myo-inositol (2-5 µg) was also added to the permethylated sample as an internal standard. The permethylated sample was then hydrolyzed using 2M trifluoroacetic acid to produce partially methylated alditol acetate (PMMA) and reduced using 10 mg/ml solution of sodium borodeuteride in 1 M ammonium hydroxide to produce partially methylated alditols. The reduction reaction was neutralized with glacial acetic acid and 20 drops of 9:1 of methlyalcohol: acetic anhydride. The partially methylated alditols were then peracetylated using acetic anhydride and TFA (230 µl) was then added and the sample was incubated at 50 ºC for 20 minutes and the reaction neutralised with 0.2 M sodium carbonate. The resulting PMAA was extracted using dichloromethane and water, and dried down using nitrogen gas prior injecting 1 µl onto the GC MS (SP2330 fused silica column, Supelco, Bellefonte) with MS operating initial temperature of 80 ºC and initial time 2.0 min.

4.3 Results

This section discusses the *Finegoldia magna* glycolipids composition data. The main objective is to understand the monosaccharides, presence of sialic acid and fatty acid on *F. magna* glycolipids. In order to achieve this objective, the techniques explored were in house developed easy and cost efficient ELLA and the expensive yet robust methods such as the GC/MS and HPAEC. Simple traditional methods such as Warren's assay were also used for determining the presence or absence of sialic acid on different anaerobes.

4.3.1: Glycans expressed by *Finegoldia magna* as determined by ELLA method

ELLA method discussed in section 3.22 was used to determine the glycans expressed on *F. magna* glycolipids. As seen in Appendix XIC based on lectin specificity the glycans likely to be expressed by *F. magna* glycolipids include Galβ1-3GalNAc specified by *Soybean agglutinin* lectin, *Peanut agglutinin* lectin and *Ricinus communis agglutinin* I lectin, GlcNAc as specified by the reaction with succinylated Wheat germ agglutinin lectin and structures containing mannose specified by *Lens culinaris agglutinin*, *Pisum sativum agglutinin* and *Phaseolus leucoagglutinin*. The reaction with *Sambucus nigra* which is specific for sialic acid attached to the terminal galactose was also positive as shown in Appendix XIC. Inhibition assay was carried out to confirm the specificity of binding of the biotinylated lectins with *F.magna* glycans shown in chapter 3 in section 3.3.5 in figure 3.4.

4.3.2 Carbohydrate and Protein assay

The quantification of carbohydrates and proteins of glycolipids of *Finegoldia magna* was determined by reference standard curve generated using commercially available *Escherichia coli* LPS in the range of 0.1-1.4 mg/ml for proteins and 0 -100 µg/ml for carbohydrates.

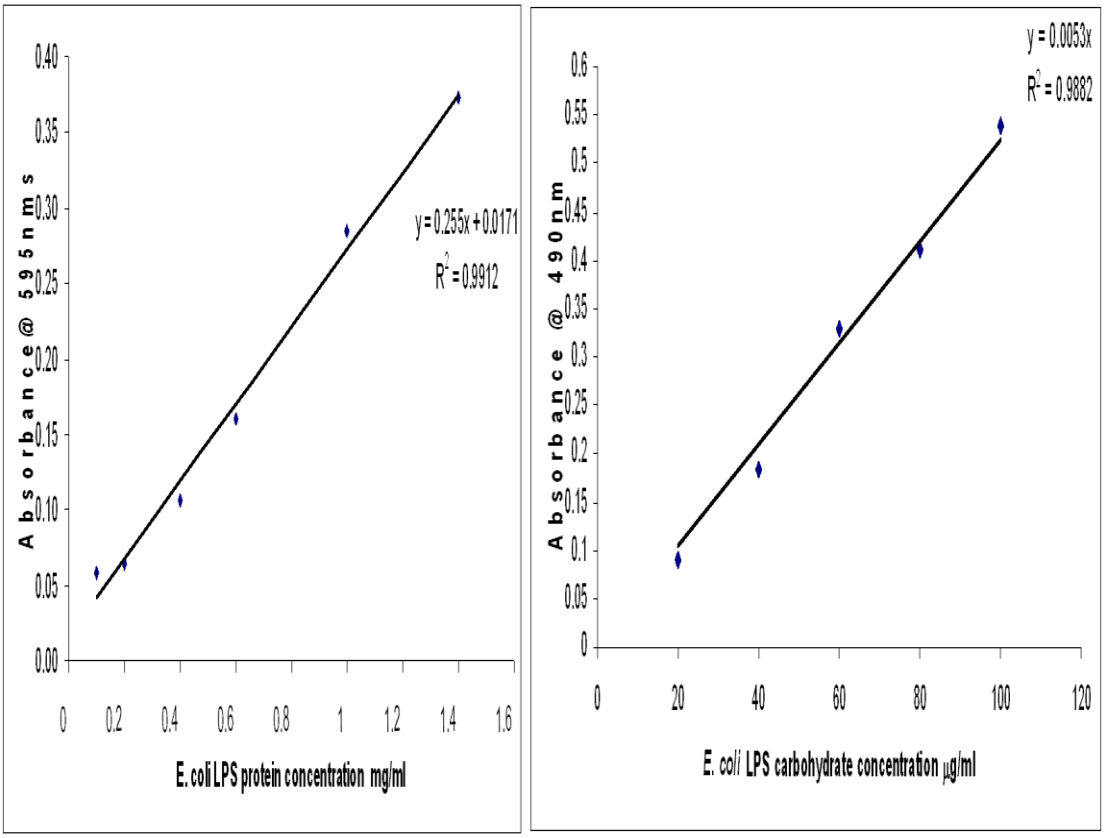


Figure 4.1: Bradford and Phenol – sulphuric acid assay for *E. coli* LPS

The reference standard curve constructed on *E. coli* LPS as seen in figure 4.1 and the equation shown in figure was used to calculate the protein and carbohydrate content of *F. magna* glycolipids

From the absorbance at 595 nm and using the equation from the figure 4.1 the protein content of *F. magna* was below the reference range of the assay (0 – 1.6 mg/ml) and due to the high amount of carbohydrate the *F. magna* glycolipids was diluted 1 in 100 times and then the carbohydrate content was determined using the phenol – sulphuric assay. It was found that *F.magna* glycolipids contained more carbohydrate than the reference range of the assay (0 – 120 µg/ml).

4.3.3 Composition analysis of glycans of *Finegoldia magna* glycolipids

Gas chromatography coupled with mass spectrometry was used to analyse the monosaccharide composition of the glycolipids by preparation of alditol acetates. Following the derivatisation procedure as described in section 4.2.5A, the sample was injected onto the DB -1 column of GC/MS for analysis. Samples were derivatised to convert the non – volatile metabolites to volatile metabolites prior to injecting on to the column of GC/MS analyser.

Initially, a range of monosaccharides were run on the column to determine their retention times (R_t). Standard monosaccharides included fucose, arabinose, galactose, glucose, mannose, rhamnose and xylose and then the sample was injected to obtain a chromatogram of the *F. magna* glycolipids (Figure 4.2). The chromatogram of the standards in shown in the (Appendix XVII). Inositol was used as an internal standard such that Inositol was the last eluted fraction.

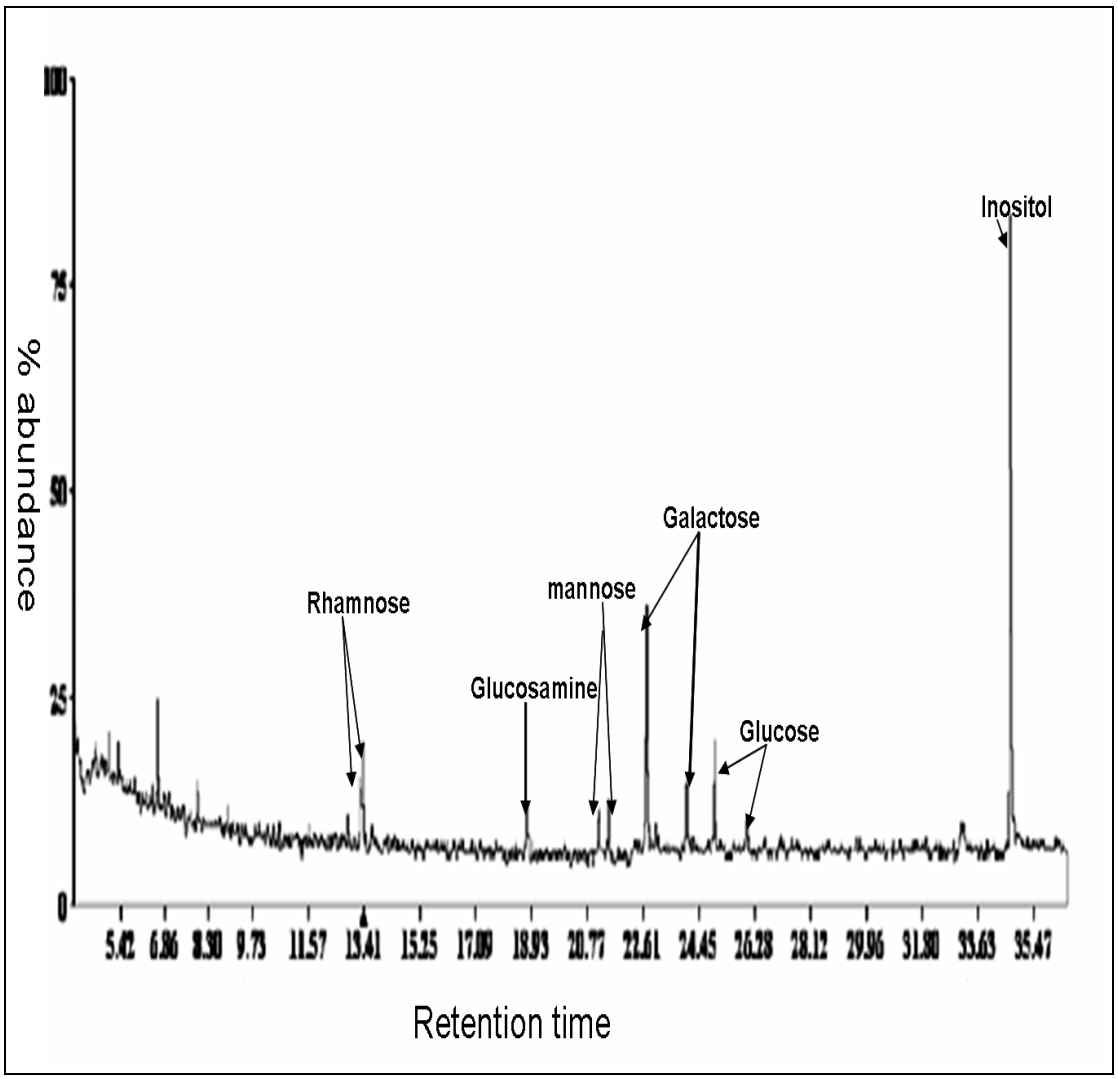


Figure 4.2: Composition analysis of glycans of *Finegoldia magna*
Figure 4.2 illustrates the peaks corresponding to the respective monosaccharides on the glycolipids of *Finegoldia magna* obtained after derivatisation by GC-MS analysis. Mass spectra of the individual standard monosaccharides were used to compare and identify the sample components.

GC-MS showed the presence of two peaks representing the alpha and the beta forms of monosaccharides. The GC/MS of *F. magna* glycolipids showed the presence of rhamnose, glucosamine, glucose, mannose and galactose with retention times (13.30, 13.4), (18.80), (24.98, 26.02), (21.16, 21.49) and (22.76, 24.07) minutes respectively. The monosaccharide composition obtained by GC-MS is similar to the glycan analysis performed by ELLA with a panel of 22 biotinylated lectins as shown in appendix XIC. It is noteworthy that this method cannot be used to detect sialic acids. Table 4.1 shows the composition of glycolipids along with their retention time and % abundance.

Table 4.1: Composition of glycolipids of *F. magna*

Monosaccharides	**% Abundance**	**GC- R_t (min)**
Glucosamine	4.39%	18.807
Glucose	15.21%	24.98 and 26.02
Mannose	15.85%	21.16 and 21.49
Galactose	39.26%	22.76 and 24.07
Rhamnose	25.28%	13.30 and 13.4

GC-Rt is peak retention time of ion on the respective GC column. GC ion abundance is used to express % abundance for each monosaccharide as function of total carbohydrate in separate experiments.

4.3.4 Characterisation of lipids by Gas chromatography – Mass spectrometry

GC/MS was also used to identify the fatty acids making up the lipid part of the *F. magna* glycolipids after the derivatisation step discussed in section. 10 µgs of the *Finegoldia magna* lipids were extracted and derivatised to obtain alditol acetates and volatilised to obtain a GC/MS chromatogram.

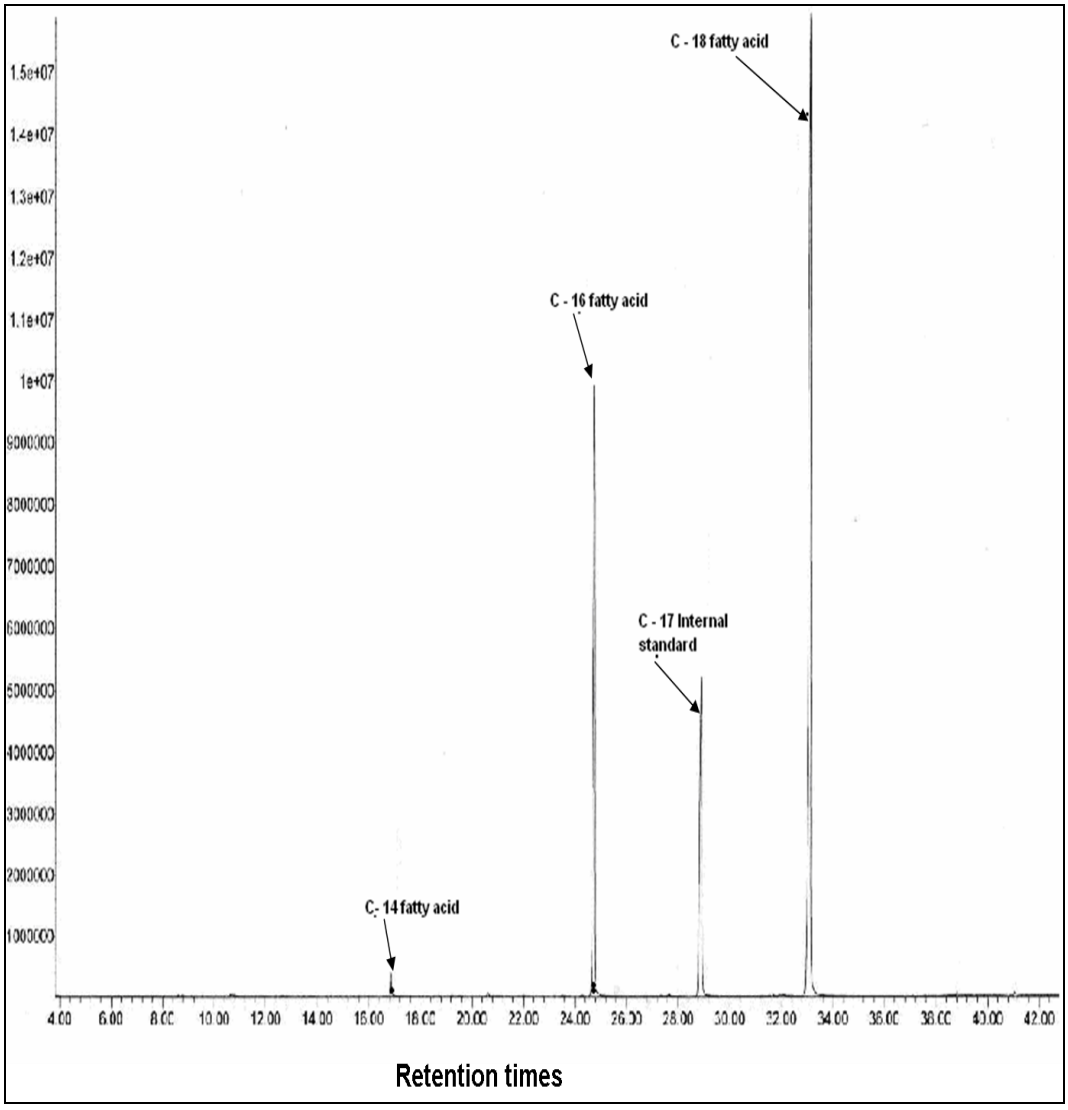


Figure 4.3: Fatty acid composition of *F. magna* lipids

Figure 4.3 shows the saturated fatty acids seen on the *Finegoldia magna* glycolipids analysed by GC-MS. The retention times and the annotation of the fatty acids expressed by *F. magna* glycolipids are shown in the table. C-17 fatty acid was used as an internal standard.

Table 4.2: Fatty acids on the glycolipids of *F. magna*

The total fatty acid composition of 10 µg of *Finegoldia magna* glycolipids is shown in the table 4.2.

Saturated fatty acid	Common name	Retention time	Ratio to standard nmol
C 14	Myristic acid	16.90	3.975
C16	Palmitic acid	24.79	70.122
C18	Stearic acid	33.16	135.749

At retention times 16.90, 24.79 and 33.16 the fatty acids expressed by the lipids of *Finegoldia magna* were myristic acid (3.975 nmol), palmitic acid (70.122 nmol) and stearic acid (135.749 nmol) respectively. Heptadecanoic acid (C-17) was used as an internal standard.

4.3.5 Linkage analysis by partially methylated alditol acetates of *Finegoldia magna* glycans

Glycosyl linkage analysis was carried out by the preparation of PMAA (Partially Methylated Alditol Acetates) in accordance to Hakomori methylation method (Hakomori, 1964). On successful permethylation, reduction and acetylation as described in section 4.2.6, the resulting partially methylated alditol acetates (PMAA) were injected on to the SP2330 (Supelco, Bellefonte) column of the GC/MS (Fig 4.4).

