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Investigation into the measurement of cotton biomarkers as an indicator of risk to respiratory health and detriment to structural quality

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Presented by

Samantha Rosemary Lane BSc. (Hons)

Department of Pharmacology

Welsh School of Pharmacy

Cardiff University

Cardiff

UK

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789 East Eisenhower Parkway
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Why have silk when you can have cotton?

SUMMARY

The aim of this study was to analyse a range of biological contaminants on cotton fibres. These fibres become contaminated with bacteria and fungi as the crop plants are growing in the field. The contamination entrained in the cotton fibres, as well as the products released from them, is inhaled by industrial workers along with the dust created during cotton processing. Prolonged exposure to these dusty environments can result in the chronic and irreversible lung disease, byssinosis.

The biological toxicants investigated during this study were endotoxin, a lipopolysaccharide released from the outer membrane of Gram-negative bacteria and glucan, a glucose polymer occurring in the cell walls of common cotton fungi. Despite the known effects of these airborne agents, there are currently no threshold limits in place for their control within the working environment. This study involved the quantification of these toxicants and their source organisms, on numerous cotton samples from different countries.

The results displayed a number of patterns; cotton samples from every country analysed, including samples that had been stored for up to 6 years, contained significant levels of all contaminants. Production factors appeared to influence the contamination levels. Cotton samples from countries within Africa, were significantly more contaminated than those originating in Asian regions, and *Gossypium hirsutum* cotton from Sudan contained higher mean levels of Gram-negative bacteria, endotoxin and fungi than *G. barbadense*.

A limited range of bacterial species were identified on cottons from across the world; the most common was *Enterobacter*. Fungal genera were also similar on all cotton samples analysed, the most prevalent being *Aspergillus*. Cotton trash material often contained 10-100 times higher contamination levels than associated lint samples. Significant positive correlations existed between the contamination parameters, particularly endotoxin and glucan. Several of these findings suggest that the cotton production environment is more hazardous than previously thought.

Cotton is the overcoat of a seed that is planted and grown in the Southern States to keep the producer broke and the buyer crazy. The fibre varies in colour and weight, and the man who can guess the nearest length of the fibre is called a cotton man by the public, a fool by the farmer and a poor businessman by his creditors.

The price of cotton is fixed in New York and goes up when you have sold and down when you have bought. A buyer for a group of mills was sent to New York to watch the cotton market and after a few days' deliberation wired his firm to this effect – "some think it will go up, some think it will go down, I do too. Whatever you do will be wrong. Act at once."

Cotton is planted in the spring, mortgaged in the summer and left in the field in the winter. There are other definitions but none better than this.

Boston News Bureau

CHAPTER 1

GENERAL INTRODUCTION

1.1 OCCUPATIONAL LUNG DISEASE

"Those who card flax and hemp so that it can be spun and given to the weavers to make fabric find it very irksome. For a foul and poisonous dust flies out from these materials, enters the mouth, then the throat and lungs, makes the workmen cough incessantly, and by degrees brings on asthmatic trouble".

This was written in 1713, by the Italian physician Bernardo Ramazzini in his treatise '*De Morbis Artificum Diatriba*"; nearly three hundred years later, its sentiment is still relevant. Many occupational environments contain a range of hazardous substances to which working populations are continually exposed. Where effective control procedures are lacking, this results in a spectrum of disease symptoms across many working disciplines. The dusts created from processing vegetable fibres such as the flax and hemp described in 1713, remain one of the most widespread causes of occupational lung disease in the present day world. These materials have been exploited by man for centuries, for a variety of purposes including textile manufacture and agricultural production. Despite this history, the nature of occupational dust exposure and associated lung disease is still under scrutiny, largely because many aspects remain unclear.

Vegetable dusts are heterogeneous mixtures of biological and physical contaminants; this heterogeneity yields considerable variability between

individuals in their response to dust inhalation, which has frustrated identification of responsible dust components. The main factors influencing the make-up of organic dusts relate to the fibre crop and include climate, soil, harvesting practice, storage conditions and processing techniques. The effect of dusts on the lungs is dependent on the size of inhaled particles and this influences ease of entry into the respiratory tract, governing the depth of penetration. The pattern of exposure to particular dusts is an added factor, for example, farm workers have seasonal cycles of dust exposure. Variables directly relating to exposed individuals, such as age, length and type of employment, atopy and smoking history also determine exposure risk. In addition, the human respiratory tract may respond to inhaled dusts in a number of ways, giving rise to several distinct diseases, the most common of which are described below.

1.1.1 Hypersensitivity pneumonitis

When an immunological reaction is observed in response to organic dust exposure, it is classified either as hypersensitivity pneumonitis (HP) or as occupational asthma (OA). Hypersensitivity pneumonitis (extrinsic allergic alveolitis), refers to a group of diseases characterised by inflammation of the peripheral airways and alveoli. This occurs when particles small enough to reach deep into the respiratory tract (< 7μm aerodynamic diameter) are inhaled, causing rhinitis, cough, fever, rigors and breathlessness. The resulting reduction in airflow reaches its peak after about 8 hours, although the symptomology varies between individuals, and with respect to the causative agent (Nicholls, 1992). HP may be acute, subacute or chronic; the

acute symptoms disappear following exposure cessation, although on repeated exposure they may eventually become permanent. Low-level prolonged dust exposure has a propensity to induce the chronic condition, whereas a period of short exposure to high-level dust concentrations gives rise to the acute response. Progressive intrapulmonary fibrosis occurs via alveolar macrophage activation, and advanced cases can be associated with signs of pulmonary heart disease and chronic heart failure (Pickering and Newman Taylor, 1994). Examples of dusts responsible for HP are shown in Table 1.01.

Disease	Source of dust
Bagassosis	Mouldy sugar cane
Bird fancier's lung	Bird droppings
Farmer's lung	Mouldy hay, straw, grain
Humidifier fever	Contaminated water
Malt worker's lung	Mouldy barley
Mushroom worker's lung	Mushroom compost
Suberosis	Mouldy cork bark

Table 1.01: Types of Hypersensitivity Pneumonitis. Adapted from Pickering and Newman Taylor, 1994.

It is likely to be microbial contamination of the dusts by bacteria or fungi, which are the ultimate common cause of the disease, but not everyone exposed to these agents succumbs to disease, and only certain individuals display sensitivity. Diagnosis requires clinical, radiological and pathological

investigation. The histological triad indicating HP includes bronchiolitis, interstitial lymphocytic infiltration and sarcoid type granulomas in the alveoli (Yi, 2002). The symptom patterns with this group of diseases show elements of both a non-immunological response and a cell-mediated allergic response, which cannot readily be categorized by any of the four types of human allergic reaction. Farmer's lung is the best characterised of this group, whereby continued exposure to hay dust leads to the formation of an antigen-antibody type III reaction. Nevertheless, HP diseases indicate that both alveolar macrophages and T cells play a pivotal role in the immune responses, and the formation of granulomas is more compatible with a type IV reaction, explained by a late-phase cell-mediated response (Rose, 1996; Yi, 2002).