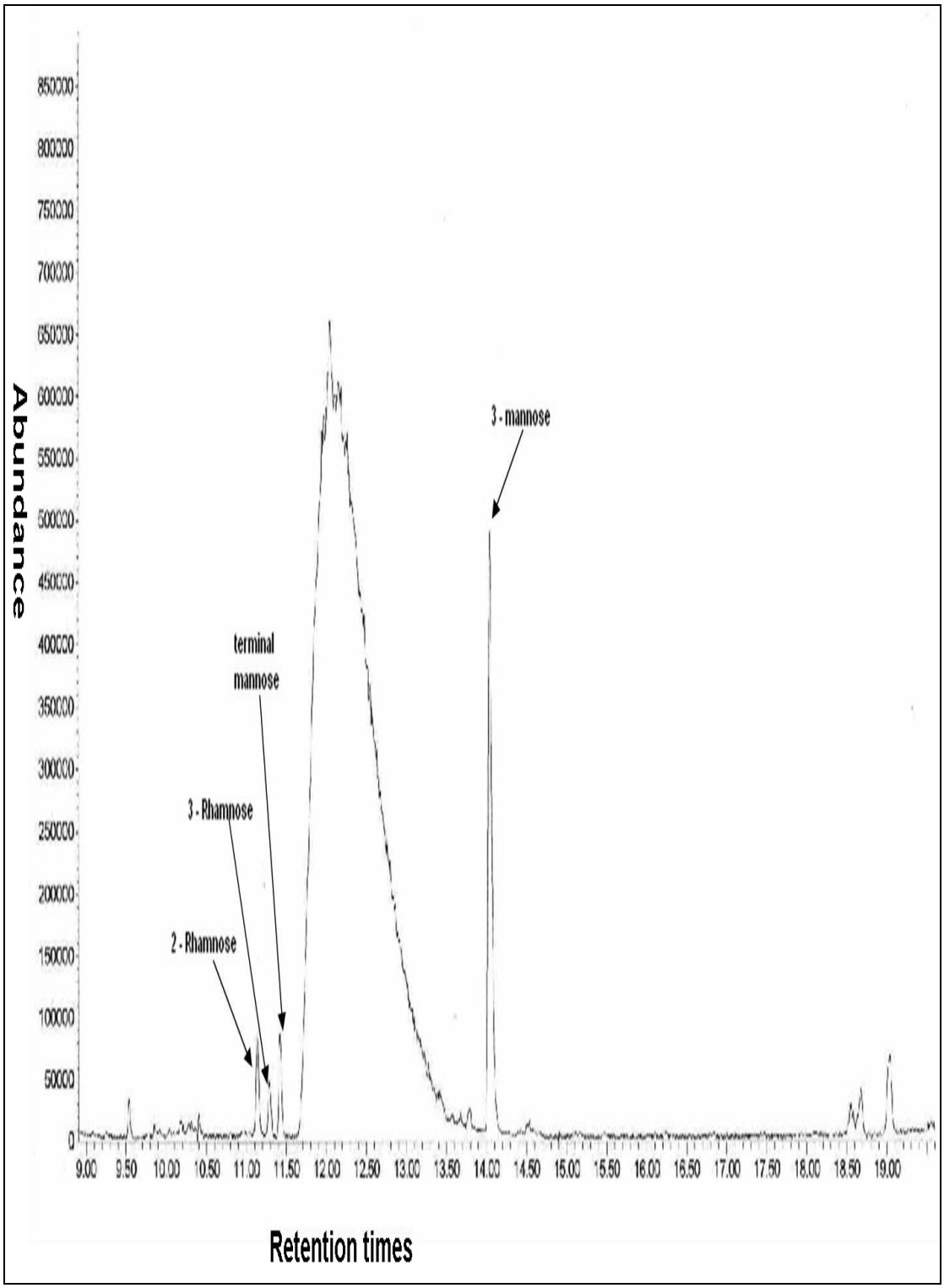


Figure 4.4: Linkage analysis of glycans of *F.magna*

PMMA derivatives of glycolipids of *F. magna* showed the linkages seen in figure 4.4. These were resolved using Gas Chromatography and identified by EI generated fragments patterns by using a library of PMAA sugar standards.

Glycans expressed by *Finegoldia magna* included mannose, glucose and galactose shown in figure 4.2 with the linkage data as shown in figure 4.4 suggesting terminal and 3 - mannose linkages with retention times 11.41 and 14.03 respectively. A naturally-occurring deoxy sugar rhamnose was also seen with 2-rhamnose and 3-rhamnose linkages with retention time 11.13 and 11.29 minutes respectively. The fragmentation patterns produced from each of the peaks were compared to libraries of PMAA derivatives present on the CCRC (Complex Carbohydate Research Centre, USA) databases specifically prepared for the most common monosaccharide linkages. Deuterium was used during the derivatisation to enable the distinction between the epimers. Deuterium helped to open the cyclic ring which resulted in the alteration of the GC retention time. With the methylation step discussed in the section the methylated structures would appear on the terminal residues as the reducing end of each monosaccharide would be expected to be engaged in a linkage with the preceding monosaccharide. All linkages were identified on the basis of relative retention time and fragmentation pattern.

4.3.6 Sialic acid determination by HPAEC

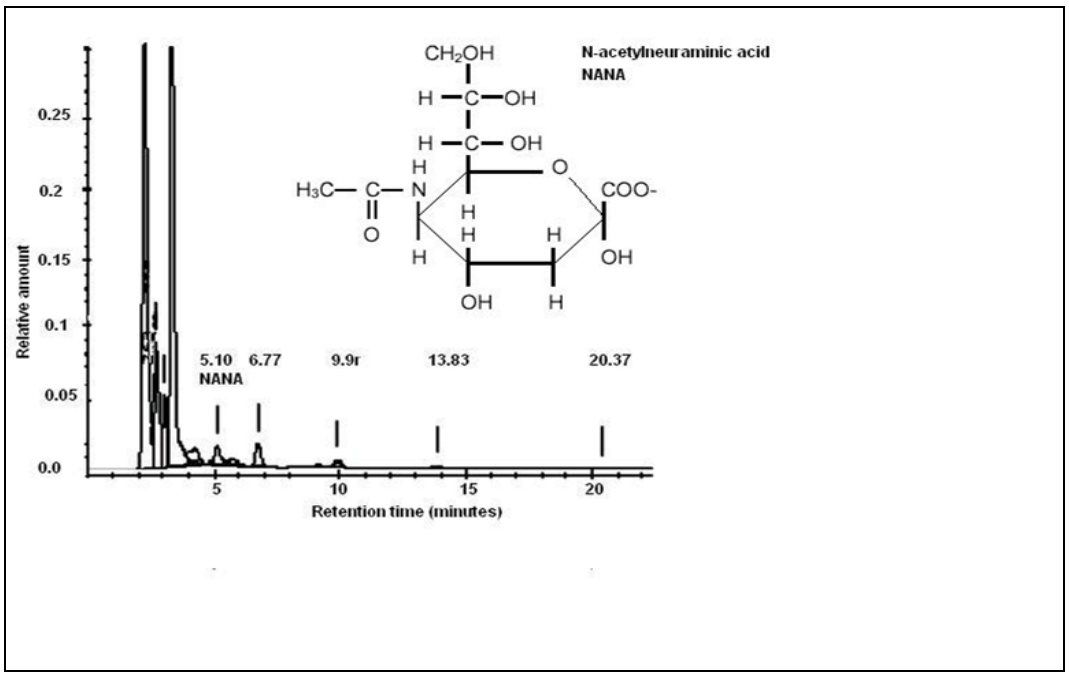


Figure 4.5: Sialic acid on the glycolipids of *F.magna*

Figure 4.5 illustrates the peak at elution time 5.10 of sialic acid obtained after hydrolysis of glycolipids of *F. magna*.

At elution time of 5.10 minutes as observed in the above figure 4.5 illustrates that the glycolipids of *Finegoldia magna* carried N-acetylneuraminic acid. 400 µg of hydrolysed *Finegoldia magna* glycolipids contained 19.94 nmoles of sialic acid (0.99 nmoles/10 µl /of injected sample volume).

4.3.7 Detection of sialic acid on glycolipids of anaerobes using the Warren assay

Warren's assay was used for the qualitative identification of sialic acid on the glycolipids of some of the most predominant anaerobes often isolated from the DF patients. Although this method can be used to quantify the amount of sialic acid, in this study due to the low amount of sample the quantification was not carried out. However the results shown below in table 4.3 show the presence or absence of sialic acid on some of the significant anaerobes.

Table 4.3: Sialic acid on the glycolipids of anaerobes

Anaerobes glycolipids	Absorbance @ 549nm	Sialic acid
Finegoldia magna	0.021	+
Peptostreptococcus asacchrolyticus	0	-
Peptostreptococcus anaerobius	0.026	+
Parvimonas micra	0	-
Prevotella bivia	0.0175	+
Prevotella prevotti	0	-
Propionibacterium acne	0.067	+
Bacteroides fragilis	0	-
Fusobacterium nucleatum	0.049	+

Table 4.3 shows the presence of absence of sialic acid for glycolipids from a number of anaerobes determined by Warren's assay.

The anaerobes shown to possess sialic acid by Warren's assay included *F. magna, P. anaerobius, Prevotella bivia*, *Propionibacterium acnes* and *F. nucleatum*.

4.4 Discussion

The characterisation of glycolipids of *Finegoldia magna* using GC/MS reveals similar glycan profiles to those obtained by ELLA of *Finegoldia magna* glycolipids shown in Appendix XIC. *Finegoldia magna* typically has a gram-positive cell envelope and monosaccharides decorate the cell surface. There have been many previous studies highlighting the importance of N-acetylneuraminic acid, N-acetyl glycol neuraminic acid and other monosaccharides in the glycopolymers of the glycocalyx of bacteria. For example, in *Lactobacillus* spp these glycopolymers are linked to the adhesive properties of the strains (Onyshchenko *et al.,* 1999). There have also been many studies highlighting the importance of bacterial lectins in adhesion and pathogenesis, but very few describing the glycans as virulence factors for anaerobes.

This study highlights the virulence factors which could play a part in the pathogenicity of the anaerobe *Finegoldia magna*. From the GC/MS carried out and the ELLA results, it is clear that glycans expressed by *Finegoldia magna* include glucose, mannose, galactose rhamnose and glucosamine with terminal mannose and 3 mannose linkages. In this study a full structure of *F. magna* glycolipids cannot be determined because the biotinylated lectins used in the ELLA can help in qualitative identification of the glycans and the possible linkages but cannot be confirmative of the exact structure of the glycolipids of *F. magna*. GC/MS can be used to build the structures but due to incomplete linkage data obtained during the derivatisation a complete structure cannot be assigned except although it can be determined from the data that *F. magna* glycolipids do contain terminal mannose, α1, 3 mannose, 2–linked rhamnose and 3-linked rhamnose.

One of the other methods used in this study namely the HPAEC which is a robust method utilises a DIONEX columns and is highly sensitive detecting as little as 1 pmole. HPAEC analysis on the *F. magna* glycolipids suggested the presence of sialic acid and thus confirmed the results

obtained using the ELLA methods with the binding of *Sambucus nigra* lectin which is specific for sialic acid attached to terminal galactose seen in Appendix IXC. Rhamnose could also play a role in virulence as there have been studies suggesting that rhamnose has been found as a component of the outer cell membrane of many clinical isolates. Studies have shown the presence of sialic acid on *Bacteroides fragilis* which is in contrast to our study (Severi *et al.,* 2007). However, there can be variations in expression of genes amongst the various strains of pathological and non pathological origin. This suggests that a number of clinical isolates should be tested for an overall comparison and for confirming the presence of sialic acids on these pathogens. Our study has demonstrated the presence of sialic acid on the ATCC strain of *F.magna* this knowledge of the composition of bacterial surface components may enable a thorough understanding of this clinically significant anaerobe which causes many skin infections and also interacts with other aerobes and anaerobes.

A study carried by Moncla *et al.* (1990) has shown many Gram - negative anaerobes differ in their sialidase activity. Sialidase is an enzyme which catalyses the hydrolysis of terminal sialic acid residues leaving sialic acid free to combine with the capsular polysaccharides or lipopolysaccharides (Moran *et al*.,1991) as seen in *Campylobacter jejuni* lipopolysaccharides. Sialic acid, due to its adhesive properties and its potential to bind to both bacterial and host sialic acid binding lectins could help anaerobes isolated from diabetic foot wound to adhere to other bacteria or fungi to form biofilms, one of the peculiar characteristics of the aetiology of diabetic foot ulcers, or to bind directly to the host cells. In diabetic patients the immune system is very weak and the presence of sialic acid on the microbes may prove to be an additional advantage for the microbes to colonise the already weak immuno-compromised diabetics. Sialic acid facilitates cell-cell adherence or cell-host adherence and the formation of biofilms which are often difficult to treat due to their lack of sensitivity to many antibiotics. Identifying the virulence factors may enable us to target treatment.

4.5 Conclusion

This study highlights the presence of sialic acid on *Finegoldia magna* and the importance of characterising the glycans on the glycolipids of other pathogenic anaerobes. The role played by sialic acid in the pathogenesis of the organism has yet to be determined.

CHAPTER – 5

GENERAL DISCUSSION

Infection is one of the major factors in the progression of foot ulcers in diabetics (Lipsky, 2004a and 2004b and Citron *et al.,* 2007). With a view to understand the complete microbiology of DFU, one part of the research focussed on identifying the aetiology of micro-organisms isolated from the DF ulcers of patients from India and the UK, determining antimicrobial sensitivity profiles and the establishment of confounding risk factors. This work also gathered other risk factors which may have contributed to the development of a DFU. From the data collected from the questionnaire designed to record the Indian diabetic foot patient's medical information it was found that the main confounding factors identified were peripheral vascular disease, neuropathy, ischaemia, trauma and neuroischaemia. These confounding factors were often found to be present in combinations of 2 or more signifying the severity of the condition suggestive in many studies (Tesfaye, Chaturvedi and Eaton, 2005; Pendsey, 2010 and Norgren *et al.,* 2007). The statistical significance of each risk factor to the risk of DFU could not be determined due to the low numbers with individual factors among the Indian diabetic foot patients. All the Indian diabetic foot patients had infection due to the presence of aerobes and/or anaerobes and/or fungi and many had mixed infections. Among the aerobes the most commonly cultured from the ulcers were Gram - positive aerobes mainly MRSA, MSSA and *Streptococcal* species while Gram - negative aerobes included those from the Enterobacteriaceae family. Among the fungi the most common included the *Candida* species with *Candida tropicalis* the most frequently isolated fungus among the India DF patients. *Clostridium* species were the most common aetiological agents isolated from the DF patients from India and among the Gram - negative organisms the *Fusobacterium* species and *B. ureolyticus* were most commonly seen. Similar microbial aetiology was seen in many other

studies carried out in India (Shankar *etal.,* 2005; Varaiya *et al.,* 2008 and Gupta *et al.,* 2009). Interestingly there were multi-drug resistant anaerobes isolated that had led to treatment failure with the worsening of the ulcer, causing some level of amputation either any toes of complete foot. There have been many studies in India suggesting that high glycaemic levels, lack of education, compromise in personal hygiene, poverty and superstitions to be the factors that may have contributed to the development of ulcer and added to the worsening of the ulcers (Ramachandran *et al.,* 2004; Pradeepa *et al.,* 2002 and Jayasinghe *et al.,* 2007). In contrast, the study carried out in the UK where patients received treatment at an early stage of the disease revealed less organisms resistant to antibiotics as compared to the multi-drug resistant organisms from the Indian DFU patients. Linezolid was the only antibiotic to which a high number of organisms were susceptible among the Indian patients while, based on antibiotic sensitivity testing, the combination antibiotic piperacillin/tazobactam followed by other antibiotics such as amikacin and levofloxacin were the antibiotics of choice for the DF patients from the UK. The results obtained for the Indian cohort have been used by Raheja Hospital in re-evaluating their testing and treatment regimens.

As outlined in chapter 2, the original aims of this research were to identify the glycans expressed by the bacteria and to explore their role in pathogenesis**.** The glycans expressed by bacteria were identified using commercially available biotinylated lectins on whole cells, Proteinase K treated whole cells and extracted glycolipids. However, it was very difficult to attach the bacterium onto the 96 well ELISA plates. Troubleshooting was required and an in house ELLA method was developed based on the work of Afrough *et al.,* (2007). Glycan – lectin interactions were carried out only on those aerobes and anaerobes that had been identified as predominantly found in DF. Interpretations of the structure based on lectin interaction utilised, in the main data from eukaryotic systems and should therefore be viewed with caution. Indeed, given the promiscuity of some of the lectins, caution needs to be used in assigning structures based solely

on lectin analysis. Experimental characterisation of the glycan-lectin interactions for a number of aerobes demonstrated that most of the Gram - positive aerobes such as *MRSA* (NCTC 33591) expressed β-Gal, α and β terminal GalNAc, Gal β1-3GalNAc, terminal α/ β GlcNAc, GlcNac, GlcNAc with sialic acid, α – 2,6 sialic acid Gal and α-fucose and Fucα1,2Gal as glycan structures. MSSA (NCTC 6571) expressed terminal α Gal, α–GalNac, α–GalNac with 1,2 Fuc ,α- Man, complex Man with core α-fucose, α-glucose, terminal GlcNac, β-GlcNAc, GlcNAc1,4 oligomers including chitobiose and chitotriose which is in acceptance with the study carried out by Silfkin and Doyle(1990), however, they suggested that there was no terminal β-GalNAc or Fucα1,2 Gal. MRSE however expressed complex mannose structures, along with Gal β1-3GalNAc, GlcNAc/sialic acid and GlcNac while MSSE expressed Gal β1-3GalNAc, terminal α-GalNAc, GalNAcα1,3[Fucα1,2]Gal, Gal β1-3GalNAc, GlcNAc/sialic acid, β1-4 GlcNAc and LacNAc oligomers, GlcNac and $(Glc\beta1,4NAc)_n$, chitotriose. The glycans specific to MSSE include Gal β1-3GalNAc, terminal α-N-acetylgalactosamine, GalNAcα1,3 [Fucα1,2]Gal, Gal β1-3GalNAc, GlcNAc/sialic acid, β1-4 GlcNAc and LacNAc oligomers, GlcNac and $(Glc\beta1,4NAc)_n$, chitotriose. The Gram - negative aerobes expressed α and β terminal GalNAc in *E. coli* as well as GalNAcα1-O-ser, α-fucose, Fucα1,2 Gal, β-GlcNAc, sialic acid, β1,4-GlcNAc oligomers and the complex structures of mannose. While *P. mirabilis* expresses α-Gal, α-N-GalN, Galα1,3 Gal, α and β-GalNac, terminal α-GalNAc complex structures of mannose. *S. pyogenes* shows high mannose structures including the tri and the tetra antennary. *E. faecalis* expresses Gal, α-GalNAc, terminal α-N GalNAc, GalNAcα1,3 [Fucα1,2]Gal, α-fucose, Fucα1,2Gal, β-GlcNAc, sialic acid and α – 2,6 sialic acid linked to Gal. Protein glycosylation was not considered to occur to any extent among the prokaryotes but recently, there have been studies suggesting that glycoproteins are present in bacteria and they have a role to play in cell-cell adhesion and pathogenesis (Benz and Schmidt, 2002; Bishop and

Gangneux, 2007; Tuomanen, 1996;Zhou and Wu, 2009 and Schmidt, Riley and Benz, 2003).

The identification of the glycans expressed by anaerobic bacteria was significant as there is very limited research carried out in this area. In DF patients the presence of anaerobes in particular worsens the ulcer especially when they are present in a polymicrobial mix along with the aerobes. There were differences in the glycans that were expressed by the most frequently isolated Gram - positive anaerobes such as *F.magna, Parvi.micra, Peptoni. asaccharolyticus, P.anaerobius* and the Gram - negative anaerobes. Although the aims of the research were originally to identify the glycans expressed by the bacteria, the ELLA results identified unique glycan-lectin binding patterns that could discriminate between organisms. It is clear that there is potential to explore the glycan-lectin interactions in the microbial diagnostics. The results showed the potential of glycan-lectin binding patterns in the rapid tying of *Staphylococcus* species mainly the methicillin sensitive and methicillin resistant *Staphylococcus aureus* and *Staphylococcus epidermidis*. In the ELLA the biotinylated lectins which were specifically detecting MRSA and MSSA were SBA, LC, EC, VV, LE, GI, UEA and Con A with varied levels of interaction. The lectins shown to be specific to MRSE and MSSE were Sol, SBA, DB, SWGA and PVE with different levels of activity. There were also specific binding patterns seen among the Gram - positive and Gram - negative anaerobes suggesting ELLA may be useful in the typing of anaerobes including the *Peptostreptococcus* lectin binding patterns, if sensitivity and specificity were proven with large numbers of clinical isolates, and hence could be used as a typing tool. In the current study, the glycan-lectin binding patterns reported were those seen among the ATCC, NCTC and AC strains. Clearly the study needs to be expanded to analyse statically significant clinical isolates to determine general utility. However, the results suggest that glycan analysis could be used in the development of a testing/typing kit.

Glycan analysis is a specialist area and the work was carried out under supervision of experts at the Complex Carbohydrate research Centre (CCRC) at the University of Georgia. A complete characterisation of the glycolipids of *Finegoldia magna* was achieved. This Gram - positive strict anaerobe is found in many soft tissue infections and is reported to be one of the most commonly isolated pathogens from the DF ulcers (Citron *et al.,* 2007). The glycans detected by the gas chromatography-mass spectrometry (GC-MS) were glucose, mannose, galactose and N-acetylglucosamine and these results correlated with those obtained by the ELLA. Linkage analysis by GC-MS suggested the presence of 1, 3 mannose and terminal mannose along with 2 and 3 linked rhamnose. The composition analysis of the lipids of *F.magna* by GC-MS suggested the presence of the C-14 (Myristic acid) C-16 (Palmitic acid) and C -18 (Stearic acids).

High pH anion exchange chromatography (HPAEC) suggested the presence of sialic acid, the glycan which has often been reported to be involved in the pathogenesis in bacteria. *Sambucus nigra* lectin which is specific for α – 2, 6 sialic acid linked to galactose also showed binding to the *F.magna* by the ELLA method confirming the data. These organisms probably utilise sialic acid either in attachment to lectins in the host or on to other organisms. *F.magna* is often isolated from biofilms and it is possible that glycan- lectin interactions act to stabilise these structures. Further work was therefore channelled towards determining whether other anaerobes also expressed sialic acid The Warren assay suggested the presence of sialic acid on glycolipids isolated from *Finegoldia magna, Peptostreptococcus anaerobius, Prevotella bivia* and *Fusobacterium nucleatum* but not those of *Peptoniphilus assachrolyticus, Parvimonas micra, Anaerococcus prevottii* and *Bacteroides fragilis.* Interestingly other studies have shown the presence of sialic acid on *Bacteroides fragilis* groups in contrast to our study (Severi *et al*., 2007). This could be due to the different strains used or differences in culture and harvesting that affected gene expression (Takashashi *et al.,* 2006 and Ruivo *et al.,* 2008).

Hence in the work described should be expanded to look at sialic acid expression by various strains organisms of pathological and non pathological origin grown in standardised conditions.

CHAPTER – 6

FUTURE WORK

The analysis of the glycans expressed by micro-organisms would provide a clearer picture of the glycome of bacteria and allow us to determine whether glycans have any role in pathogenesis. The increase in antibiotic resistant bacterial pathogens makes it vital to find new means of treating such bacteria Sharon, (1987) have proposed anti-adhesion therapy, and using carbohydrates to block lectin-glycan mediated attachment or adhesion of the bacteria to the host tissues at the early stages of infection. This strategy can be utilised without fear of toxicity as the method utilises saccharides which are non-toxic, cannot induce bacterial resistance and are relatively less harmful than chemicals (Ofek, Hasty and Sharon, 2003). Some however can stimulate immune response in the host (Tuomanen, 1996). This approach could be used as a preventive measure rather than the treatment measure in those infections that are glycan – lectin mediated. There are number of infections that are initiated by adhesion of the pathogenic organisms to the cells and the mucosal surfaces of the host such as (Sharon and Ofek, 2000). Adhesion is required so that the organism remains unaffected by the host clearing mechanisms and natural immune defence. If this adhesion is targeted then many infections could be treated using a similar strategy. These methods may work best when the treatment is topically rather than systemically applied as the blocking molecules would have direct access to the bacteria.

Treatment of diabetic foot infections relies on antibiotics and on surgical intervention (Lipsky, 2004a). These methods can lead to long - term impairment of normal routine life and increased hospital stay without a complete cure. There is a need to explore other ways that the wound can be treated effectively, including targeting directly by inhibiting the organisms from adhering to the host. By understanding the mechanisms of carbohydrate specific adhesion, bacterial infections *in vivo* could be

prevented (Ofek, Hasty and Sharon, 2003 and Battin *et al.,* 2007). The presence of the sialic acid on the anaerobe *F.magna* suggests an involvement in pathogenicity (Takahashi *et al.,* 2006; Severi, Hood and Thomas, 2007 and Ruivo *et al.,* 2008). If proven, sialic acid analogues in a topical cream could be prepared which would block the glycan binding sites of the lectins and prevent attachment of *F.magna*. Indeed, if sialic acid – lectin binding is also a key feature of biofilm production through binding of sialic acid on one microbe to a sialic acid binding residue on another, saccharides could be used to disrupt these structures and hence improve access of other antimicrobial agents (Rickard *et al*., 2003; Battin *et al*., 2007 and Lloyd *et al.,* 2007). Further work to understand how they may be utilised needs to be explored.

The cohort study carried out in India and the UK could be further extended to recruit more patients and then identify the risk factors and statistically analyse the contribution of individual factors to the risk of DFI. The antibiotic susceptibility testing also could be carried out in such as way that there are more number of organisms belonging to the same species to obtain statistically significant results and is a confirmative conclusion based on a high number of organisms belonging to the same species. The research design could also be extended by including the microbial flora before and after amputations along with the difference in the risk factors. Surveillance studies could be carried out to compare the antibiotic susceptibility patterns from India and UK with the sample size of 100 organisms belonging to the same species. However, given the low numbers of some organisms isolated, thousands of patients would need to be recruited.

Diabetic foot infections often are polymicrobial with a peculiar characteristic of biofilm (Dowd *et al.,* 2008) It would be interesting to identify the glycans expressed using the in house developed ELLA method with biotinylated lectins on biofilms produced by combination of 2 or more organisms (1 facultative aerobe and 1 strict anaerobe) from India and UK. Biofilms could be created in the laboratory using a bioreactor (Goeres *et*

al., 2005) or Calgary^{Tm} 96 well polystyrene ELISA plate system with pegs (Ceri *et al*., 1999, Olfson *et al.,* 2002). Confocal microscope can be used to visualise the exopolysaccharides layer on the formed biofilms using fluorescent lectins (Windernder *et al.,* 2001). 'LIVE/DEAD Baclight Bacterial Viability Kits' would be utilised to determine the presence of biofilms (Harrison *et al*., 2006 and Neu, Swerhone and Lawrence, 2001).

In vitro studies would also allow research into the interactions of keratinocyte cell lines and bacterial glycans. On identification of glycans from the outer membranes of pathogens from India and UK DF patients, *in vitro* studies using cell culture could then be used to determine whether:

a) Pathogens bind to keratinocyte cell lines in a culture system.
b) Pre - treatment of cells with sugars (i.e. blocking of host lectins) prevent bacteria binding.
c) Pre - treatment with lectins (i.e. blocking of host sugars) stop bacteria binding.
d) Cells pre-treated with specific glycans bind differently.
e) There is a difference in glycan expression in single colony culture, biofilms and patient samples and if the interaction is mediated through lectin binding and most importantly if there are differences in the glycan – lectin expressions seen from India and the UK.

The development of a rapid typing and identification method is now feasible with the preliminary information provided by this study. Further work could be channelled towards obtaining the glycan-lectin profile on clinical strains and to compare them with the already obtained glycan-lectin profiles of individual organisms. Finally strains vary from different geographical locations could be analysed to determine, for example, whether those organisms in the Indian cohorts had the same glycan profiles as those from the cohort in the UK.

A diagnostic kit with a panel of biotinylated lectins can be created which is practical as well as economical and affordable in clinical laboratories. The reproducibility of the kit can be determined statistically by carrying out the tests on a number of strains from different genus. Automation in clinical microbiology is still in a very early stage of development compared to the level of automation in other disciplines of science. Glycan–lectin microarrays can be developed with the aim to introduce automation along with user friendly microbial diagnostics.

In conclusion, therefore this work has increased our understanding of anaerobes and their interactions. This study has provided us with preliminary information on the glycan-lectin profiles of number of aerobes and anaerobes. It has highlighted the potential of glycan-lectin interactions in microbial diagnostics. The AST data has identified the multi - drug resistant organisms and would alert the clinicians to limit the usage and to introduce alternative therapy to target infections.

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APPENDICES

Appendix I
Ethics approval letters from UK and India

Appendix II
Patient's consent form

Appendix III
Patient's questionnaire for the Indian Cohort study

Appendix IV
Society for General Microbiology President's fund

Appendix V
Indian diabetic foot patients' wound pictures (Wagner's grades)

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Glycan profile of whole cells of MSSA by ELLA
Appendix VIB
Glycan profile of Proteinase K treated whole cells of MSSA by ELLA
Appendix VIC
Glycan profile of glycolipids of MSSA by ELLA

Appendix VIIA
Glycan profile of whole cells of MRSA by ELLA
Appendix VIIB
Glycan profile of Proteinase K treated whole cells of MRSA by ELLA
Appendix VIIC
Glycan profile of glycolipids of MRSA by ELLA

Appendix VIIIA
Glycan profile of whole cells of MSSE by ELLA
Appendix VIIIB
Glycan profile of Proteinase K treated whole cells of MSSE by ELLA
Appendix VIIIC
Glycan profile of glycolipids of MSSE by ELLA

Appendix IXA
Glycan profile of whole cells of MRSE by ELLA
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Appendix IXC
Glycan profile of glycolipids of MRSE by ELLA

Appendix XA
Glycan profile of whole cells of *Escherichia coli* by ELLA
Appendix XB
Glycan profile of whole cells of *Pseudomonas aeruginosa* by ELLA
Appendix XC
Glycan profile of whole cells of *Proteus mirabilis* by ELLA
Appendix XD

Glycan profile of whole cells of *E. faecalis* by ELLA

Appendix XE

Glycan profile of whole cells of *Streptococcus pyogenes* by ELLA

Appendix XIA

Glycan profile of whole cells of *Finegoldia magna* by ELLA

Appendix XIB

Glycan profile of Proteinase K treated whole cells of *Finegoldia magna* by ELLA

Appendix XIC

Glycan profile of glycolipids of *Finegoldia magna* by ELLA

Appendix XIIA

Glycan profile of whole cells of *Prevotella bivia* by ELLA

Appendix XIIB

Glycan profile of glycolipids of *Prevotella bivia* by ELLA

Appendix XIIIA

Glycan profile of whole cells of *Parvimonas micros* by ELLA

Appendix XIIIB

Glycan profile of glycolipids of *Parvimonas micros* by ELLA

Appendix XIVA

Glycan profile of whole cells of *Propionibacterium acnes* by ELLA

Appendix XIVB

Glycan profile of glycolipids of *Propionibacterium acnes* by ELLA

Appendix XVA

Glycan profile of whole cells of *Anaerococcus prevottii* by ELLA

Appendix XVB

Glycan profile of glycolipids of *Anaerococcus prevottii* by ELLA

Appendix XVIA

Glycan profile of glycolipids of *Fusobacterium nucleatum* by ELLA

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Glycan profile of glycolipids of *Peptostreptococcus asaccharolyticus* by ELLA

Appendix XVIC

Glycan profile of glycolipids of *Bacteroides fragilis* by ELLA

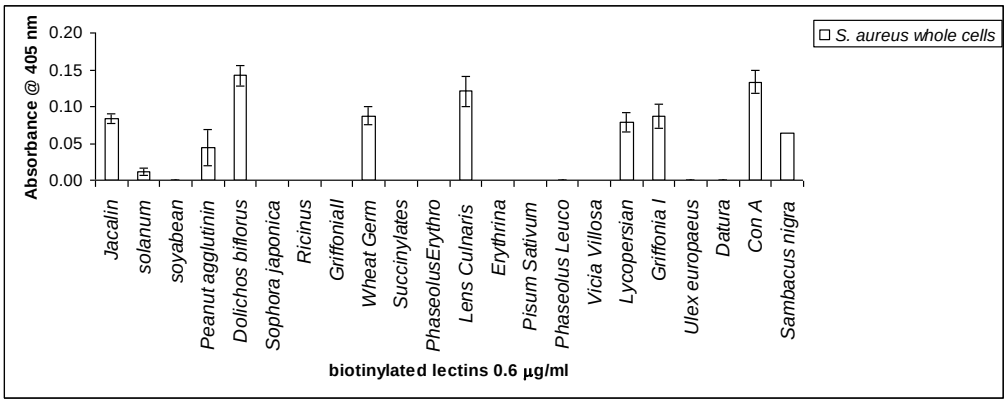
Appendix XVII

Standard chromatogram of the different monosaccharides by GC-MS

Appendix XVIII

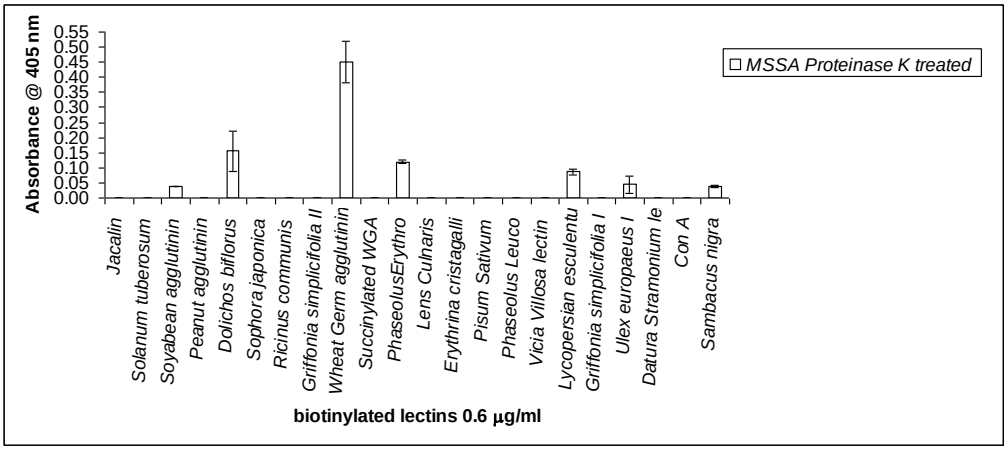
Posters, presentations and potential publications

Appendix VI

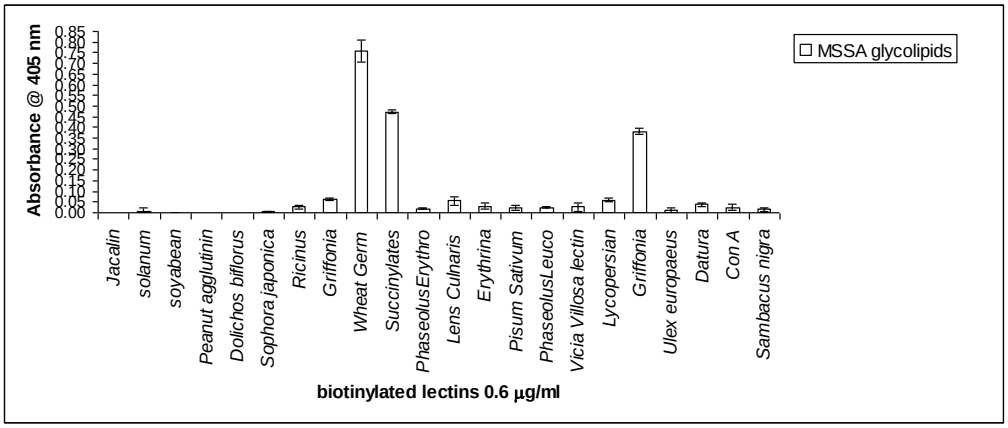


ELLA was also carried out on tests with no lectin and no streptavidin added which were negative controls. The data is the mean result of three independent experiments