1.1.2 Occupational Asthma

Occupational asthma (OA) is the most common form of occupational disease in the developed world (Lombardo and Balmes, 2000). It is associated with diffuse, intermittent, reversible airway obstruction in response to the inhalation of a wide range of dusts such as wood dusts and grains. Unlike idiopathic asthma, OA is dependent on sensitization to the workplace antigen, though exposure may exacerbate symptoms in patients with pre-existing asthmatic conditions. The disease is characterised by shortness of breath, chest tightness, wheezing, cough and bronchoconstriction. Symptoms may appear immediately with no latency period, and this form of OA is also known as reactive airways dysfunction syndrome (RADS) or irritant-induced asthma. Symptoms may also emerge 18 months to 15 years

after initial exposure, during which time sensitization occurs (Bernstein, 1997). It is a serious condition, which is potentially fatal when a massive dose causes a severe acute attack. Over 250 agents have been identified as possible causes of OA, including animal proteins, plants, a variety of chemicals, antibiotics, enzymes, dyes and metals, affecting a large range of occupations (Newman Taylor and Pickering, 1994). Putative mechanisms by which sensitization occurs can be arbitrarily defined by the molecular weight of the agent responsible. Most high molecular weight compounds induce OA by specific IgE antibody-dependent reactions. However, the disease mechanism may not be either a type I or a type III response, as complex non-immunologic reactions have also been suggested with some agents.

OA may eventuate after exposure to dust from a number of hardwoods such as the Western Red Cedar, where it is thought the dust allergens release histamine and other mediators by a non-immune response. Wheat is the most common cause of OA in response to grain and flour dusts, inhalation of which causes rhinitis and asthma in a large number of workers. Rice is a major agricultural product in many parts of Asia where it is dried and milled, a process which creates large aerosol clouds. The dust causes irritation to the eyes, skin and upper respiratory tract as well as allergic-type reactions such as asthma and eosinophilia, though non-specific reactions also occur. Rice husks have high silica content, which could be a causative factor in OA. The husks also possess microscopic needle-like spines, which are capable of inducing corneal scarring. Husk fragments, which are inhaled may thus cause damage by this physical process. Acute and chronic diseases occur in

a number of workers exposed to common tea dust. Tea-worker's asthma has also been described in those exposed to herbal tea dusts such as sage and chamomile, although it has not been established whether the causal agent is derived from the plants themselves or contaminating fungi. Green and roasted coffee beans are also capable of causing asthma, rhinitis, conjunctivitis and bronchitis. The resulting acute reduction in respiratory flow rate can be eased by cromoglycate, which implies that the reactions are IgE-mediated (Lane, *et al.*, 2005; Nicholls, 1992).

1.1.3 Organic Dust Toxic Syndrome (ODTS)

ODTS (also known as toxic pneumonitis) involves febrile episodes that occur in the absence of other signs of alveolitis on first contact with agricultural dusts such as cotton and grain. Acute fever, chills, joint pain and other usually mild, flu-like symptoms develop in the afternoon or evening after exposure during the working day and subsequently last for 24-48 hours. Tolerance to the dust develops and symptoms disappear completely upon continued exposure, though they may return after a prolonged absence from the allergen or an episode of very heavy exposure (Kirkhorn and Garry, 2000). ODTS is not progressive and sensitization does not occur, hence it is probably attributable to a toxic reaction rather than an immune response. It typically occurs with mouldy hay and grains and is common in swine containment units (Malmberg and Rask-Andersen, 1993). Synonymous with mill fever, inhalation fever and factory fever, this condition is rare in modern mills due to improved ventilation (Schilling and Rylander, 1994). It is not known why some individuals are pre-disposed to this condition while others

remain unaffected, but it has been suggested that those who develop mill fever may be more susceptible to byssinosis following prolonged exposure to dusts (Gill, 1947).

1.1.4 Byssinosis

Byssinosis is the traditional disease of the cotton industry - the term was first used in 1877, derived by the English author Proust from the Greek 'byssos' meaning a fine, flaxen substance. Also known as brown lung and cotton worker's lung, it is a disease specific to those employed in the textile processing industry. Byssinosis is a worldwide problem, which has been reported in every country where textile fibres are processed industrially. Onset of the disease normally occurs after at least five years exposure to the mill atmosphere and regularly only after fifteen years employment. It is characterised by a sensation of chest tightness and breathlessness, experienced on the first day back to work after the weekend break (the so-called Monday phenomenon) or a holiday period (Rylander, *et al.*, 1985). Acute byssinosis is associated with changes in lung function suggestive of reversible airway narrowing. The term describes a range of Monday-related symptoms, including acute chest tightness, shortness of breath, coughing and wheezing, as well as non-specific malaise (Rylander, 1992). These symptoms can spread to other days of the week upon further exposure, until they are continuous throughout the week, resulting in the chronic state of the condition.

The textiles known to cause byssinosis include; cotton, flax, hemp, jute, sisal and kapok. It is most often described in the context of cotton production since here, the highest numbers of workers are at risk due to the scale of the industry. Prevalence of byssinosis in cotton mills has been reported to be as high as 30-40% (Cloutier and Rohrbach, 1986), although this varies greatly according to the quantity and composition of the causative dust, which is dependant on many factors. Assessing disease prevalence also depends on the correct recognition of symptoms. Consequently, accurate diagnosis of byssinosis may be complex, since it is compounded by inter-individual symptom variability. During onset, the disease is indistinguishable from non-occupational chronic bronchitis. However, it is discernable as a separate condition since it is associated with a linear fall in maximum mid-expiratory flow (MMF) and an increase in airway resistance during the working day; these functional changes are reversible by bronchodilator drugs.

There are well-defined stages of byssinosis, which were originally clinically graded by symptom profile (Schilling, *et al.*, 1955) (Table 1.02). Few physical signs accompany the condition; the only common one being expiratory wheeze with grades C½-C2 although patients suffering from C3 byssinosis may also exhibit impaired breath sounds. Therefore, diagnosis of C½-C2 relies on history of industrial exposure, history of clinical grades and fall in FEV_1 or MMF, during the working day or week. Individuals with C½ and C1 invariably recover completely after leaving the industry, and this also applies to C2 workers, although there may be patients with grade C2 byssinosis such

as heavy smokers, who have persisting symptoms (Newman Taylor and Pickering, 1994).

Grade (C)	Symptoms
Grade 0	No symptoms of byssinosis
Grade ½	Occasional chest tightness on first day of working week
Grade 1	Chest tightness on every first day of working week
Grade 2	Chest tightness on the first and other days of working week
Grade 3	Grade 2 symptoms accompanied by evidence of permanent respiratory disability

Table 1.02: The Schilling clinical grades of byssinosis

Prevalence data on cotton dust-induced lung disease tends to be outdated and often originates from different and sometimes conflicting sources (Bates, *et al.*, 1992). Table 1.03 presents estimates of US workers at risk from lung disease in a number of industries. Although relevant over a decade ago, these data highlight cotton dust exposure as one of the highest risks for developing airway disease, largely due to the sheer size of the cotton industry and hence the numbers of employees. In response to this, the implementation and monitoring of cotton dust exposure limits has reduced byssinosis prevalence in developed countries to minor proportions.

Agent	Workers
Ammonia	500,000
Arsenic	1,500,000
Cadmium oxide	2,000
Chlorine	15,000
Chromium	175,000
Coal mine dust	200,000
Coke oven emissions	10,000
Cotton dust	**800,000**
Osmium tetroxide	3,000
Nitrogen oxides	1,500,000
Phosgene	10,000
Toluene diisocyanate	40,000
Vanadium	10,000

Table 1.03: Number of workers at risk of non-specific airway disease. Adapted from Bates, *et al.*, 1992.