VIA) Glycan profile of the whole cells of MSSA by ELLA

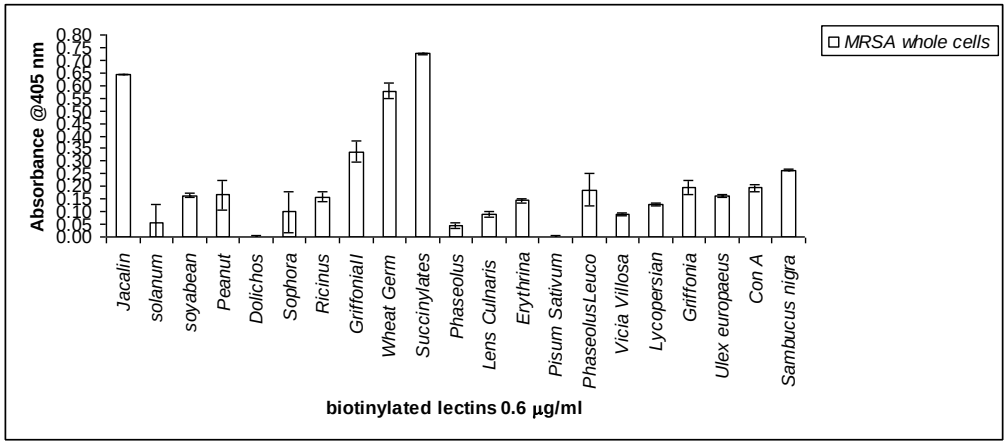


VIB) Glycan profile of Proteinase K treated whole cells of MSSA by ELLA

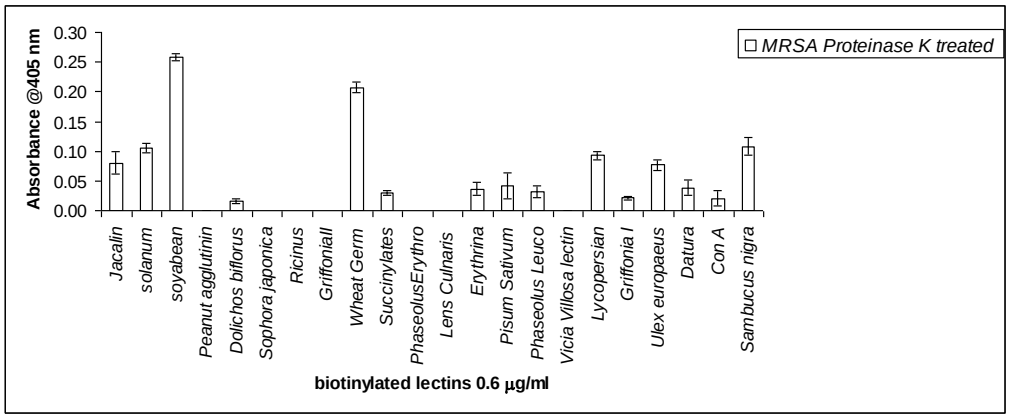


VIC) Glycan profile of glycolipids of MSSA by ELLA

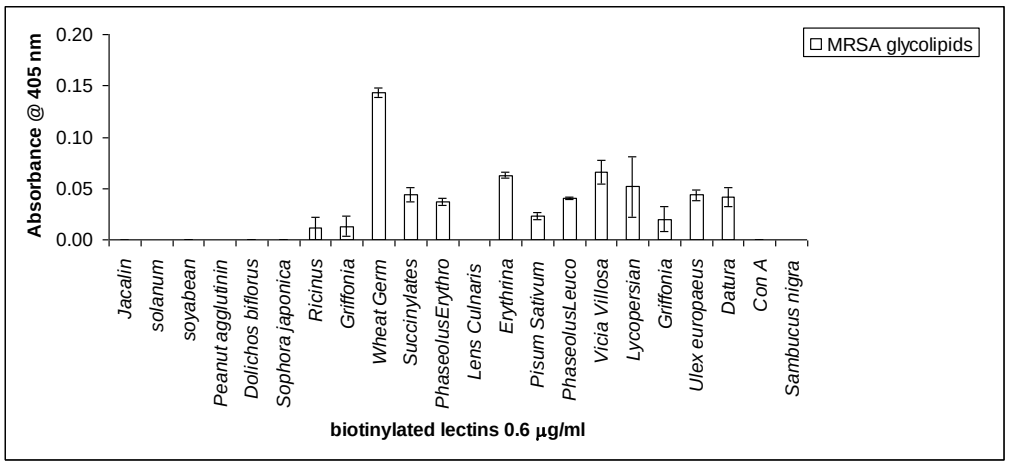
Appendix VII



VIIA) Glycan profile of the whole cells of MRSA by ELLA

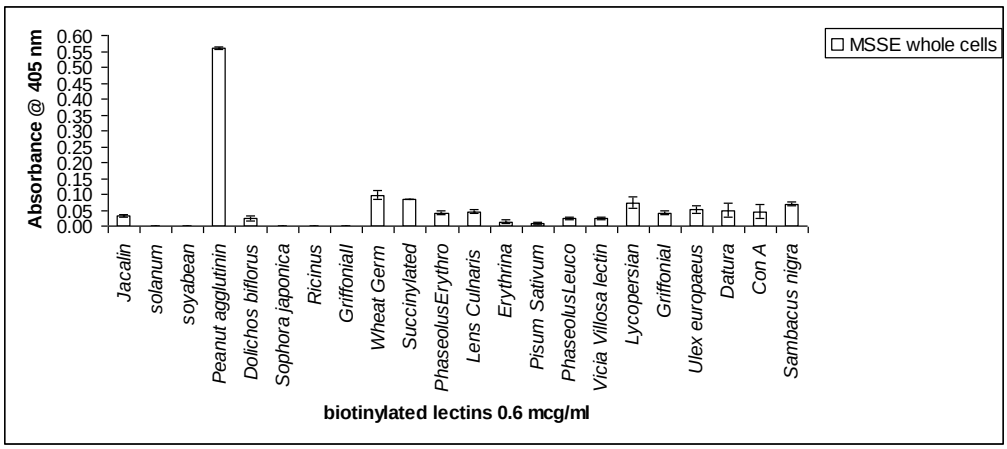


VIIB) Glycan profile of Proteinase K treated whole cells of MRSA by ELLA

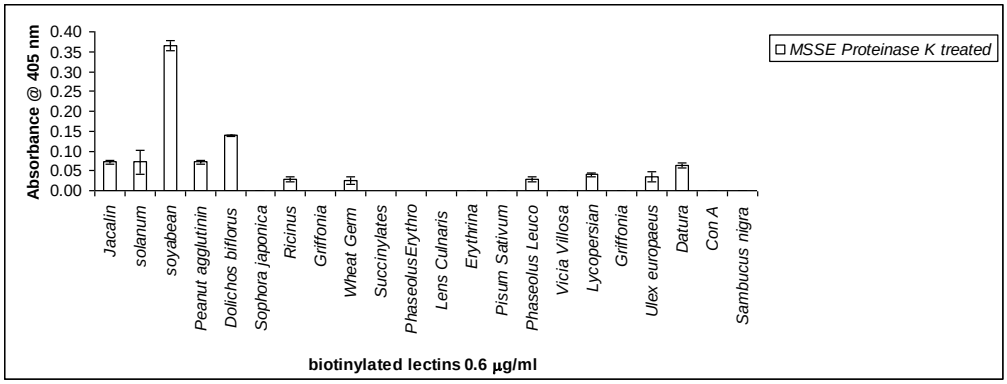


VIIC) Glycan profile of glycolipids of MRSA by ELLA

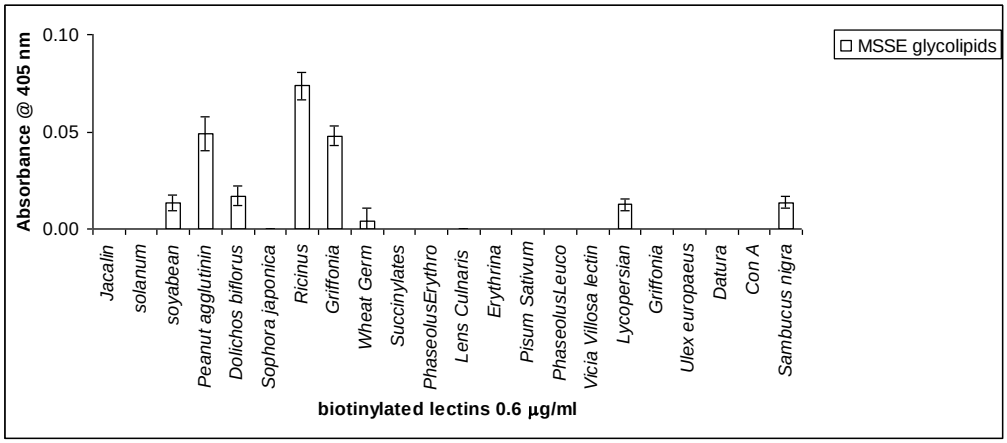
Appendix VIII



VIIIA) Glycan profile of whole cells of MSSE by ELLA

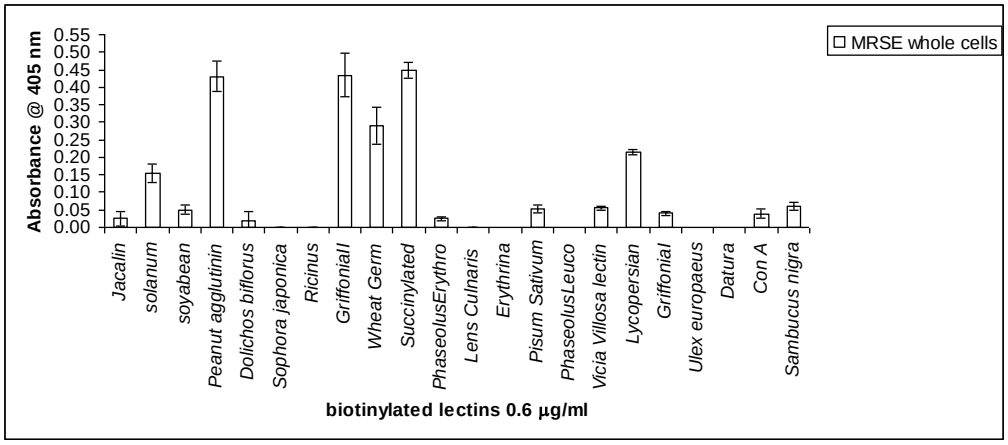


VIIIB) Glycan profile of Proteinase K treated cells of MSSE by ELLA

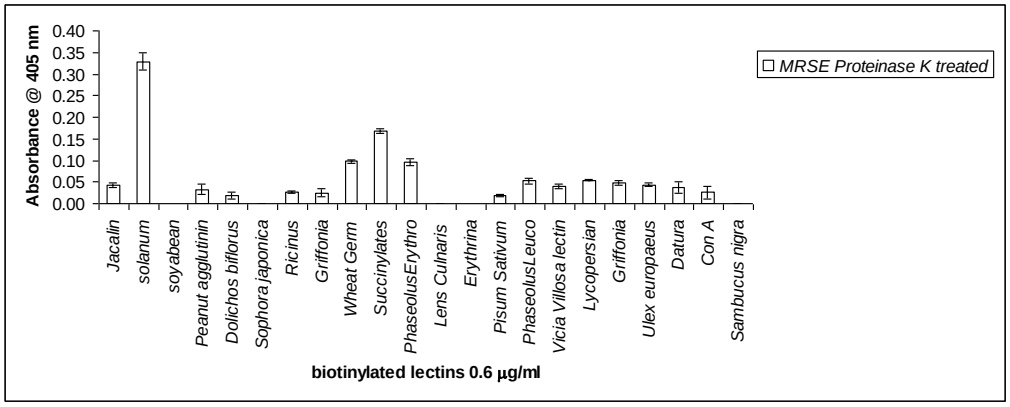


VIIIC) Glycan profile of glycolipids of MSSE by ELLA

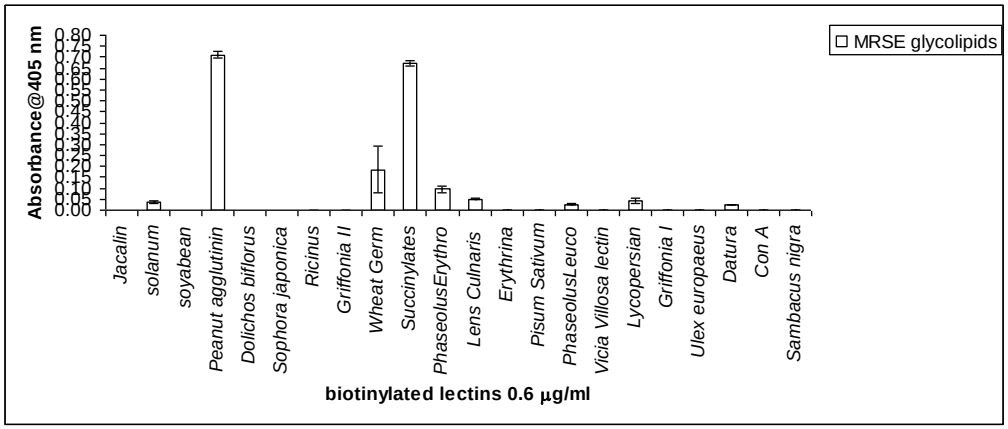
Appendix IX



IXA) Glycan profile of whole cells of MRSE by ELLA

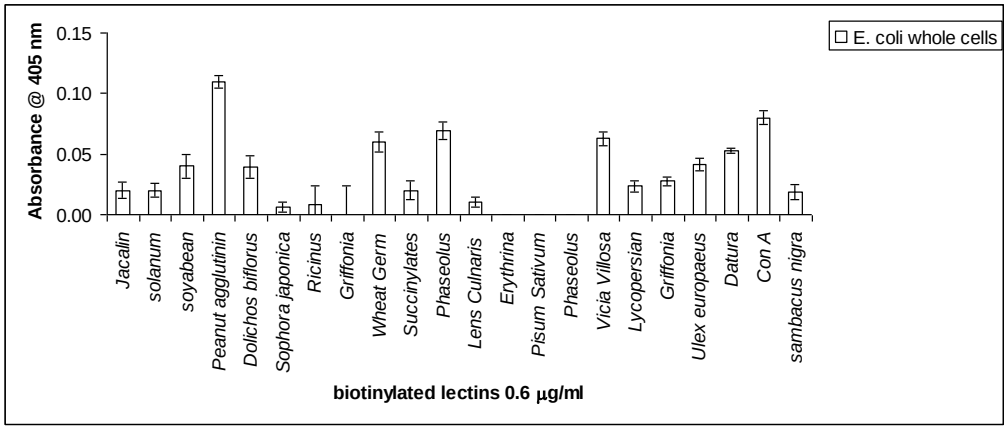


IXB) Glycan profile of Proteinase K treated whole cells of MRSE by ELLA

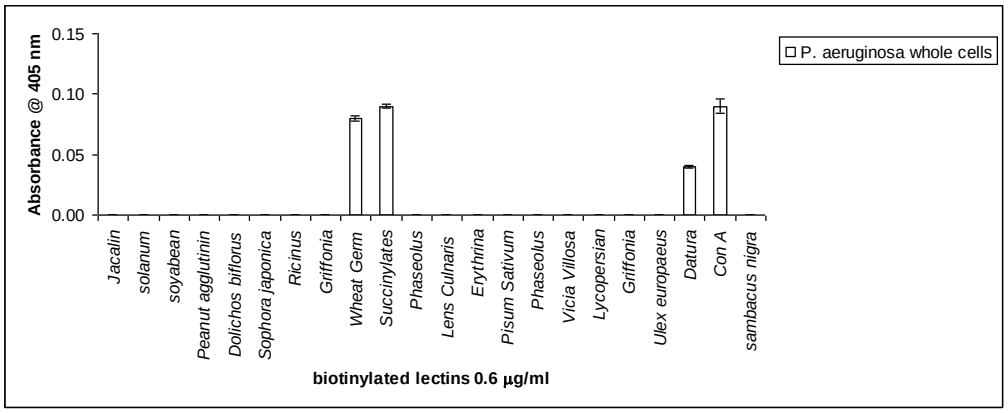


IXC) Glycan profile of glycolipids of MRSE by ELLA

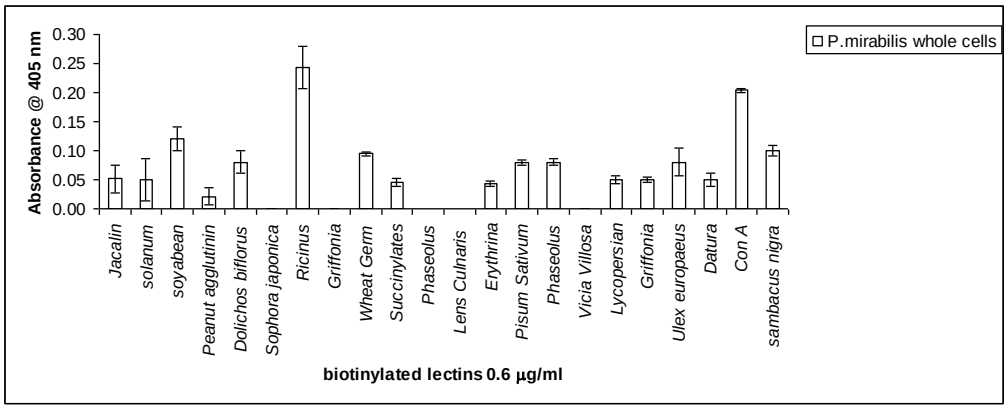
Appendix X



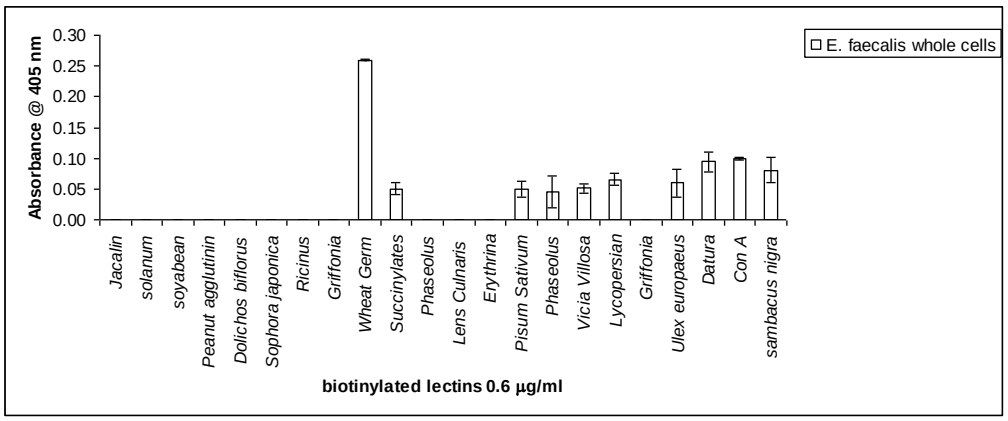
XA) Glycan profile of whole cells of *Escherichia coli* by ELLA



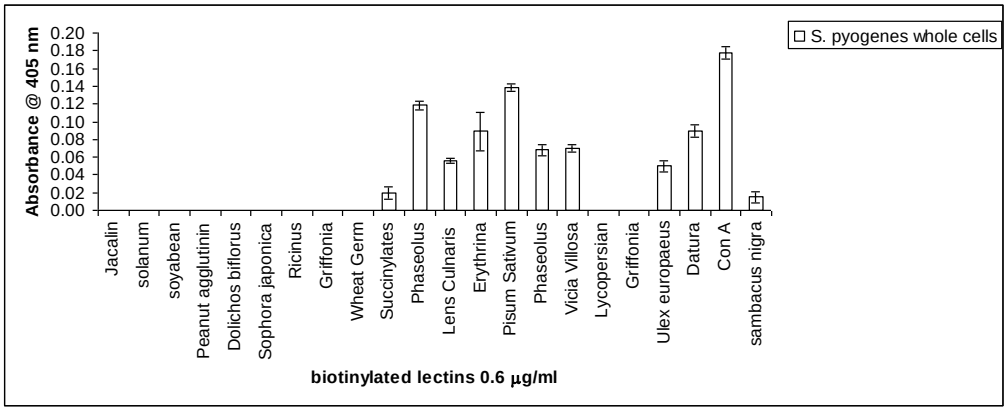
XB) Glycan profile of whole cells of *Pseudomonas aeruginosa* by ELLA



XC) Glycan profile of whole cells of *Proteus mirabilis* by ELLA

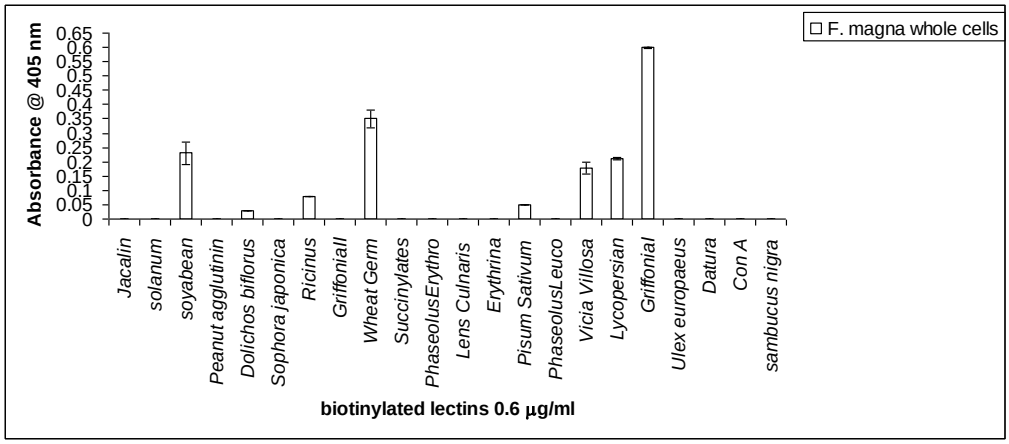


XD) Glycan profile of whole cells of *E. faecalis* by ELLA

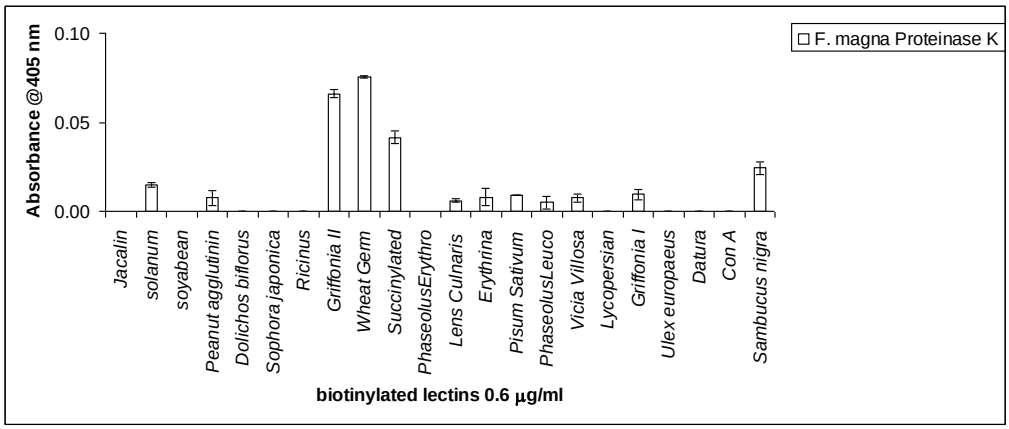


XE) Glycan profile of whole cells of *Streptococcus pyogenes* by ELLA

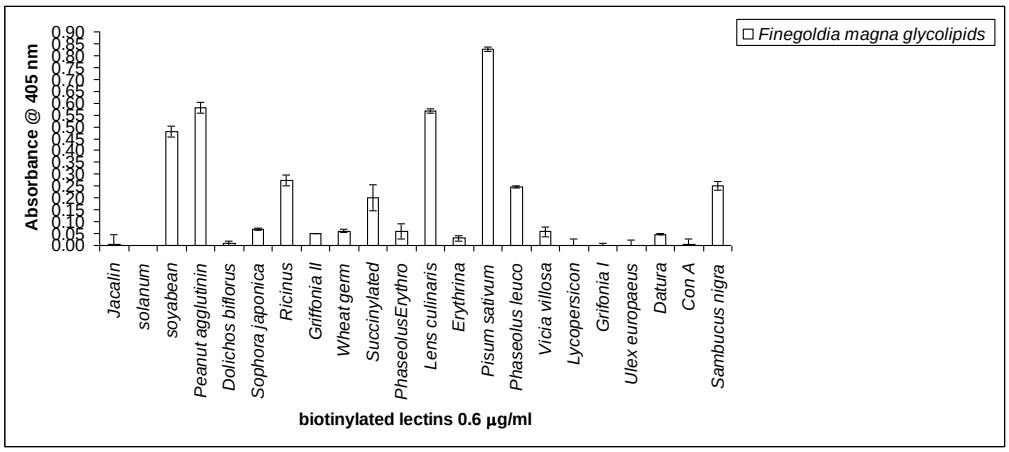
Appendix XI



XIA) Glycan profile of whole cells of *Finegoldia magna* by ELLA

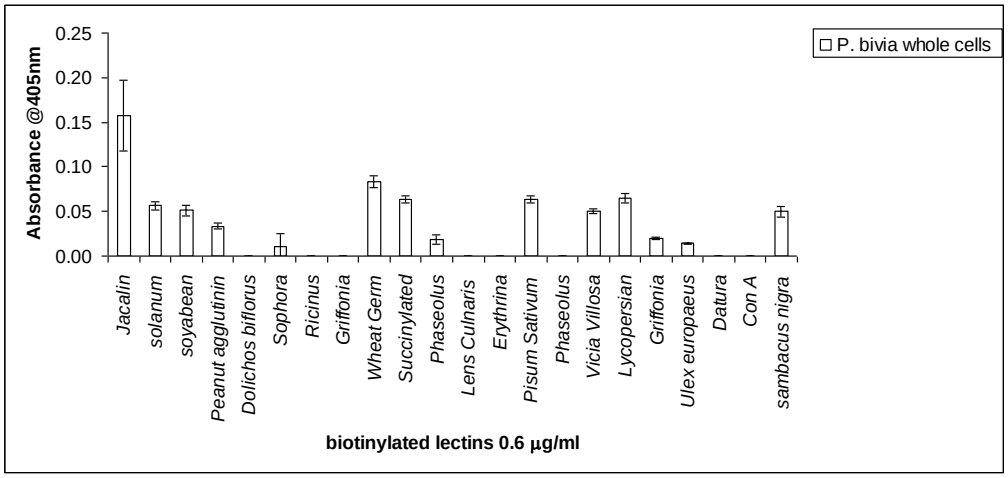


XIB) Glycan profile of Proteinase K treated whole cells of *Finegoldia magna* by ELLA

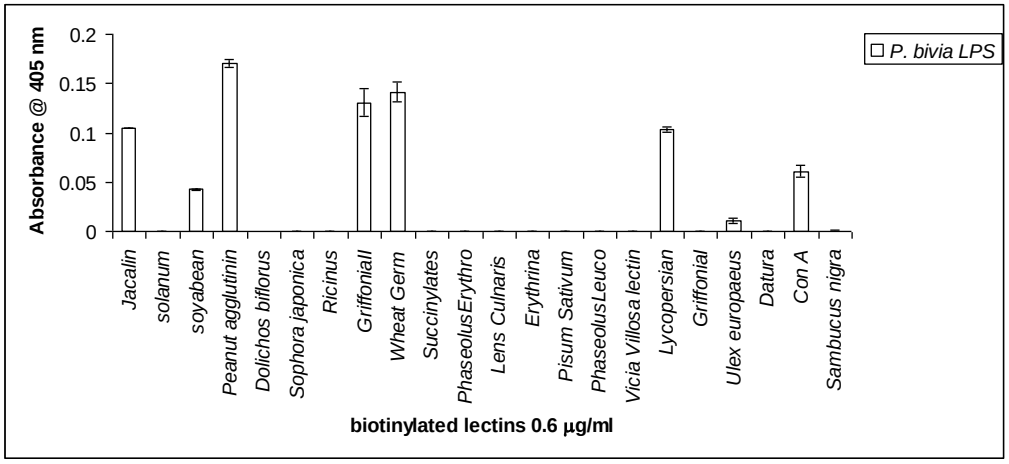


XIC) Glycan profile of glycolipids of *Finegoldia magna* by ELLA

Appendix XII

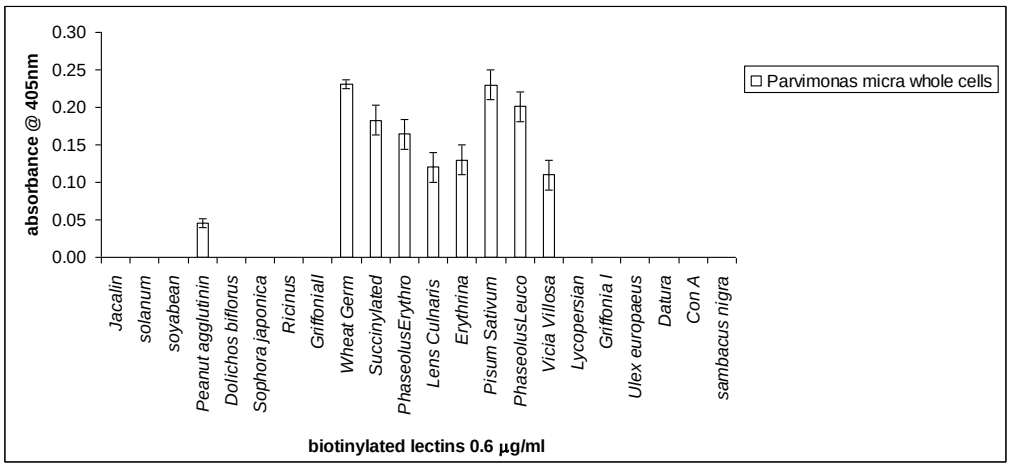


XIIA) Glycan profile of whole cells of *Prevotella bivia* by ELLA

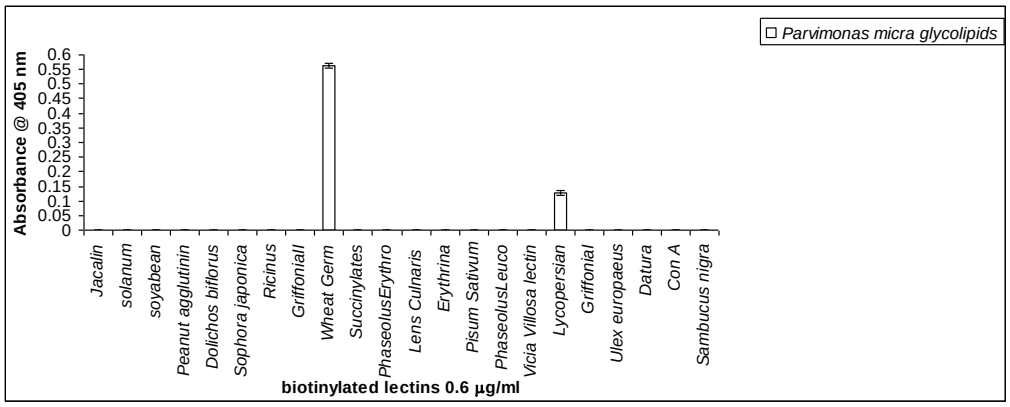


XIIB) Glycan profile of LPS of *Prevotella bivia* by ELLA

Appendix XIII

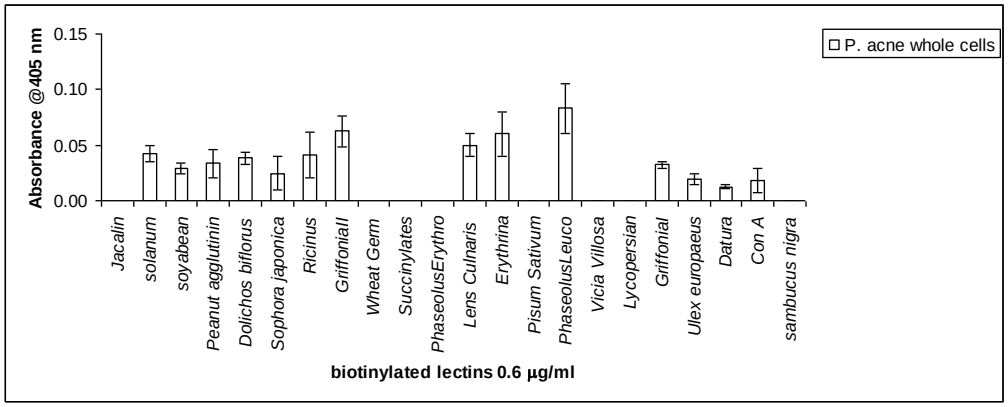


XIIIA) Glycan profile of whole cells of *Parvimonas micros* by ELLA

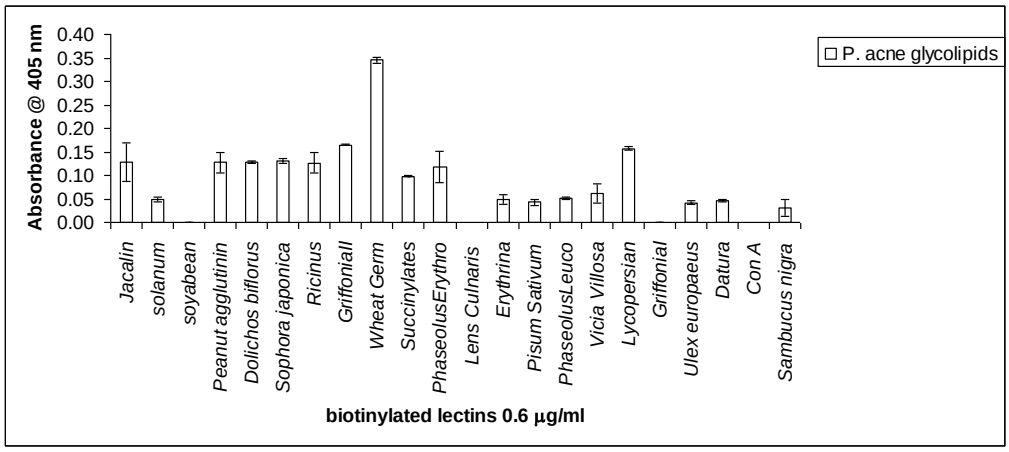


XIIIB) Glycan profile of glycolipids of *Parvimonas micros* by ELLA

Appendix XIV

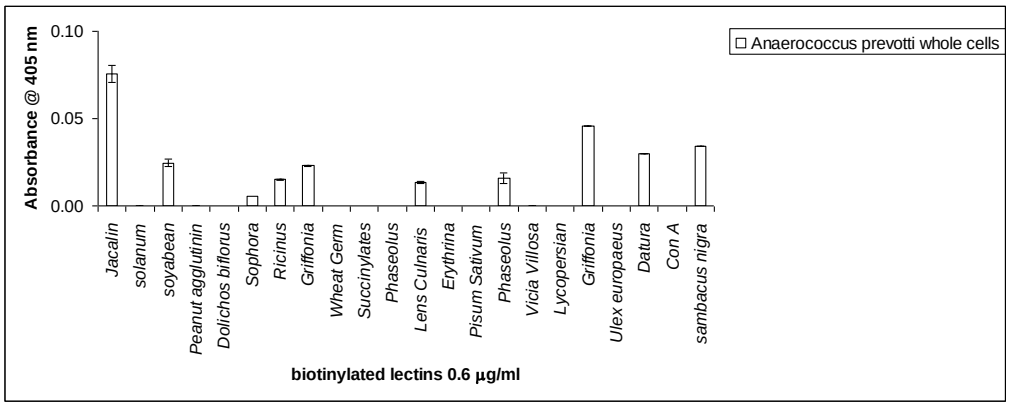


XIVA) Glycan profile of whole cells of *Propionibacterium acnes* by ELLA

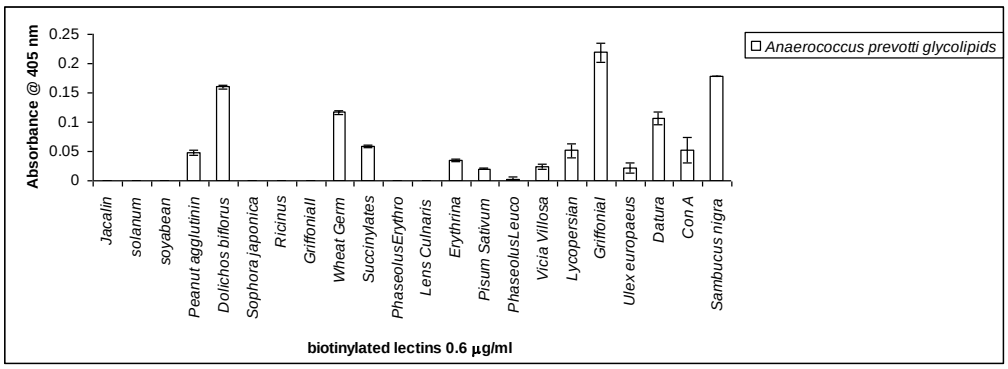


XIVB) Glycan profile of glycolipids of *Propionibacterium acnes* by ELLA

Appendix XV

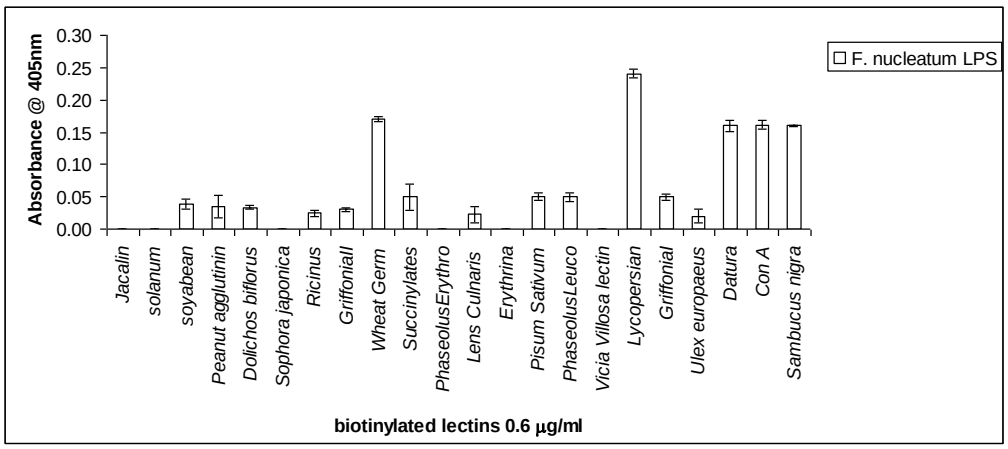


XVA) Glycan profile of whole cells of *Anaerococcus prevottii* by ELLA

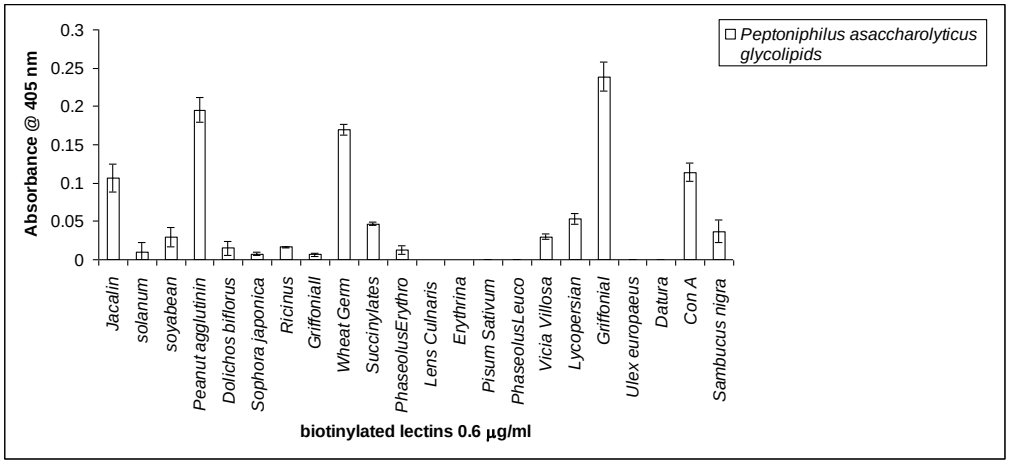


XVB) Glycan profile of glycolipids of *Anaerococcus prevottii* by ELLA

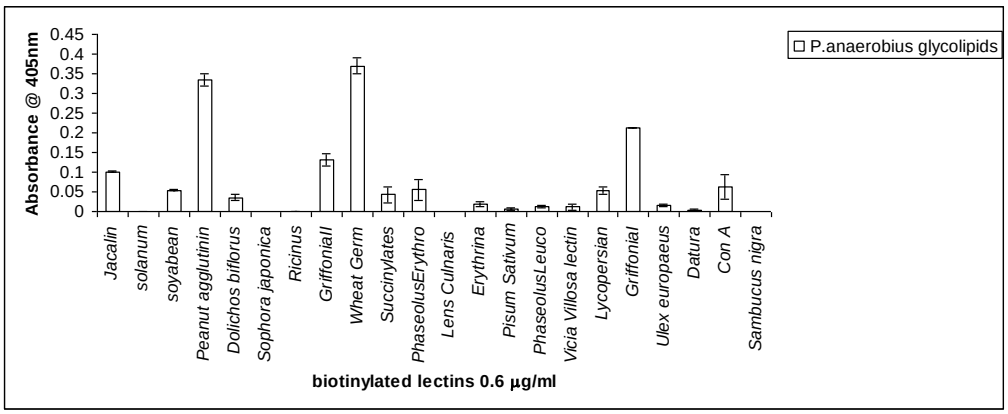
Appendix XVI



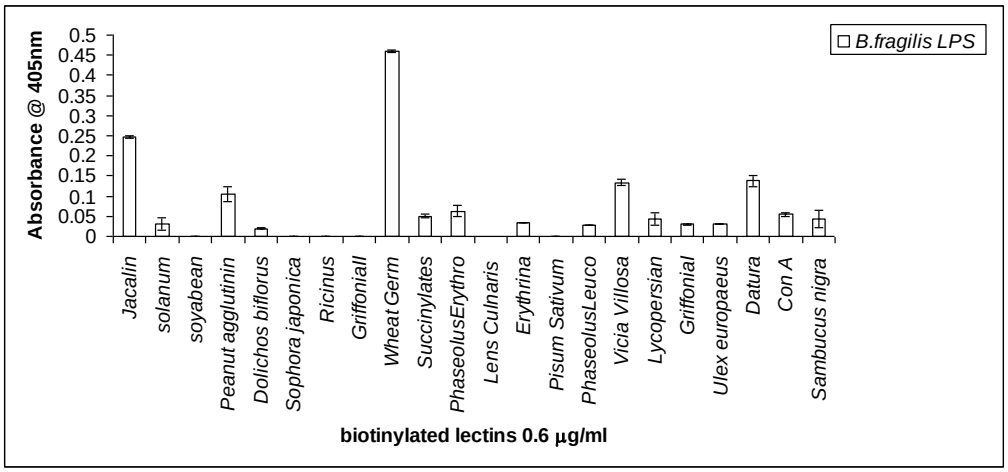
XVIA) Glycan profile of glycolipids of *Fusobacterium nucleatum* by ELLA



XVIB) Glycan profile of glycolipids of *Peptoniphilus asaccharolyticus* by ELLA



XVIC) Glycan profile of glycolipids of *Peptostreptococcus anaerobius* by ELLA



XVID) Glycan profile of glycolipids of *Bacteroides fragilis* by ELLA

Appendix XVII
Standard chromatogram of the different monosaccharides by GC-MS

Peak (Std)	Retention time	Width	Area	Start time	End time
Rhamnose	13.404	0.054	3783129	13.315	13.535
Rhamnose	13.706	0.059	330543	13.645	13.795
Xylose	15.669	0.065	2611185	15.532	15.877
Xylose	16.419	0.066	1336197	16.278	16.557
Mannose	21.170	0.069	4465559	20.966	21.308
Mannose	21.498	0.067	622749	21.388	21.594
Galactose	22.742	0.068	2454944	22.607	22.856
Galactose	23.018	0.076	195671	22.945	23.122
GluNAc	25.320	0.071	956160	23.953	24.122
GluNAc	25.610	0.087	495968	25.091	25.436
GalNAc	29.662	0.074	511284	29.507	29.758
GalNAc	31.731	0.070	903089	31.615	31.852
Inositol	34.731	0.089	3403077	34.601	34.872

The table below in Appendix XVII represents the standard of different monosaccharides and internal standard with their retention times, area, width, start time and their end time that were run on the column SP2330 fused silica, Supelco. The retention times of these standards were used to determine the composition of *Finegoldia magna* glycans.