Table 1.04 (below) shows some overall byssinosis prevalence rates in textile workers reported in the literature from a wide range of studies conducted in different countries. Prevalence varies considerably, from 1.1% in Australia to 45.5% in Ethiopia. Byssinosis prevalence in developing countries where cotton production is increasing, remains high, and has reached approximately the levels recorded in the UK in the 1950s (Nakládalová, 2000), although again, standardised, current prevalence data are largely unavailable.

Country	Prevalence (%)	Reference
Australia	1.1	Gun, *et al.*, 1983
Cameroon	18	Takam & Nemery, 1988
China	5.6	Lu, *et al.*, 1987
	9	Markham, 1993
	2-15	Wang & Christiani, 2003
Ethiopia	45.5	Abebe & Seboxa, 1995
Hong Kong	2.7	Han, 1982a
India	30-40	Bhagia & Parikh, 1990
	30	Murlidhar, *et al.*, 1995
Indonesia	30	Baratawidjaja, 1990
	30	Han, 1982b
Sudan	37	El Karim & Onsa, 1987
Sweden	20	Haglind, 1984
Turkey	14.2	Altin, *et al.*, 2002

Table 1.04: Reported prevalence of byssinosis in various countries, data taken from a range of literature.

Byssinosis therefore remains a significant disease worldwide, which despite its long history still demands considerable scientific attention. In order to gain a fuller understanding of the disease, a description of the situation in which it arises, its causative factors and prevention follows.

1.2 COTTON

Cotton is a white vegetable fibre formed as an outgrowth from the epidermal trichome of seeds from the shrub plant family *Malvaceae* (mallow) and genus *Gossypium*. As the fibre develops, secondary wall thickening takes place as

cellulose is deposited inwardly in successive layers. The fibre forms within a protective capsule called the cotton boll, which expands with heat from the sun until bursting open, each boll contains around 30 seeds and up to 500,000 fibres. When the fibres in the open boll become exposed to air (Figure 1.01), the protoplasmic contents dry out leaving a lumen, the fibre then collapses into a ribbon, which is harvested, spun into yarn and eventually used to make fabric (Anthony, 1991).

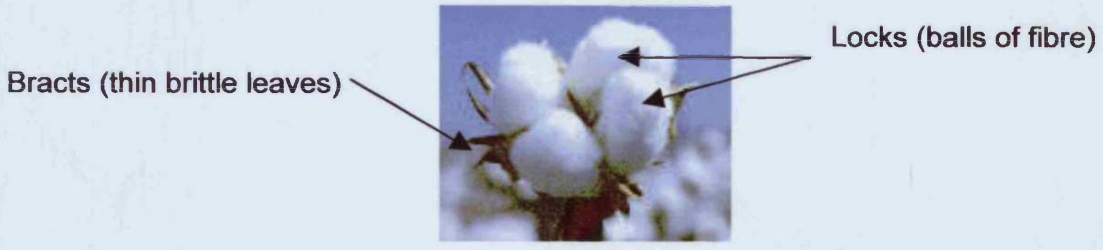


Figure 1.01: Exposed cotton fibres, after the surrounding boll has opened. Adapted with permission from ACCRC, [WWW] 2004.

Cotton has been cultivated for its fibre for over 5000 years and today still accounts for approximately half of the world market of textile fibres. It is grown world-wide in over seventy countries in a band stretching between latitude 42°N in the former Soviet Union, and latitude 30°S in Australia and Argentina (Anthony, 1991), covering a total estimated area of 34 million hectares (Bremer Baumwollbörse, 2004). For a good cotton crop, a long sunny growing season is required with at least 160 frost-free days and ample water supply. In most regions, the large quantities of water required must be supplied by irrigation. The largest producers of cotton in the world today are China, USA, India, and Pakistan (see Table 1.05).

Rank	Country	Cotton production '000 tons
1	China	4,900
2	USA	3,957
3	India	2,850
4	Pakistan	1,615
5	Brazil	1,032
6	Uzbekistan	915
7	Turkey	900
8	Syria	295
9	Australia	265
10	Mali	250

Table 1.05: The top 10 cotton producing countries in the world, 2003/2004. Data adapted from Bremer Baumwollbörse, 2004.

Cotton plants are all of the genus *Gossypium,* but cultivated cotton species fall into three main species groups; Group I is the extra long staple Egyptian, Sea Island and Pima cotton *Gossypium Barbadense*. Group II consists of American and African Upland medium staple *G. hirsutum* and group III contains two species of Asiatic or Old World short staple cotton, *G. arboreum* and *G. herbaceum.* Group III cottons are grown commercially in India, Pakistan and other parts of South East Asia. These groups account for 8%, 90% and 2% of world production respectively (Geocities, [WWW] 2003). Each of the commercially important species contain many varieties of cotton, developed through breeding programmes to produce cottons which are faster

maturing, have improved insect and disease resistance (hardiness) and are of greater yield and strength, hence benefiting the industry (Nicholls, 1992).

1.2.1 Cotton Production; from field to fabric

Raw seed cotton is harvested from the plant by either manual (hand) or mechanical (machine) picking methods. The majority of world cotton is still picked by hand, with only a limited number of countries including Australia, USA, Spain and Israel using machine picking methods for all cotton harvesting. Hand picking is slower but has advantages in that the fibres are treated more carefully than by the high-speed combing action of the picking machine. Machine picking also introduces more contamination into the harvested product, as it is less discriminatory than manual picking. Once harvested, seed cotton is taken to the ginnery where the cotton fibres (lint) must be separated from the seeds, this is done either by a saw or roller gin, both of which use force to rip the fibres away from the seeds. Removed cottonseeds are not wasted, they are either saved for seeding the following years crop or processed to obtain their oil. Cottonseed oil is used as cooking oil; margarine is produced from the highly refined components, whereas less refined oil is used for soap, candles and detergent manufacture. The hulls removed during processing are used as fertiliser and fuel as well as in packing; short fibres (linters) provide padding for mattresses and furniture. Even the fibre from the stalk waste is utilised to make pressed paper and cardboard, hence there is very little wastage from the whole cotton plant (NCPA, [WWW] 2004).

The cotton lint is the highest value componant of the crop, and after separation in the ginnery, it is compressed into bales of approximately 500lbs (227kg) weight (Figure 1.02), then transferred to the cotton mill.

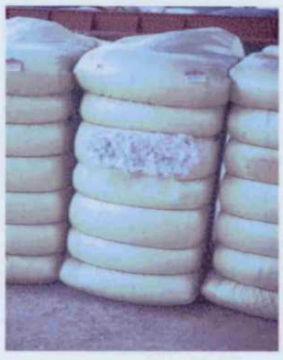


Figure 1.02: Raw cotton lint bales consisting of around 500lb of cotton. Adapted with permission from MEMBG, [WWW] 2004.

At the mill, bales are opened and lint from several bales is blended together. The blended cotton is then cleaned; this procedure is required since during harvesting cotton acquires various contaminants including fragments of seed, leaf, stem, soil and other particles. The cleaning process requires the cotton to pass through a blowing room and carding room. In the blowing room, impurities are removed using air currents and different fibres are thoroughly mixed. At the next stage, the carding room is where steel-wire teeth rotate on a metal cylinder and comb the cotton into parallel lines and short cotton lengths are taken out. The aligned fibres are then drawn into a single strand called a sliver, a roving machine draws the slivers out further and twists them to give strength, the rovings are spun into yarn by a spinning machine, which continues to draw and twist the strands. The yarn is then wound onto bobbins ready for weaving into cloth.

There are a number of different weaving patterns; all have lengthwise yarn (the warp) and crosswise yarn (the weft). Plain weave, where the weft is passed alternately over and under the warp is used for gingham and chambray. Sturdier fabrics such as denim require the twill weave; this interlaces the yarns to form diagonal ridges, whereas fine, smooth cloth involves the satin weave, which has fewer yarn interlacings. The cotton fabrics produced from weaving are known as grey goods, they pass to the finishing processes of dyeing, bleaching or printing. Irrespective of the final destination, the overall quality of cotton cloth is ultimately dependent on characteristics of the original fibre harvest. This determines a number of features, including fibre length, strength and colour, which in turn affect how easily and quickly the fibre can be spun, how much wastage there is and whether the cloth is evenly dyed. Due to the importance of the crop quality, cotton lint is graded by testing a large number of parameters.

1.2.2 Industrial History

Cotton is an interesting product historically, in that its increased production during the industrial revolution, lead to the first large-scale mechanised industry, the textile industry. In the UK, the cotton industry became concentrated in Lancashire, with Manchester at the centre. Expansion was rapid – Manchester had a population of 13,787 in 1773, and of 270,363 by 1831. This paralleled the increased demand for cotton from 5 million lbs in 1778 to 274 million lbs in 1831 (Jacobs, 1997). Following industrialisation, cotton mills were built to process vast quantities of fibre. This lead to large numbers of workers being confined in a single space, a situation that laid the

foundation for poor industrial working conditions, which increased the likelihood of disease, morbidity and mortality among working populations (Jacobs, 1997). A typical mill environment was inherently unhealthy, "the heat, the lack of movement in the air, the complete lack of ventilation, and this without taking into consideration that in some departments of the mill the dust was so thick that it was impossible to see across the room" (Rooke, 1976). It is the dust clouds, created during the physical agitation of the cotton fibres, which have the most serious implications for occupational health, because long-term inhalation may induce respiratory impairment, including byssinosis.

1.3 COTTON DUST

Heterogeneous in nature, cotton dust contains a myriad of particles (Table 1.06); because of this, it has been difficult to fully elucidate the dust components, which are causal factors for byssinosis. Cotton dust is defined by the Occupational Safety and Health Administration (OSHA) as particles "present in the air during the handling or processing of cotton, which may contain a mixture of many substances including ground up plant matter, fiber, bacteria, fungi, soil, pesticides, non-cotton plant matter and other contaminants, which have accumulated with cotton during growing, harvesting and subsequent processing or storage periods" (OSHA, 1995).

The components of cotton dust
8-10% H_2O
Up to 2% NO_3
10-20% inorganic compounds (sand, grit etc.)
Carbohydrates: cellulose and hemicelluloses
Lignins
Condensed tannins
Hydrolysable tannins
Phenolic pigments
Porphyrins
Lipids
Proteins and peptides
Glycoproteins and peptides
Free sugars, amino acids, aminosugars, and amines
Miscellaneous compounds (from insecticides etc.)

Table 1.06: The composition of cotton dust. Adapted from Wakelyn, *et al.*, 1976.

There was a flurry of research into the nature of cotton dust exposure 20-30 years ago, probably in response to the prevalence of dust-related illness at this time, which remained high despite increased awareness of the problem. Experimental exposure to cotton dust demonstrates an inflammatory response, characterised by invasion of neutrophils and other cells into the lungs and airways, along with production of inflammatory mediators from alveolar macrophages such as thromboxanes, prostaglandins, interleukin 1

and platelet activating factor (PAF) (Rylander, 1990). Spirometry studies using model cardrooms have shown that following several hours controlled exposure to artificially created cotton dust; acute decrements in lung function can be observed in young, healthy non-smokers. Mean FEV_1 decline in atopic subjects is significantly higher (Sepulveda, *et al.*, 1984).

As specific toxic agents present in the dust remained unidentified until relatively recently, exposure limits for general dust levels were implemented. This followed recommendation by organisations such as the Health and Safety Executive (HSE) in the UK and the National Institute for Occupational Safety and Health (NIOSH) and the Occupational Safety and Health Administration (OSHA) in the USA (HSE, 2000; OSHA, 1995). These limits are monitored in terms of mass of dust collected gravimetrically by vertical elutriator dust sampler. However, several dust components with toxic activity have now been isolated, suggesting that more specific exposure limits may be more appropriate to protect against the development of lung disease.

1.4 THE BIOLOGICAL CONTAMINATION OF COTTON

There are several agents, which have been investigated with respect to byssinosis causality, and these can be divided into two groups: those naturally present in cotton plants, and those of microbial origin, from external sources (Table 1.07). The toxicants present on cotton fibres, as a result of microbial contamination are of particular interest. Bacterial endotoxin, the most extensively studied toxicant has shown direct evidence of causality in

numerous experiments (Castellan, *et al.*, 1987; Haglind and Rylander, 1984; Milanowski, *et al.*, 1995; Sandström, *et al.*, 1992). There is also mounting evidence of the role of fungal-derived glucan in occupational lung disorders (De Lucca, *et al.*, 1992; Rylander, *et al.*, 1989; Rylander, 1996; Thorn, *et al.*, 2001), hence these two toxicants and their source organisms continue to be scrutinised.

Plant Origin	Microbial Origin
Epoxides	Gram-positive bacteria (proteases)
Histamine	Gram-negative bacteria (endotoxin)
Phenols	Fungi (1-3-β-glucan)
Tannin	
Terpenoids	

Table 1.07: Agents present on cotton fibres, investigated in the context of byssinotic symptoms. Adapted from Rylander, 1990.

Exposure to field weathering after boll opening is the main cause of high microbial contamination of cotton fibres (Millner, *et al.*, 1984); which can ultimately affect cotton quality. In the case of hand picking, it is usual to carry out 3-4 pickings in a season to harvest cotton locks as soon as possible after boll opening. However, machine harvesting does not discriminate between open and closed bolls, hence harvesting cannot commence until all bolls have opened. A further factor in machine picking is the need to wait for the green leaves surrounding the boll to drop, in order to fully expose the cotton fibres and avoid picking large amounts of leaves. As time is an important

factor, when conditions do not promote natural defoliation prior to harvesting, a chemical defoliant can be used on the plants, and this is routinely carried out in Australia and Israel. The defoliant is applied when 60-80% of bolls have opened, and subsequently harvest commences around 10 days later (Encyclopedia, [WWW] 2004).