Appendix XVIII
List of poster, presentations and potential publications

Posters and presentations

1) Pathogenesis of diabetic foot Infections; The role of glycans – poster presented at the conference held by International society of chemotherapy and infection in Canada.

2) Antibiotic study of infected flora of diabetic foot ulcers in UK and Indian populations – poster presented at the International society of chemotherapy and infection in Canada.

3) Role of glycans in the pathogenesis of diabetic foot Infections – poster presented at the conference organised by the Institute of biomedical science.

4) Role of glycans in the pathogenesis of diabetic foot Infections – oral presentation at the conference organised by the Institute of biomedical science.

5) Antibiotic resistance and the role of glycans in Diabetic Foot infections – oral presentation at the conference organised by the Society for General Microbiology.

Potential publications

1) Aerobes, anaerobes and fungi from the diabetic foot patient's from India and the UK

2) Comparison of the antibiotic susceptibility profiles from diabetic foot cohorts from India and UK

3) A comparative view on distribution of aerobes, anaerobes and fungi on the pus and tissues specimens from the Indian diabetic foot patients

4) Glycans of *Finegoldia magna*: a role in pathogenesis of diabetic foot infections?

5) Glycan – Lectin interactions: any scope for diagnostics

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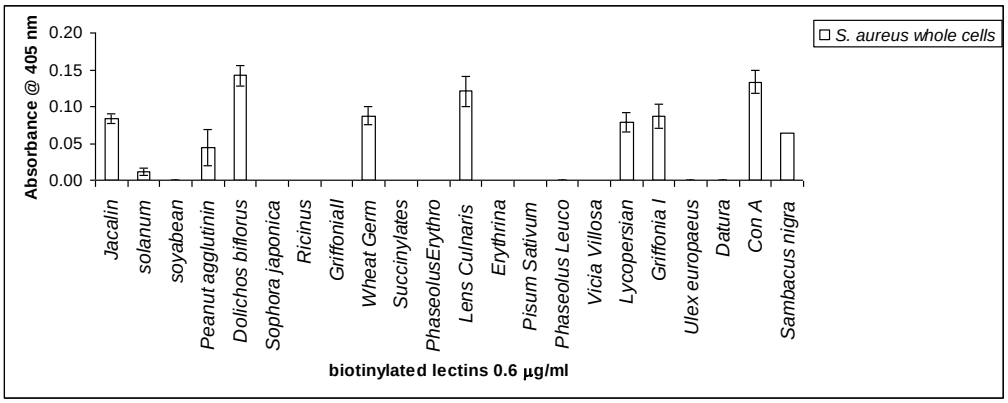
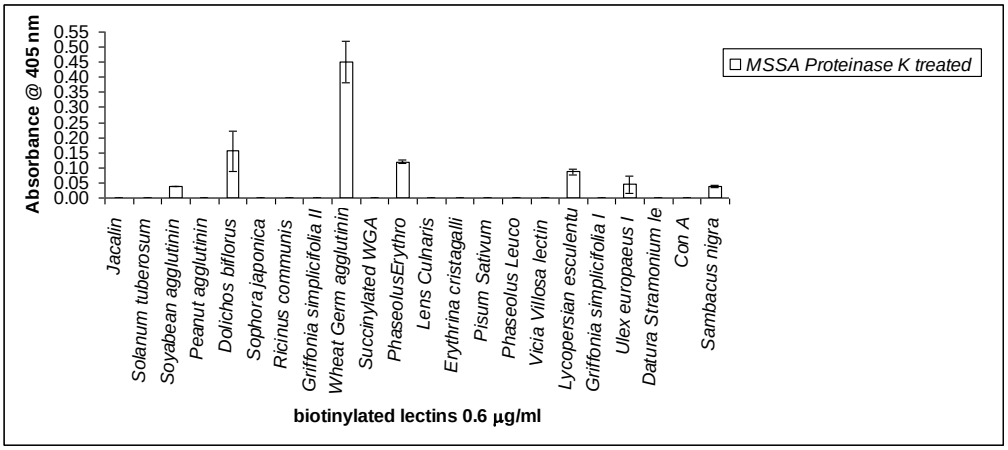
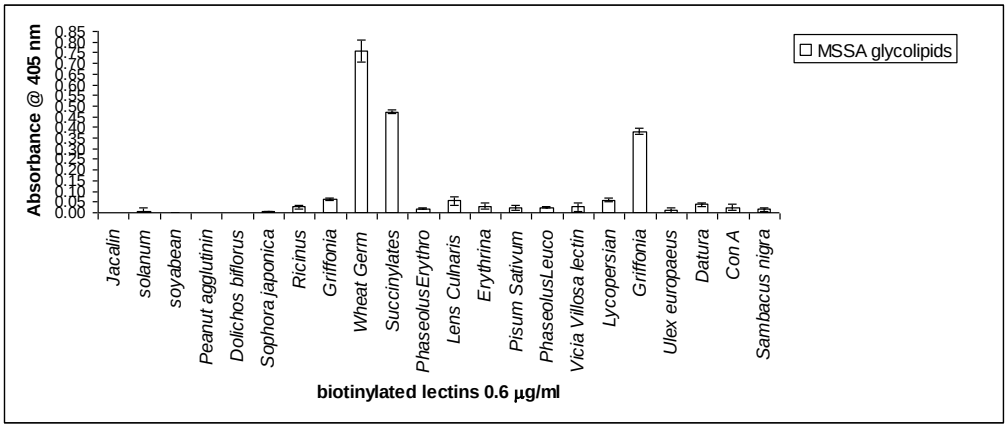


ELLA was also carried out on tests with no lectin and no streptavidin added which were negative controls. The data is the mean result of three independent experiments

VIA) Glycan profile of the whole cells of MSSA by ELLA

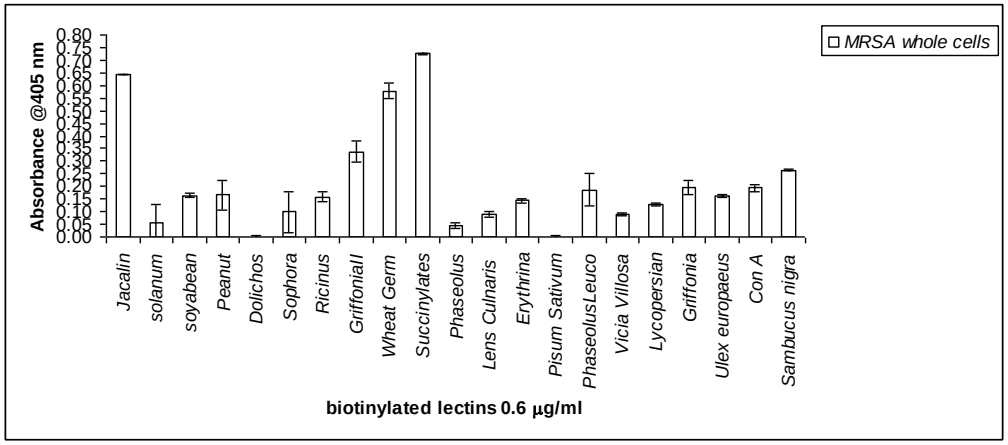


VIB) Glycan profile of Proteinase K treated whole cells of MSSA by ELLA

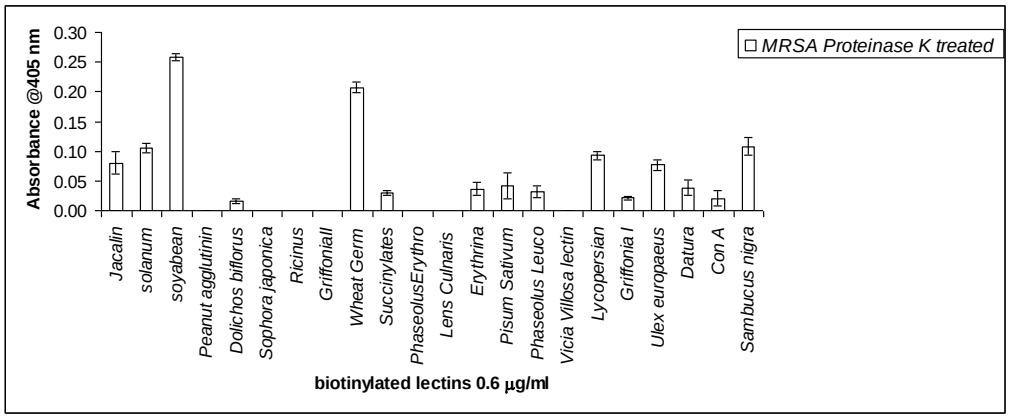


VIC) Glycan profile of glycolipids of MSSA by ELLA

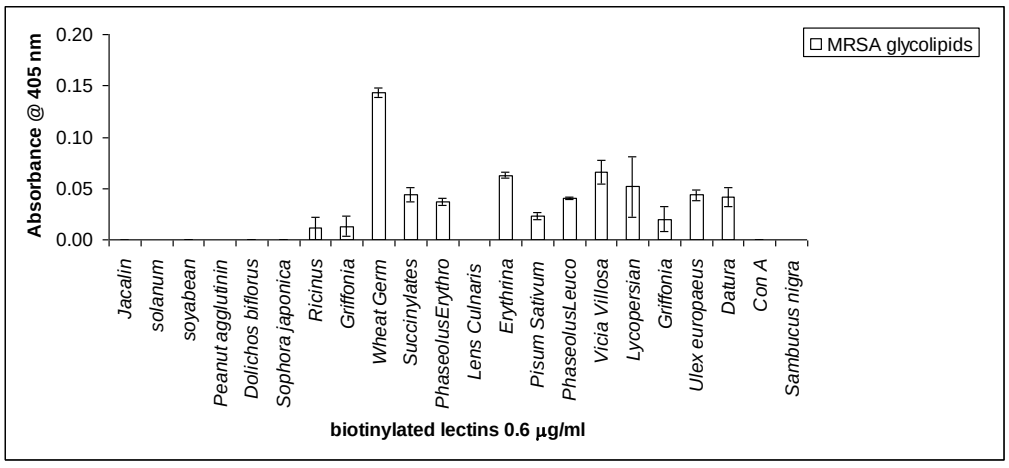
Appendix VII



VIIA) Glycan profile of the whole cells of MRSA by ELLA



VIIB) Glycan profile of Proteinase K treated whole cells of MRSA by ELLA



VIIC) Glycan profile of glycolipids of MRSA by ELLA

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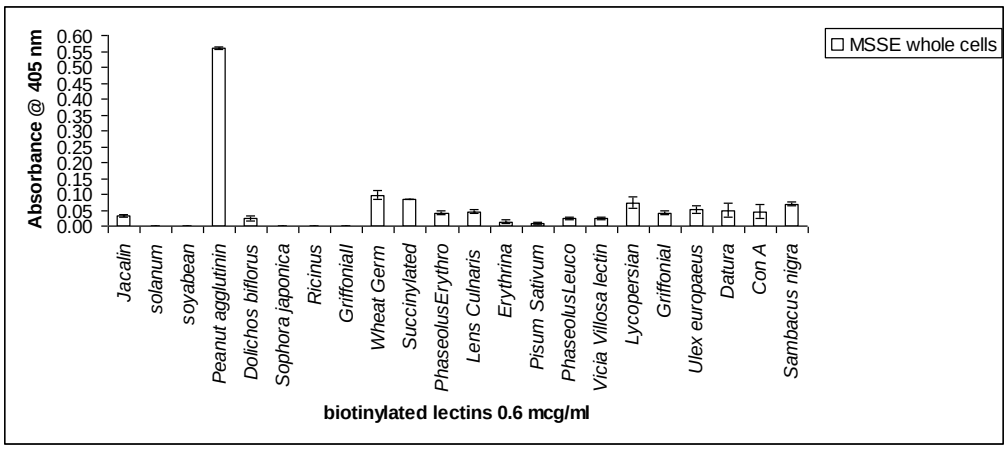
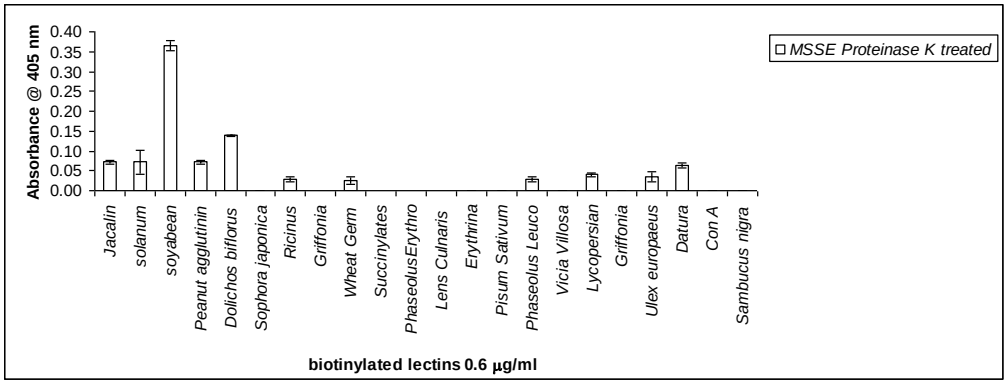
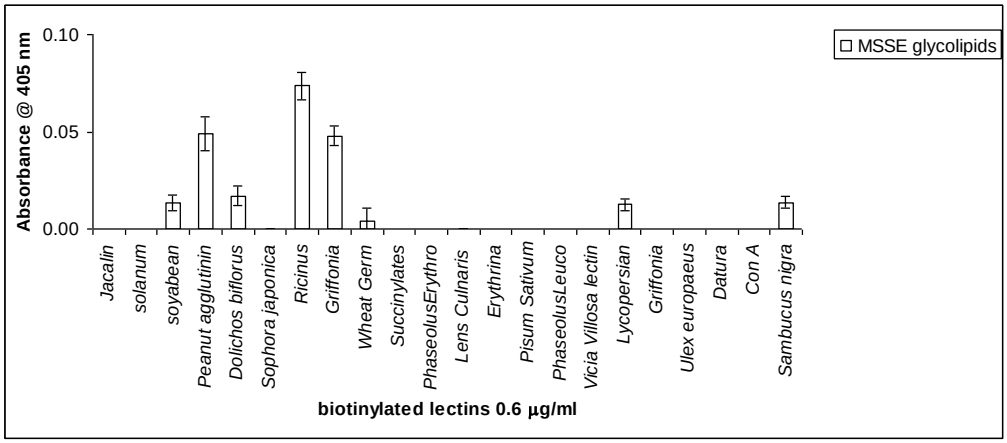


VIIIA) Glycan profile of whole cells of MSSE by ELLA

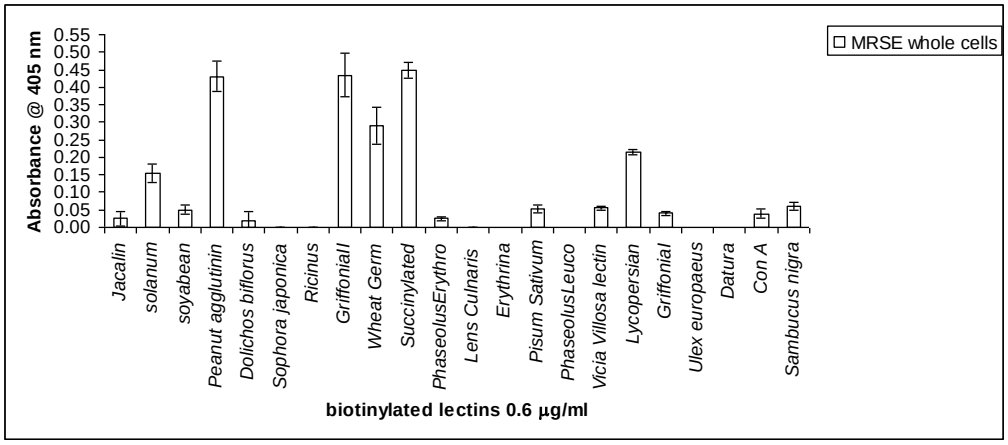


VIIIB) Glycan profile of Proteinase K treated cells of MSSE by ELLA

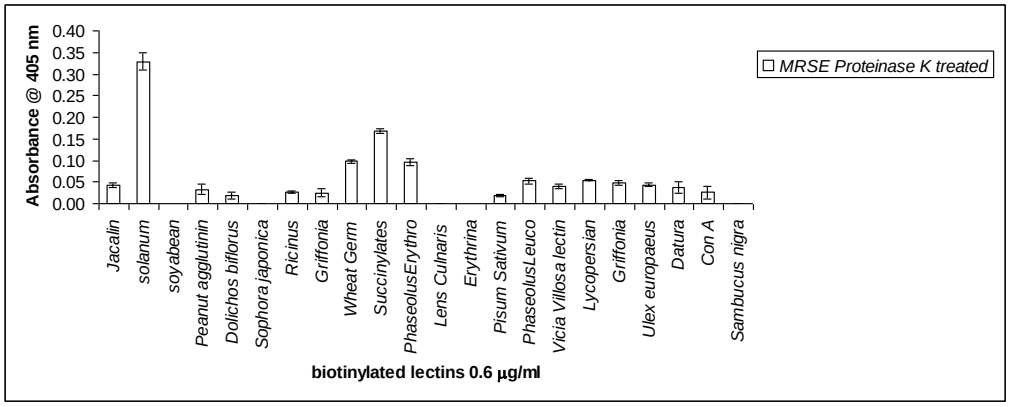


VIIIC) Glycan profile of glycolipids of MSSE by ELLA

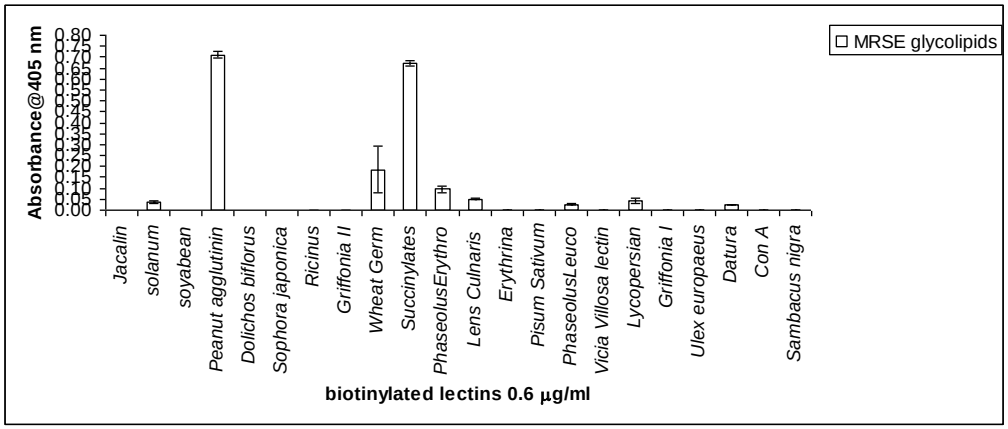
Appendix IX



IXA) Glycan profile of whole cells of MRSE by ELLA

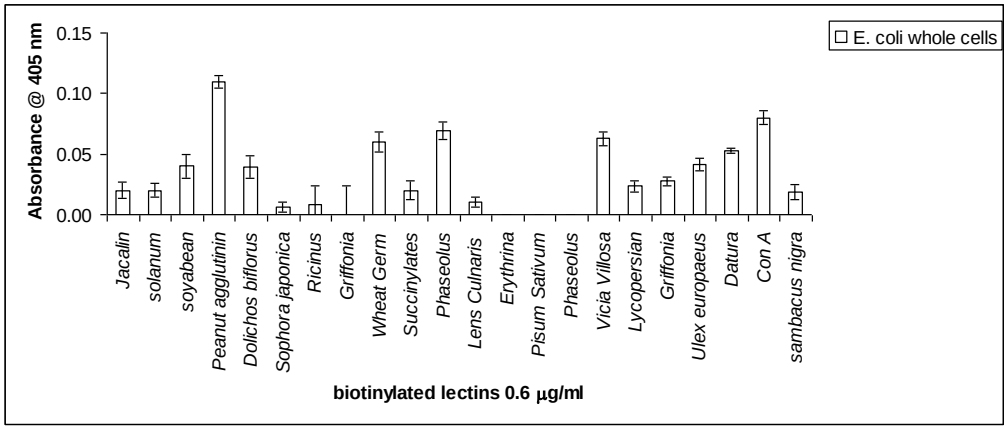


IXB) Glycan profile of Proteinase K treated whole cells of MRSE by ELLA

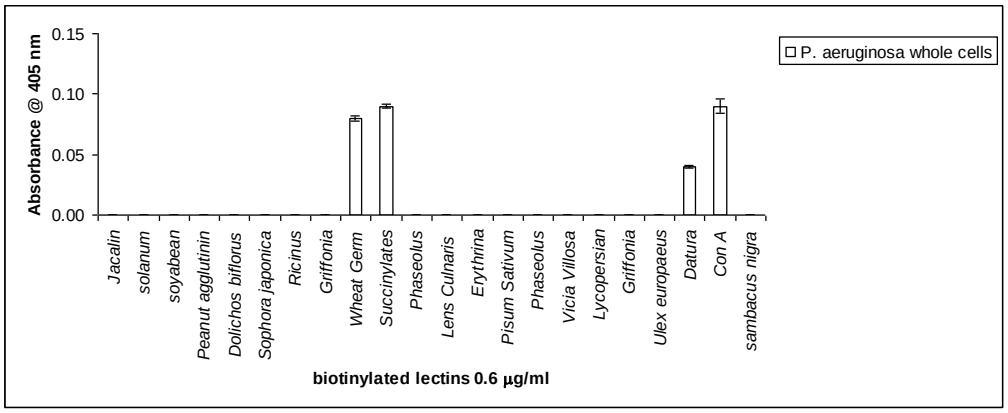


IXC) Glycan profile of glycolipids of MRSE by ELLA

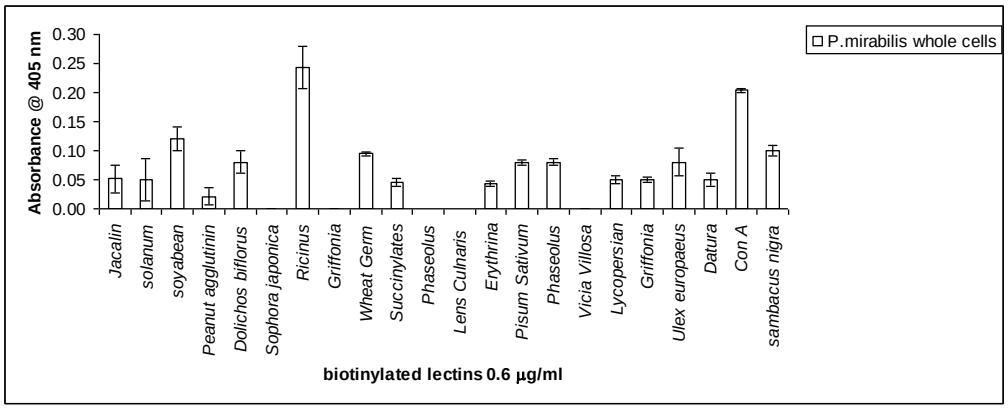
Appendix X



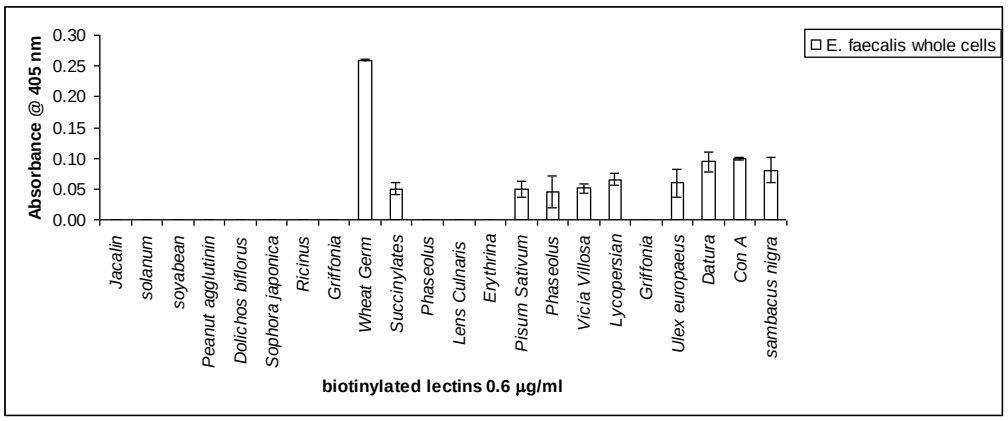
XA) Glycan profile of whole cells of *Escherichia coli* by ELLA



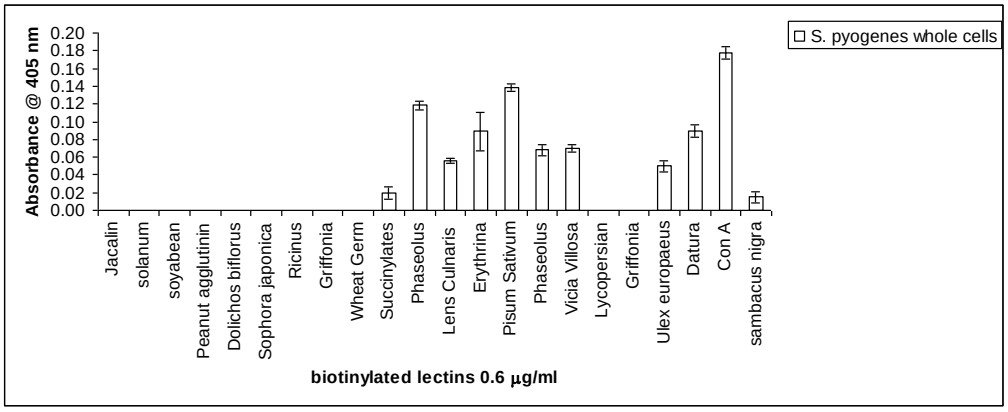
XB) Glycan profile of whole cells of *Pseudomonas aeruginosa* by ELLA



XC) Glycan profile of whole cells of *Proteus mirabilis* by ELLA

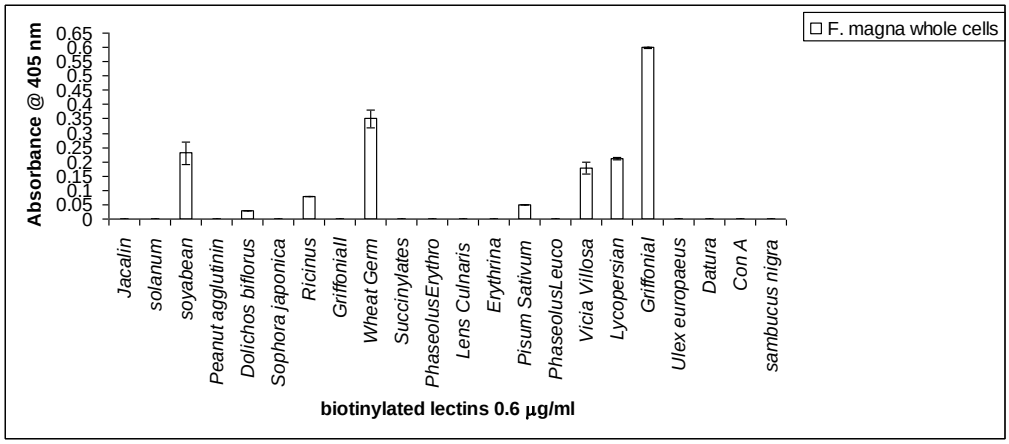


XD) Glycan profile of whole cells of *E. faecalis* by ELLA

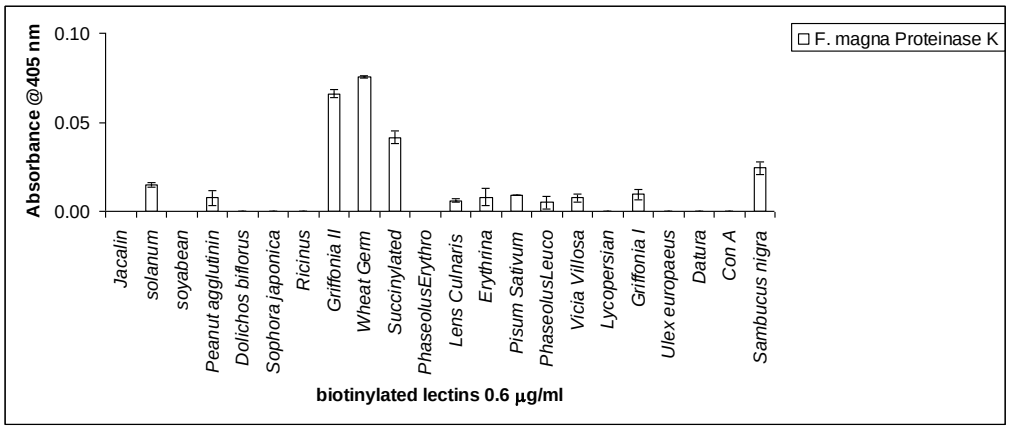


XE) Glycan profile of whole cells of *Streptococcus pyogenes* by ELLA

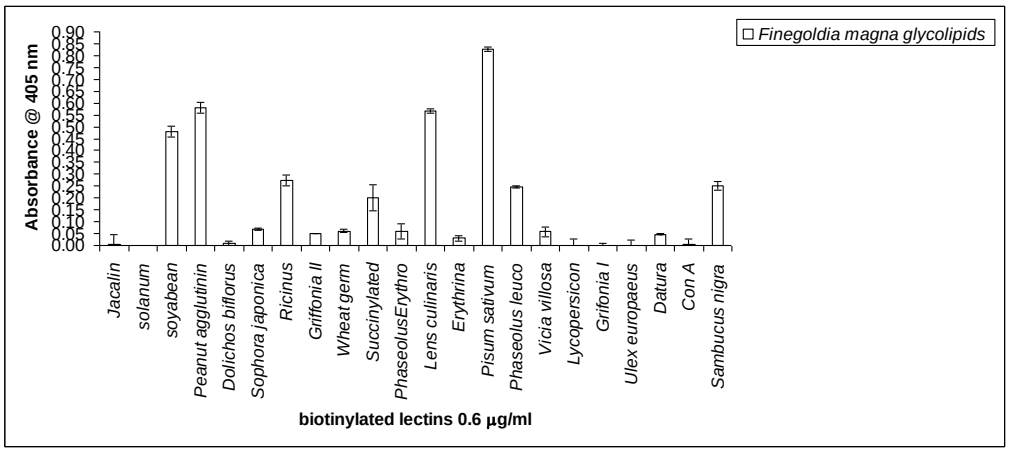
Appendix XI



XIA) Glycan profile of whole cells of *Finegoldia magna* by ELLA

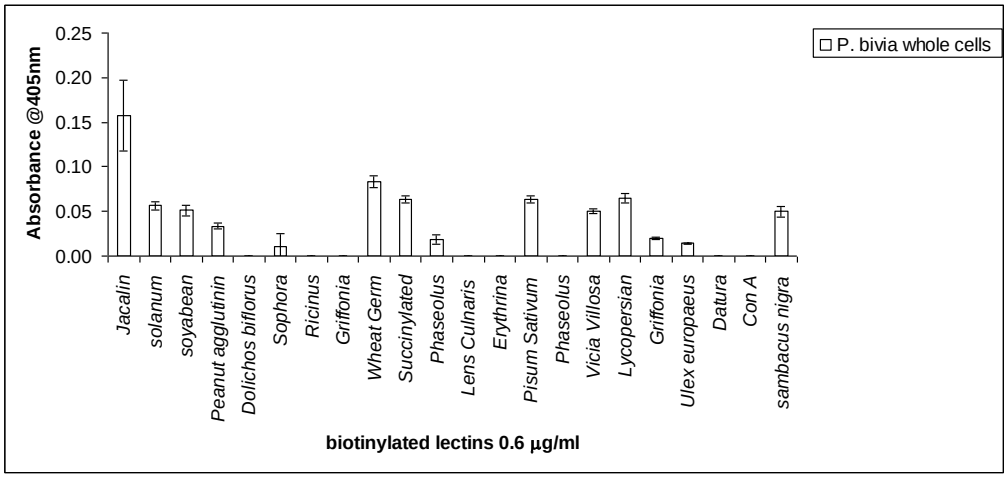


XIB) Glycan profile of Proteinase K treated whole cells of *Finegoldia magna* by ELLA