Prior to boll opening, cotton fibres are in a sealed, sterile environment. However, in the weathering period of days or weeks prior to harvesting, cotton fibres are openly exposed to environmental conditions. During this time, it is not only climatic factors that affect the fibres, but also living organisms present in the surrounding environment. There are numerous insect pests to which cotton is vulnerable during this time, the two most feared of which are the phloem feeding whitefly (*Bemesia tabaci*) and cotton aphid (*Aphis gossypii*). After ingesting the sucrose that constitutes 90% of cotton phloem sap, these insects excrete numerous sugars as honeydew (Gamble, 2001). When large quantities of honeydew build up, cotton fibres can become 'sticky'. Problems associated with sticky lint include higher costs of insect control, increased trash in seed cotton, special handling requirements at cotton gins, and reduced efficiency at textile mills. When these deposits accumulate on processing machinery, they can cause yarn breakage, shutdowns, loss of production, and hence reduced profits.

The high levels of sugars, both of plant and insect origin, along with warm temperatures during cotton production provide ideal conditions for microbial growth. The soil surrounding the cotton plants (the rhizosphere), and

surfaces of surrounding plants (the phyllosphere), act as a source of microbial contamination, transferred to newly exposed fibres via insects, physical contact when plants rub together in the wind, or in droplet splash from rain or heavy dew. In this way when cotton fibres are harvested they are already colonised by a range of bacteria and fungi. As cotton is initially processed without being subjected to treatments that would remove this contamination such as heating or washing, it remains throughout the early stages of processing.

1.5 COTTON BACTERIA

Bacteria are found on all parts of cotton plants including roots, stems, leaves, branches and lint. Both Gram-positive (GPB) and Gram-negative (GNB) species have been identified on cotton; most of these organisms live as epiphytes that produce no visible symptoms. Although there are some bacteria, which cause severe infection of the cotton plant, for example, angular leaf spot disease, also known as bacterial blight, is caused by the bacterium *Xanthomonas malvacearum*. The microbial contamination of cotton can lead to structural damage to the fibres, a condition called cavitoma, which can seriously affect the quality and hence price of a cotton harvest. Quantitative tests used to assess fibre quality can measure the parameters affected by microorganisms.

During opening and carding, large numbers of microorganisms and their fragments are released along with the dust, into the mill atmosphere.

Although some bacteria may die, Gram-positive spores are resistant and remain in the environment for long periods (Fisher and Domelsmith, 1997). However, it is the Gram-negative bacteria isolated from cotton, including *Enterobacter* and *Pseudomonas* that stimulate respiratory responses, for example when inhaled by guinea-pigs *in vivo* (Salkinoja-Salonen, *et al.*, 1982). The two major bacterial groups, Gram-negative and Gram-positive, are based on properties of the cell walls, which govern whether they stain positive or negative under the Gram stain. The Gram-positive cell wall consists largely of a thick layer of peptidoglycan, whereas the Gram-negative cell wall is more complex, containing an additional outer layer, consisting of lipopolysaccharide (endotoxin) (Brock, 2000). The range of Gram-negative species commonly isolated from cotton, are rarely human pathogens, and hence do not cause lung infections in those exposed to them. Instead, fragments of the outer membrane, which become airborne during cotton processing, stimulate an immune response in the respiratory system of industrial workers.

1.6 ENDOTOXIN

Endotoxin is a component of the external membrane of all Gram-negative bacteria where it is needed to maintain cell integrity and function (Holst, *et al.*, 1996). Bacteria naturally release small quantities of endotoxin as they replicate, and the whole membrane content is released upon death and subsequent cell lysis. The term 'endotoxin' describes the molecule *in situ*, i.e. when still associated with proteins and other molecules of the bacterial

membrane. However, the pure molecule can be obtained via purification steps, and is referred to as 'lipopolysaccharide' (or LPS) due to its chemical composition, which consists of both lipid and saccharide moieties. The structure of LPS is well characterised; it can be divided into three different regions, the O-specific side chain, the core region and Lipid A (Figure 1.04).

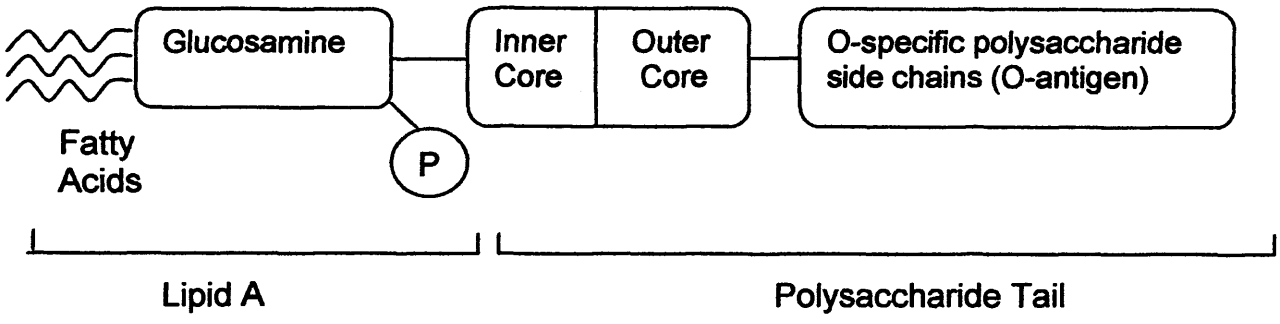


Figure 1.04: Diagrammatic representation of the basic structure of the LPS molecule.

The O-specific side chain (or O-antigen) is a heteropolysaccharide consisting of repeating units up to eight sugar monomers long. A large range of residues may constitute these chains, including amino sugars, deoxy sugars, neutral sugars, sugar acids and sugar phosphates (Holst, *et al.*, 1996). The chain protrudes extracellularly from the membrane and is the most structurally variable segment of the molecule. Its diversity in structure is thought to help the toxin evade the host immune system via stimulation of specific immune responses. The core region is a small string of sugars displaying limited variation between different bacterial species (Holst, *et al.*, 1996). This region can be subdivided into the inner (linked to Lipid A) and the outer core. The inner core segment contains KDO (2-keto-3-deoxy-D-manno-octonic acid), an eight-carbon sugar, which anchors the oligosaccharide

section of the molecule to lipid A. The lipid A region is responsible for the toxicity of the molecule and has specific roles in the assembly and function of outer membranes. This molecule is unique to Gram-negative bacteria since it consists of the sugar glucosamine, phosphate, and several long chain fatty acids.

Whole animal studies on guinea pigs using plethysmography have revealed that endotoxin exposure produces a dose-dependent decrease in specific airway conductance, and an increase in vascular permeability (Gordon, *et al.*, 1988). Increased production of free lung cells, particularly macrophages and neutrophils following inhalation of aerosolised LPS, has also been demonstrated in guinea pigs (Fogelmark, *et al.*, 1994; Snella and Rylander, 1982). The effect of LPS on the airways is mediated through the alveolar macrophage as a primary target, which initially stimulates an inflammatory response in the respiratory system. Neutrophil and eosinophil invasion into lung tissue occurs a few hours after exposure, and these cells subsequently enter the airways 12-24 hours after activation has occurred (Rylander, 1992). Endotoxin is a potent pyrogen, and tests have shown that macrophages are activated at concentrations of LPS as low as 1ng/ml (see Michel, 2000).