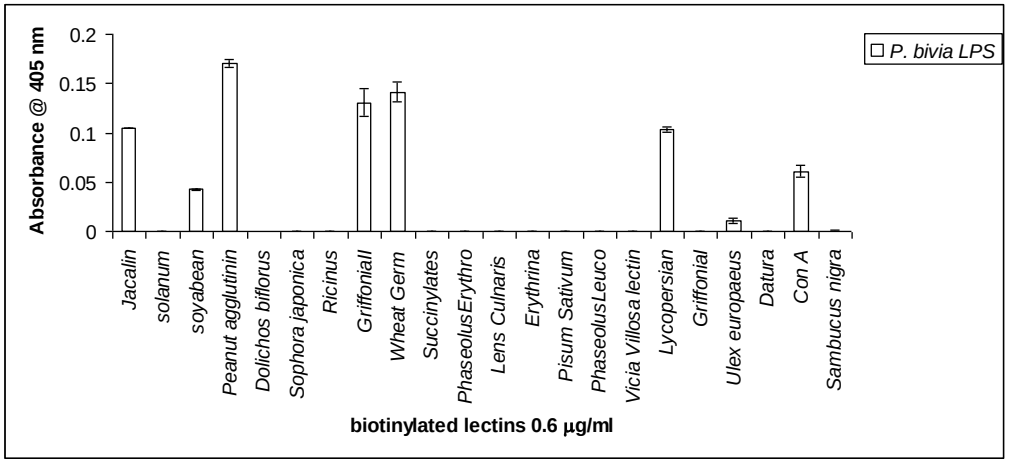


XIC) Glycan profile of glycolipids of *Finegoldia magna* by ELLA

Appendix XII

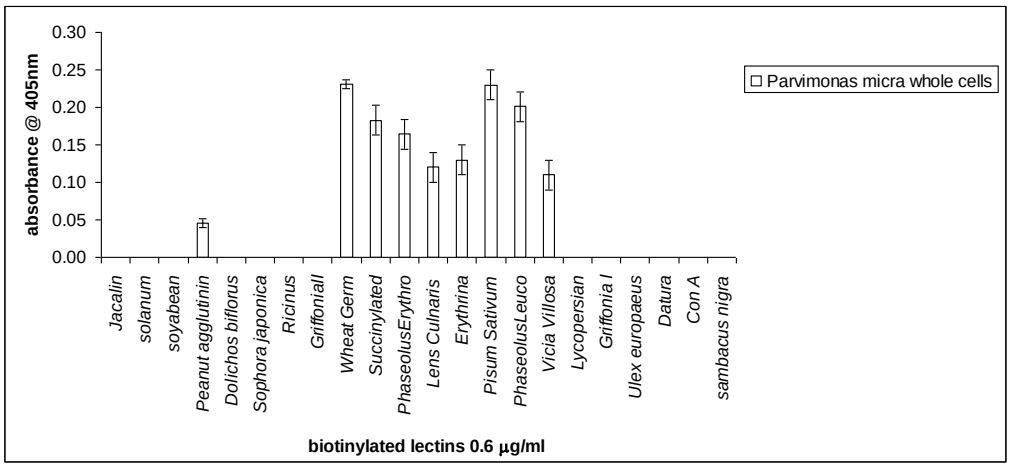


XIIA) Glycan profile of whole cells of *Prevotella bivia* by ELLA

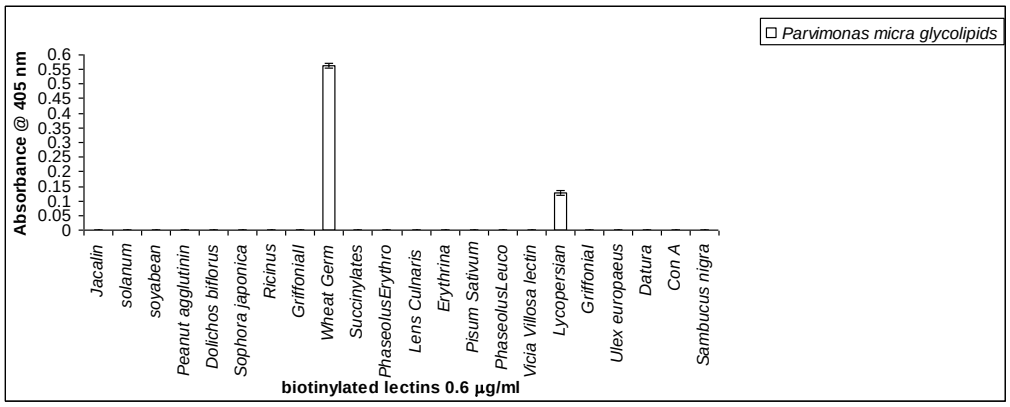


XIIB) Glycan profile of LPS of *Prevotella bivia* by ELLA

Appendix XIII

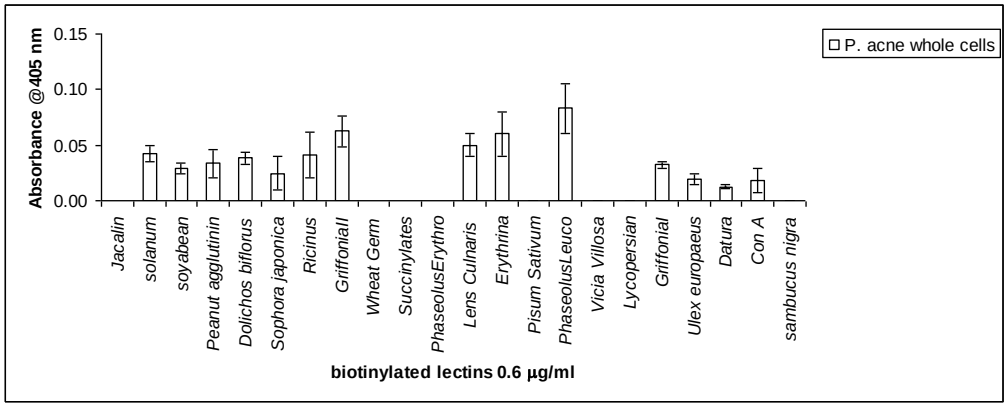


XIIIA) Glycan profile of whole cells of *Parvimonas micros* by ELLA

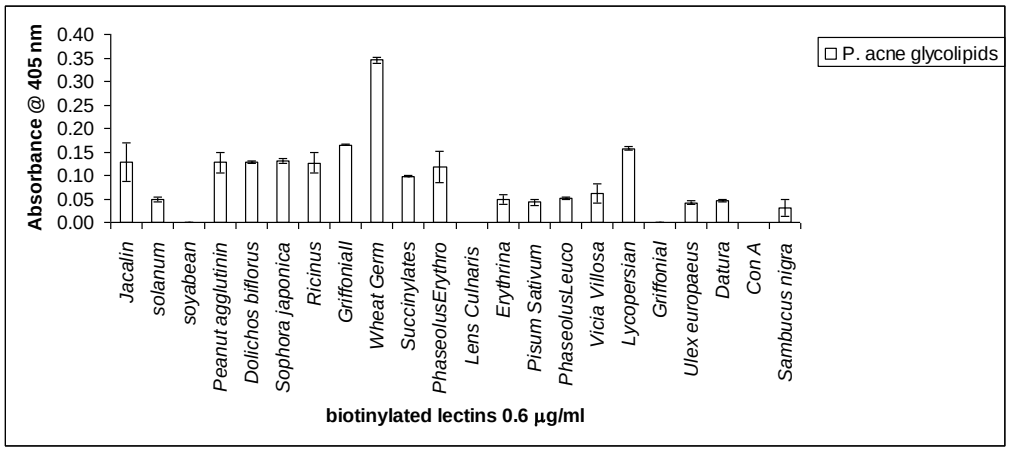


XIIIB) Glycan profile of glycolipids of *Parvimonas micros* by ELLA

Appendix XIV

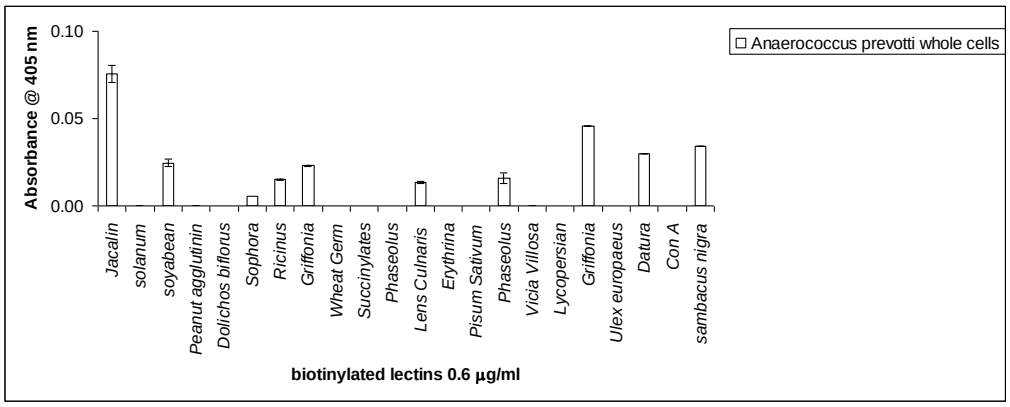


XIVA) Glycan profile of whole cells of *Propionibacterium acnes* by ELLA

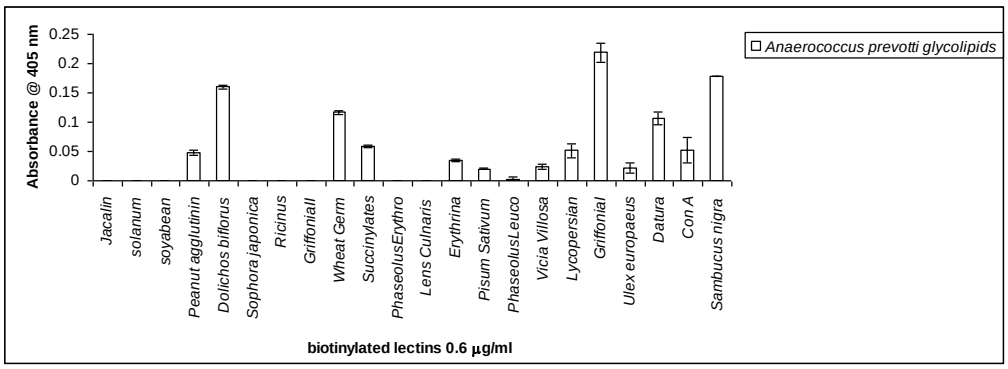


XIVB) Glycan profile of glycolipids of *Propionibacterium acnes* by ELLA

Appendix XV

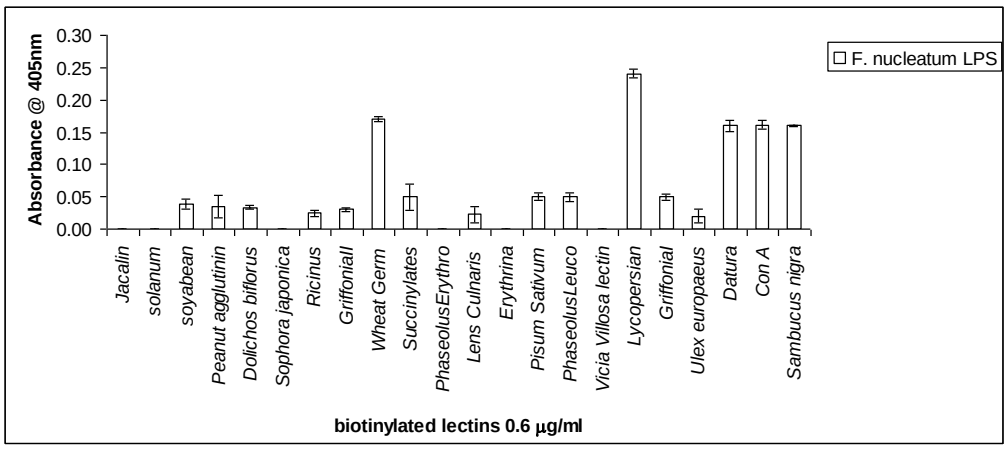


XVA) Glycan profile of whole cells of *Anaerococcus prevottii* by ELLA

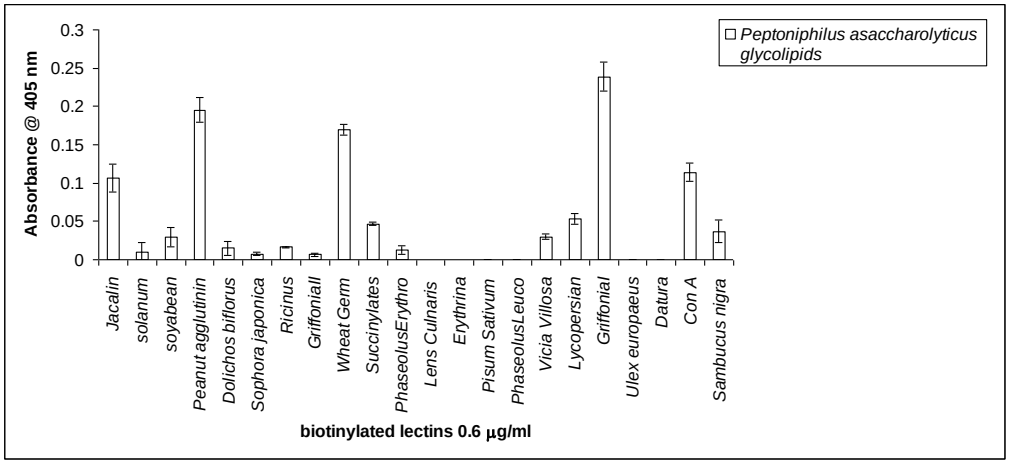


XVB) Glycan profile of glycolipids of *Anaerococcus prevottii* by ELLA

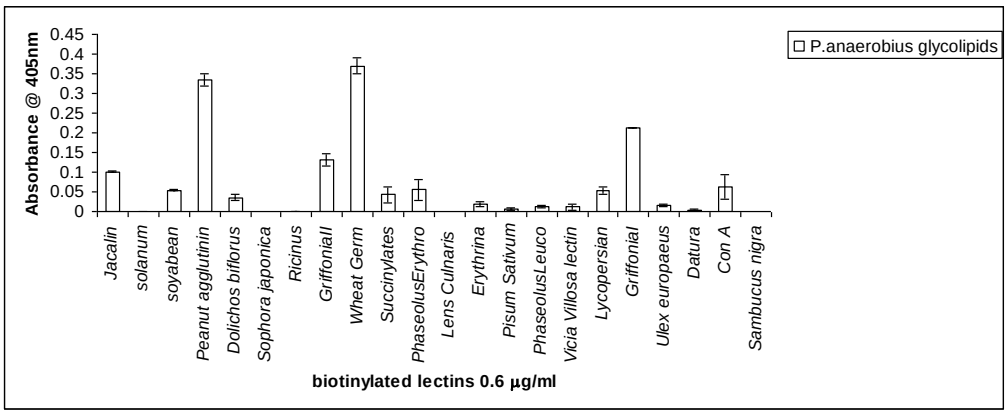
Appendix XVI



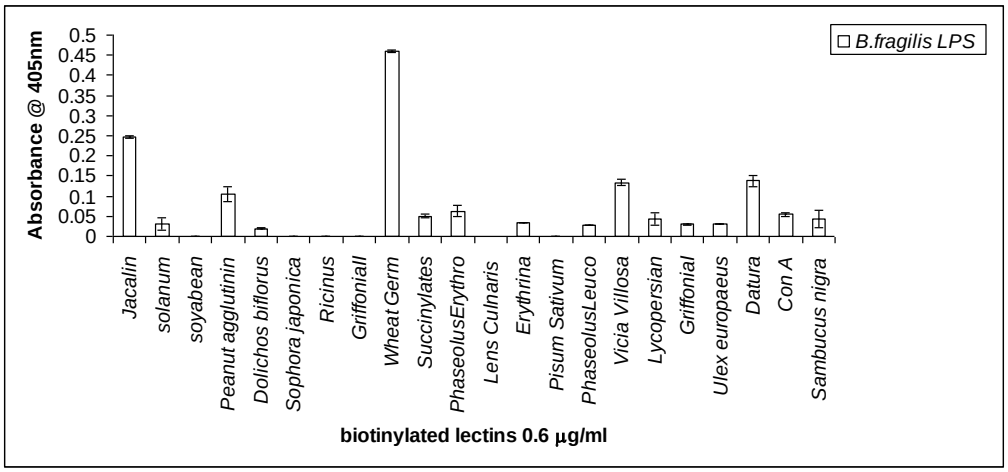
XVIA) Glycan profile of glycolipids of *Fusobacterium nucleatum* by ELLA



XVIB) Glycan profile of glycolipids of *Peptoniphilus asaccharolyticus* by ELLA



XVIC) Glycan profile of glycolipids of *Peptostreptococcus anaerobius* by ELLA



XVID) Glycan profile of glycolipids of *Bacteroides fragilis* by ELLA

Appendix XVII
Standard chromatogram of the different monosaccharides by GC-MS

Peak (Std)	Retention time	Width	Area	Start time	End time
Rhamnose	13.404	0.054	3783129	13.315	13.535
Rhamnose	13.706	0.059	330543	13.645	13.795
Xylose	15.669	0.065	2611185	15.532	15.877
Xylose	16.419	0.066	1336197	16.278	16.557
Mannose	21.170	0.069	4465559	20.966	21.308
Mannose	21.498	0.067	622749	21.388	21.594
Galactose	22.742	0.068	2454944	22.607	22.856
Galactose	23.018	0.076	195671	22.945	23.122
GluNAc	25.320	0.071	956160	23.953	24.122
GluNAc	25.610	0.087	495968	25.091	25.436
GalNAc	29.662	0.074	511284	29.507	29.758
GalNAc	31.731	0.070	903089	31.615	31.852
Inositol	34.731	0.089	3403077	34.601	34.872

The table below in Appendix XVII represents the standard of different monosaccharides and internal standard with their retention times, area, width, start time and their end time that were run on the column SP2330 fused silica, Supelco. The retention times of these standards were used to determine the composition of *Finegoldia magna* glycans.

Appendix XVIII
List of poster, presentations and potential publications

Posters and presentations

1) Pathogenesis of diabetic foot Infections; The role of glycans – poster presented at the conference held by International society of chemotherapy and infection in Canada.

2) Antibiotic study of infected flora of diabetic foot ulcers in UK and Indian populations – poster presented at the International society of chemotherapy and infection in Canada.

3) Role of glycans in the pathogenesis of diabetic foot Infections – poster presented at the conference organised by the Institute of biomedical science.

4) Role of glycans in the pathogenesis of diabetic foot Infections – oral presentation at the conference organised by the Institute of biomedical science.

5) Antibiotic resistance and the role of glycans in Diabetic Foot infections – oral presentation at the conference organised by the Society for General Microbiology.

Potential publications

1) Aerobes, anaerobes and fungi from the diabetic foot patient's from India and the UK

2) Comparison of the antibiotic susceptibility profiles from diabetic foot cohorts from India and UK

3) A comparative view on distribution of aerobes, anaerobes and fungi on the pus and tissues specimens from the Indian diabetic foot patients

4) Glycans of *Finegoldia magna*: a role in pathogenesis of diabetic foot infections?

5) Glycan – Lectin interactions: any scope for diagnostics

List of Abbreviations

ATCC	American type culture collections
AD	After death of Jesus
BC	Before Christ birth
BUN	Blood urea nitrogen
CLD	Chronic liver disease
DFI	Diabetic foot infections
DMSO	Dimethyl sulphoxide
ELISA	Enzyme linked immunosorbant assay
ELLA	Enzyme linked lectin sorbent assay
Gal	D-Galactose
GalNAc	2-acetamido-2-deoxy-D-galactopyranose
GC-MS	Gas Chromatography – Mass spectrometry
GDM	Gestational Diabetes mellitus
GlcNAc	2-acetamido-2-deoxy-D-glucopyranose
H2S	Hydrogen sulphide
HCl	Hydrochloric acid
HPAEC	High pH anion exchange chromatography
IgG	Immunoglobulin G
LB broth	Luria Bertani broth
LPS	Lipopolysaccharide
MALDI	Matrix assisted laser desorption/ionisation
MODY	Diabetes Maturity Onset Diabetes of the Young
MS	Mass spectrometry
NANA	N-acetyl neuraminic acid
NCCL S	National committee for clinical laboratory standards
NCTC	National collection of type cultures
NI	Neuroischemic
NMR	Nuclear magnetic resonance
O.D	Optical density
PCV	Packed cell volume
PG	Peptidoglycan

PMAA	Partially methylated alditol acetates
PN	Peripheral neuropathy
PNP	Paranitrophenol
PPA	Phenylalanine deamination test
PVD	Peripheral vascular disease
RC	Renal complications
RT	room temperature
Sia	Sialic acid
SPSS	Statistical package for social sciences
T1DM	Type 1 Diabetes mellitus
T2DM	Type 2 Diabetes mellitus
TFA	Trifluoro acetic acid
TSB	Tryptone soya broth
TSI	Triple sugar iron test
UCLH	University college London hospital
VP	Voges–Proskauer test
WBC	White blood cells

Microbes Abbreviations

A. baumannii	*Acinetobacter baumannii*
C. albicans	*Candida albicans*
C. botulinum	*Clostridium botulinum*
C. butyricum	*Clostridium butyricum*
C. difficile	*Clostridium difficle*
C. glabrata	*Clostridium glabrata*
C. guilliermondii	*Clostridium guilliermondii*
C. histolyticum	*Clostridium histolyticum*
C. innocuum	*Clostridium innocuum*
C. kefyr	*Candida kefyr*
C. krusei	*Candida krusei*
C. limosum	*Clostridium limosum*
C. paraputrificum	*Clostridium paraputrificum*
C. perfringens	*Clostridium perfringens*
C. pseudotropicalis	*Candida pseudotropicalis*
C. putrificum	*Clostridium putrificum*
C .rugosa	*Candida rugosa*
C .stellatoid	*Candida stellatoid*
C .tertium	*Clostridium tertium*
C .tetani	*Clostridium tetani*
C .tropicalis	*Candida tropicalis*
E.coli	*Escherichia coli*
F .necrogenes	*Fusobacterium necrogenes*
F .varium	*Fusobacterium varium*
F.m	*Finegoldia magna*
H. ducreyi	*Haemophilus ducreyi*
H. influenzae	*Haemophilus influenzae*
K .pneumoniae	*Klebsiella pneumoniae*
MRSA	Methicillin resistant *Staphylococcus aureus*
MRSE	Methicillin resistant *Staphylococcus epidermidis*
MSSE	Methicillin sensitive *Staphylococcus epidermidis*

N. gonorrohoeae	*Neisseria gonnorrohoeae*
N.meningitides	*Neisseria meningitides*
P .mirabilis	*Proteus mirabilis*
P .vulgaris	*Proteus vulgaris*
P. asaccharolyticus	*Peptoniphilus asaccharolyticus*
P. glycinophilus	*Peptoniphilus glycinophilus*
P. indolicus	*Peptoniphilus indolicus*
P. magnus	*Peptoniphilus magnus*
P. niger	*Peptoniphilus niger*
P. prevottii	*Peptoniphilus prevottii*
P.a	*Pseudomonas aeruginosa*
Pst. micros	*Peptostreptococcus micros*
Pst. anaerobius	*Peptostreptococcus anaerobius*
Pst. asaccharolyticus	*Peptostreptococcus asaccharolyticus*
Pst. heliotrinreducens	*Peptostreptococcus heliotrinreducens*
Pst. indolicus	*Peptostreptococcus indolicus*
Pst. magnus	*Peptostreptococcus magnus*
Pst. parvulus	*Peptostreptococcus parvulus*
Pst. prevottii	*Peptostreptococcus prevottii*
Pst. productus	*Peptostreptococcus productus*
Pst. tetradius	*Peptostreptococcus tetradius*
S. artitae	*Staphylococcus artitae*
S. capitis	*Staphylococcus capitis*
S. epidermidis	*Staphylococcus epidermidis*
S. intermedius	*Staphylococcus intermedius*
S. klossi	*Staphylococcus klossi*
S. marcescens	*Salmonella marcescens*
S. pisa fermentans	*Salmonella pisa fermentans*
S. schleiferi	*Staphylococcus schleiferi*
S. typhi	*Salmonella typhi*
S.a	*Staphylococcus aureus*
Spp	Species
TB	*Tuberculosis*