Endotoxin preparations obtained by isolating bacteria from cotton have also been shown to cause decreased pulmonary function, as demonstrated by a fall in forced expiratory volume in one second (FEV_1) when inhaled by naïve human subjects (Rylander, *et al.*, 1989). Thorn, (2001a) also described human responses to endotoxin including decreased FEV_1, increased airway

hyperresponsiveness, and elevated blood neutrophil levels. Increased levels of bronchoalveolar lavage (BAL) neutrophils, lymphocytes, IL-1, IL-8 and TNFα, have also been reported in human challenge studies (see Thorn, 2001a). Endotoxin has been implicated as a causative or at least exacerbating factor in not only byssinosis but also ODTS (Lang, 1996). Hypersensitivity pneumonitis, specifically farmer's lung, has also been linked with endotoxin (Nicholls, 1992). However, the exact role of endotoxin and nature of the immune response in these types of disease remain unresolved.

1.7 COTTON FUNGI

Fungi are also prolific contaminants of cotton plants; they are spore bearing heterotrophic organisms, which rely on their surrounding environment for survival. In order to absorb nutrients, fungi must grow through and within their substrate. They can form a number of relationships with their host, commonly saprophytic (feeding on dead or decaying matter), or parasitic (feeding on living organisms). There are several serious diseases of cotton caused by fungal infection. Fusarium wilt and Verticillium wilt caused by *Fusarium oxysporum* and *Verticillium dahliae* respectively, are soil borne organisms, which attack the water-conducting vascular system of the plant stem; stems and veins turn brown and become inactive, resulting in wilted foliage (Arizona University[a], [WWW] 2004). Alternaria leaf spot is caused by *Alternaria macrospora*, a fungus that infects the leaves, bracts and bolls, it survives in plant debris and on weeds. Under high humidity or rainfall, spores are produced that are windblown or splashed onto cotton plants (Arizona

University[b], [WWW] 2004). Boll rot is caused by *Diplodia gossynia* and some *Fusarium* species, which also survive on plant debris in the soil, (Issac, 1992). Along with these specific pathogenic organisms, cotton plants can be colonised by fungi without exhibiting symptoms of disease. These may cause structural damage to fibres through the production of the cellulose degrading enzyme cellulase (Kaese, *et al.*, [WWW] 2003). As cotton fibres are 94% cellulose in their dry mass, this often visually undetectable damage may be observed in fibre quality testing, as measurements of strength and colour could be adversely affected.

Species of cotton fungi are also capable of causing human illness. The common cotton genera *Alternaria* and *Cladosporium* can cause asthma in susceptible individuals causing broncho-constrictive symptoms similar to byssinosis. *Aspergillus,* another fungus often associated with cotton can also cause respiratory illness. Allergic bronchopulmonary aspergillosis (ABPA) produces an allergy to *Aspergillus* spores, it usually occurs in asthmatics causing intermittent episodes of coughing and wheezing (H&E, [WWW] 2005). Many fungi can produce low molecular weight toxic metabolites called mycotoxins, which when inhaled in sufficient quantities cause a range of health problems. Species in the genera *Aspergillus, Penicillium* and *Cladosporium* produce mycotoxins, and these three have all been isolated from cotton fibres (Kaese, *et al.*, [WWW] 2003). *Aspergillus versicolor* is often isolated from mouldy organic matter, and has been identified on cotton fibres, it produces the mycotoxin sterigmatocystin, which can cause diarrhoea, upset stomach and is a kidney and liver carcinogen (Lawley, [WWW] 2005).

Aspergillus is also often isolated from soil and decomposed plant material. *Aspergillus flavus* is responsible for aflatoxin production; depending on the levels, this group of toxins can severely affect the liver and are human carcinogens. Aflatoxins are frequently found in agricultural material but their disease-causing potential in organic dusts is currently unknown (Sorenson, 1999).

Many of these fungi are, at best, only opportunistic or facultative human pathogens and those capable of producing toxic chemicals do so in small quantities under specific conditions. Consequently, they are not often a serious concern in the everyday cotton production environment. However, one of the most topical fungal agents attributed to occupational lung disease is glucan, a structural component of the fungal cell, which is present in large quantities in occupational environments and capable of inducing an inflammatory response.

1.8 GLUCAN

Glucans are glucose polymers consisting of glucopyranosyl subunits, which occur with α or β linkages, (Figure 1.05 shows the basic structure). The most biologically active forms have a (1→3)-β-D backbone. (1→3)-β-D-glucans are important components in the cell walls of fungi and plants. They are also present in small amounts in fungal cytosol and as polymers secreted into the environment by certain bacteria. Glucans have known immune stimulatory effects and are also implicated in occupational disease. In the fungal wall,

glucans are linked to proteins, lipids and other carbohydrates forming a network of branches.

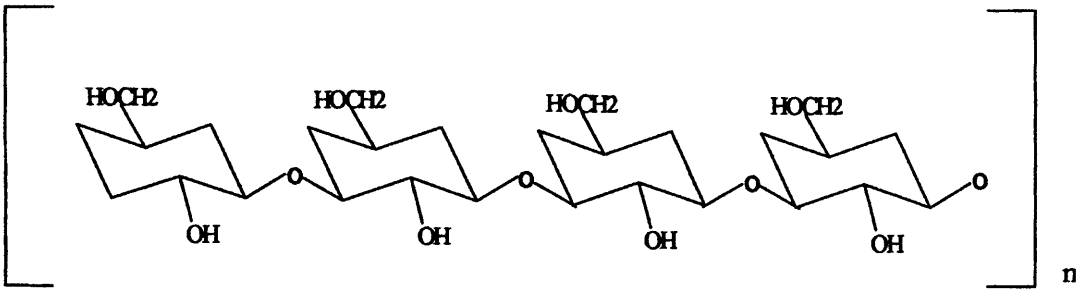


Figure 1.05: The basic structure of (1-3)-β-D-glucan

The role of glucans in fungi is thought to be structural, as they maintain the rigidity and integrity of the cell wall (Williams, 1996). Glucans can be branched or non-branched and are able to exist as a single helical polymer strand or as a complex of three polymer strands forming a triple helix. It is the triple helix that is most common in nature, probably due to its stable structure caused by extensive hydrogen bonding (Williams, 1996). The biological activity of glucans is dependent on factors such as molecular weight, degree of branching, ultrastructure and source (Young, *et al.*, 1998).

The exact mode of action of this group of toxins has not been fully elucidated, though they are known to possess a wide range of biological activities. Some glucans have the ability to enhance the function of macrophages and neutrophils, providing protection to the host against cancer, microbes and radiation and it is because of these properties that they are known pharmacologically as "biological response modifiers" (BRM) (Bohn and BeMiller, 1995). Glucans however, are also well documented as potent

immunotoxicants, and zymosan, a β-glucan containing particle prepared from the yeast *Saccharomyces cerevisiae* (bakers yeast), is a model for inducing acute inflammatory response. The insoluble and particulate glucans such as curdlan tend to have greater pharmacological effects, most importantly on macrophages, leading to severe inflammation. The binding of glucans activates macrophages leading to the production and release of inflammatory mediators. *In vitro* studies have shown that the glucan grifolan, stimulates the secretion of TNF-α and IL-6 from cultured macrophages (Thorn, *et al.*, 2001), as well as the production of chemotactic factors (Milanowski, 1997). This is further demonstrated *in vivo* by neutrophil migration (Nicholls, *et al.*, 2002). The stimulation of monocytes by β-glucan *in vitro* has also been found to cause IL-1 production (Abel and Czop, 1992).

Although the release of these mediators implies a similar response to that elicited by endotoxin, inhalation of β-glucan appears not to cause an increase in neutrophils in the lungs. As neutrophil recruitment is characteristic of endotoxin inhalation, this indicates that glucans operate by a different mechanism (Thorn, *et al.*, 2001). Glucans have been shown to potentiate ovalbumin-induced infiltration of eosinophils into guinea-pig airways on chronic exposure (Fogelmark, *et al.*, 2001). However, another study has reported that ovalbumin-induced eosinophilia was decreased by glucan (Rylander and Holt, 1998). These anomalies, which highlight the difficulties of studying such complex agents, may be due to the utilisation of different glucan types, exposure regimes and/or animal species.

1.9 PREVENTION OF OCCUPATIONAL LUNG DISEASE

There are now set procedures in place to monitor and control cotton dust levels, at least in the more developed World. Efficient industrial exhaust ventilation removes the majority of the hazardous dust. Surveillance of workers is also important so that employees are not exposed to unnecessary risks. In order to decrease this risk, worker and management education is required, especially regarding the individuals who are most at risk such as asthmatics and smokers. All prospective employees are examined by a questionnaire in which a family history of allergy or asthma is taken, along with a physical examination. Workers have annual clinical examinations to monitor any progressive fall in FEV_1 and respirators are worn to protect workers. Airborne dust of respirable size is controlled in several countries by keeping levels below highest permitted concentrations. These exposure limits vary for different regions. Table 1.08 shows allowable levels of textile dust.

However, due to a closer correlation with occupational symptoms, endotoxin and glucan measurement may confer a predictive advantage of individual exposure risk compared to dust measurement alone (Rylander, 1997). Since enzyme assays used to assess endotoxin and glucan levels essentially evaluate a biologically active component, which may fluctuate even when dust levels remain constant, the importance of establishing limits for these agents is reinforced (Olenchock, *et al.*, 1983; Rylander, 1997).

Country	Exposure limit
Czech Republic	2 mg/m^3 (PEL)
Germany	1.5 mg/m^3 (TWA)
Sweden	0.5 mg/m^3 (TWA)
Switzerland	0.3 mg/m^3 (TWA)
UK	2.5 mg/m^3 (PEL)
USA (NIOSH)	< 0.2mg/m^3 (TWA)
USA (OSHA)	0.1-1mg/m^3 (PEL)*

Table 1.08: Highest permitted concentrations of textile dust in different countries. TWA = time weighted averages, PEL = personal exposure limit, * = dependent on area of operation. Derived from Nakládalová, 2000.

Currently, the implementation of endotoxin safety limits has not progressed beyond the stages of discussion and recommendation. The Dutch Expert Committee on Occupational Standards (DECOS) of the National Health Council have recommended a limit of 50 EU/m^3 (approximately 4.5ng/m^3) (Heederik and Douwes, 1997). However, the introduction of endotoxin exposure safety levels is compounded by numerous problems. In addition to differences in measurement procedures used in various studies, fluctuations in measurement assay results and inter-individual variation in inhalation response, there is no consensus on experimental endotoxin 'no effect levels', which range from 9-170ng/m^3 (Heederik and Douwes, 1997). Hence, there is controversy surrounding the precise level of endotoxin, which should be regulated in order to achieve optimal disease prevention. Due to glucan only emerging as a significant toxicant more recently, there is a lack of information

regarding its exact pharmacological effects and responses and a threshold level for this agent has yet to even reach the recommendation stage.

Moreover, human exposure risk to these toxicants is dependent on a number of other factors such as atopy, medical history, age, duration of employment and specific production area of employment. It is also known that the exact chemical structure of the LPS encountered may affect the nature (i.e. potency and intensity) of inflammatory response (Helander, *et al.*, 1982; Kirkiae, *et al.*, 1994). Heterogeneity of the inhaled dust yields further disparity in response and adds the further complication of simultaneous inhalation of endotoxin and glucan, which may cause exacerbated effects, (Cook, *et al.*, 1980; Fogelmark, *et al.*, 1994). These complexities highlight the scale of research into these toxicants still required in order to fully understand them.

1.10 PROJECT AIMS

General aims of the project are to:

- Adapt and apply procedures to characterise the biological contamination of cotton fibre samples, including the identification, and enumeration of bacteria and fungi as well as the measurement of their inflammatory products (endotoxin and glucan).

- Investigate differences between contamination profiles of various cotton samples from a range of countries, and relate this to potential risk of developing respiratory illness.

- Consider factors that may affect the contamination of cotton fibres, including possible sources, influence of production regions and conditions, cotton variety, and sample storage.

- Explore correlations between the different contaminants measured, such as Gram-negative bacteria and endotoxin, to assess the possibility of using them as biomarkers of contamination.

- Compare microbial contamination levels with structural data relating to cotton fibres, in order to assess whether correlations exist between microbes/microbial products and fibre quality.

CHAPTER 2

MATERIALS AND GENERAL METHODS

General methodologies were employed to prepare cotton samples prior to analysis and these procedures will be described below.

2.1 COTTON SAMPLE DETAILS

Cotton lint samples were provided by the Liverpool Cotton Research Corporation (LCRC), Liverpool, UK. They were sent to Liverpool by different cotton producers and traders for arbitration and quality testing procedures. Full sample details are given in the following Tables 2.01.

Thirteen diverse samples for inter-country analysis

Origin	Variety	Harvest	Trash sample
Benin	-	2001/2002	
China 146	-	2001/2002	✓
China Xinjiang	-	2001/2002	
CIS	-	2001/2002	
Iran	-	2001/2002	✓
Ivory Coast	-	2001/2002	
Paraguay	-	2001/2002	
Syria	-	2001/2002	
Tajikistan	-	2001/2002	
Turkey	-	2001/2002	✓
USA	-	2001/2002	
Zambia	-	2001/2002	
Zimbabwe	-	2001/2002	

Four samples for intra-country regional analysis

Turkey	Bergama	2002/2003	✓
Turkey	Efes 1	2002/2003	✓
Turkey	Efes 2	2002/2003	✓
Turkey	Selçuk	2002/2003	✓

Ten samples for intra-country variety/crop year analysis

Sudan (Gezira)	Barakat G4B	1998	
Sudan (Gezira)	Barakat G4B	2000	
Sudan (Gezira)	Barakat G4B	2003	
Sudan (Gezira)	Barakat G6B	1998	
Sudan (Gezira)	Barakat G6B	2000	
Sudan (Gezira)	Barakat G6B	2003	
Sudan (Gezira)	Acala 3SG	2002	
Sudan (Gezira)	Acala 4G	2002	
Sudan (Rahad)	Acala 3SG	2002	

Two samples of seed cotton

Eritrea	SJ2G	2001/2002	
Eritrea	Gedera 5A	2001/2002	

Table 2.01: Details of all cotton samples analysed during the study; including harvest season, availability of trash material and (where known) cotton variety and grade.

2.1.1 Cotton lint samples

Each sample consisted of 30-40g raw cotton lint, taken from a bale of cotton, which had been subjected to ginning (seed removal) but had undergone no other processing stages. Samples were taken from a larger (approximately 100g) section of cotton (removed from the compressed cotton bale centre with a metal hook) originally sent to the LCRC. Upon subsequent arrival at

the School of Pharmacy in Cardiff, they were stored at room temperature in the plastic bags in which they were received (Figure 2.01). Samples remained under these conditions throughout the study period.

Figure 2.01: Cotton samples sealed in plastic wrappings for storage

Each fibre sub-sample used for individual experiments (e.g. 0.2g for endotoxin testing), was taken from a different area of the main sample, in order to establish the mean (n=6) of a cross section of fibres that would have originated from different cotton plants. This procedure was adopted to ensure the samples were as representative as possible of the bale of origin. Samples were placed in sub-groups (shown in Table 2.01 above) for inter- and intra-country studies and analyses, in order to compare samples from the same year of harvest, variety or source, to minimise the number of variables involved.

2.1.2 Trash Samples

Trash samples were also provided by the LCRC. The samples were generated by passing 100g of cotton through a Shirley Analyser. This removed plant particles and short fibres from the lint by passing it around a drum covered in bristles, which separated the fibres allowing particles to drop

down into a tray, resulting in the trash material as shown in Figure 2.02. Trash material was stored under the same conditions as lint samples.

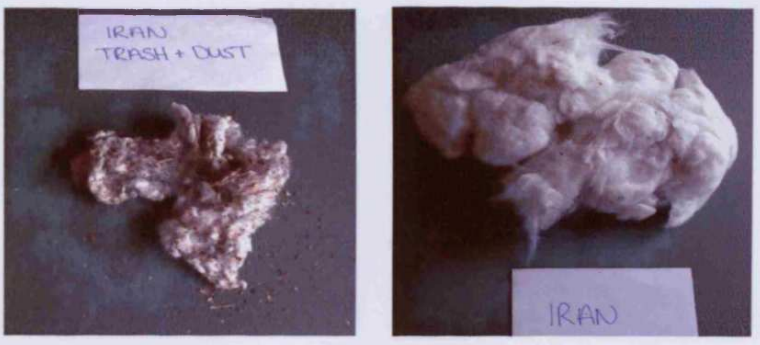


Figure 2.02: Example of trash material removed by the Shirley Analyser, compared to equivalent cotton lint sample.

A limited amount of trash material was available for only a small number of samples, however, all trash data is included in this thesis, as trash material may be an important source of respiratory toxicants.

2.1.3 Seed cotton

Seed cotton is the raw form of cotton fibre. The samples were taken prior to being ginned, and had not had the seeds removed (Figure 2.03).

Figure 2.03: Seed cotton from Eritrea

Seed cotton was stored under the same conditions as lint and trash samples. In order to weigh the seed cotton without the seeds, sterile tweezers were

used to tear the fibres away from the seeds prior to weighing each sub-sample. Although only two samples of seed cotton were available during the study, they were included in order to investigate whether this variety of cotton can be treated in a similar way to cotton lint i.e. if equivalent tests can be successfully performed on this type of sample.

2.1.4 Sub-samples

All sub-samples of lint and trash were weighed on an enclosed balance with an extractor fan attached. They were 'teased' from the main sample using sterile tweezers and weighed into pyrogen free centrifuge tubes. Specific weights were utilised for each set of experiments, determined by preliminary testing to find the optimum for each particular method.

2.2 COUNTRIES OF ORIGIN

The countries from which cotton samples originated, covered a wide range of geographical areas, from Africa (Figure 2.04), Asia (Figure 2.05) and the Americas (Figure 2.06). They represented both large and small cotton producers with varying histories of the trade.

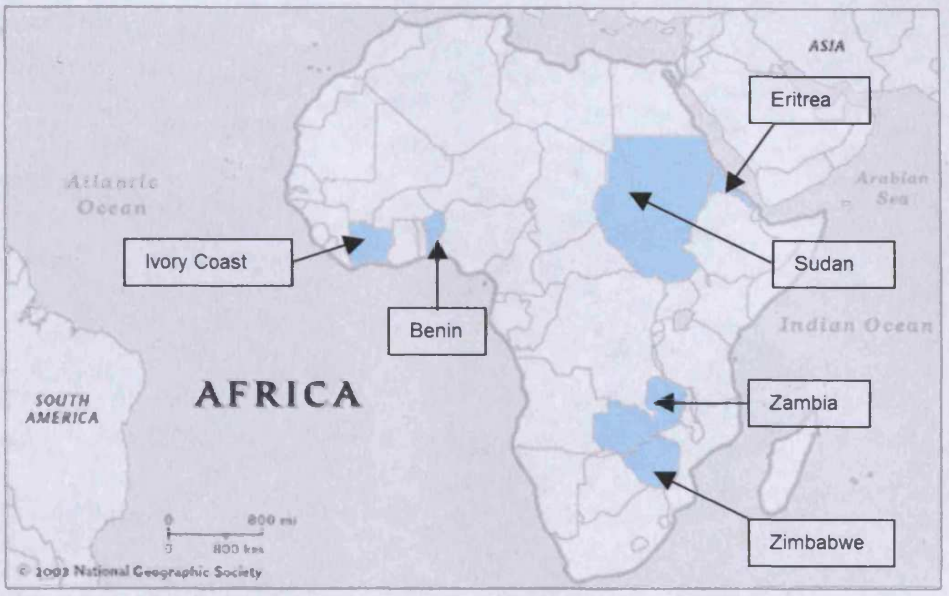


Figure 2.04: The geographical location within Africa of the countries in which cotton lint samples were produced. Adapted with permission from the National Geographic Society, [WWW] 2004.

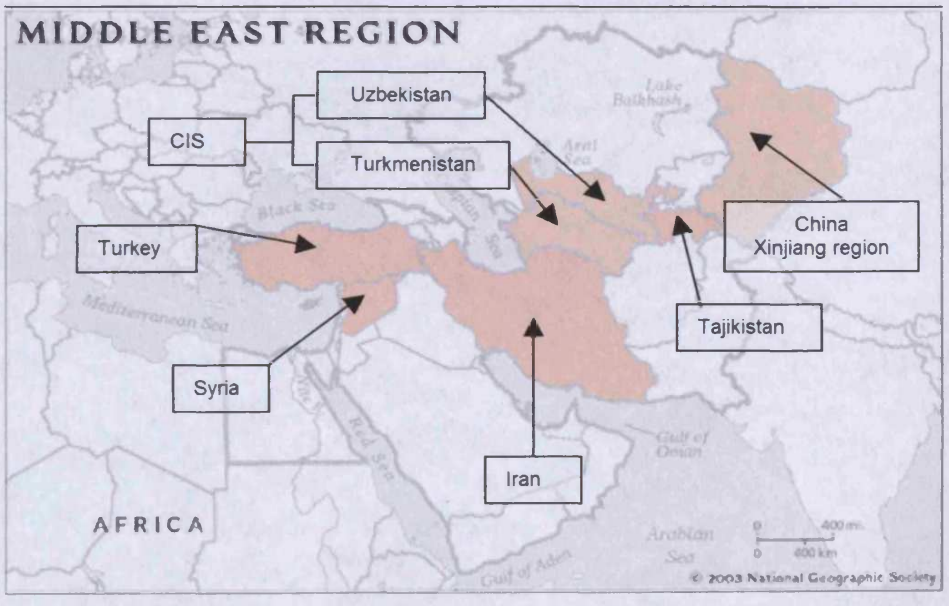


Figure 2.05: The geographical location within Asia of the countries in which cotton lint samples were produced. Adapted with permission from the National Geographic Society, [WWW] 2004.

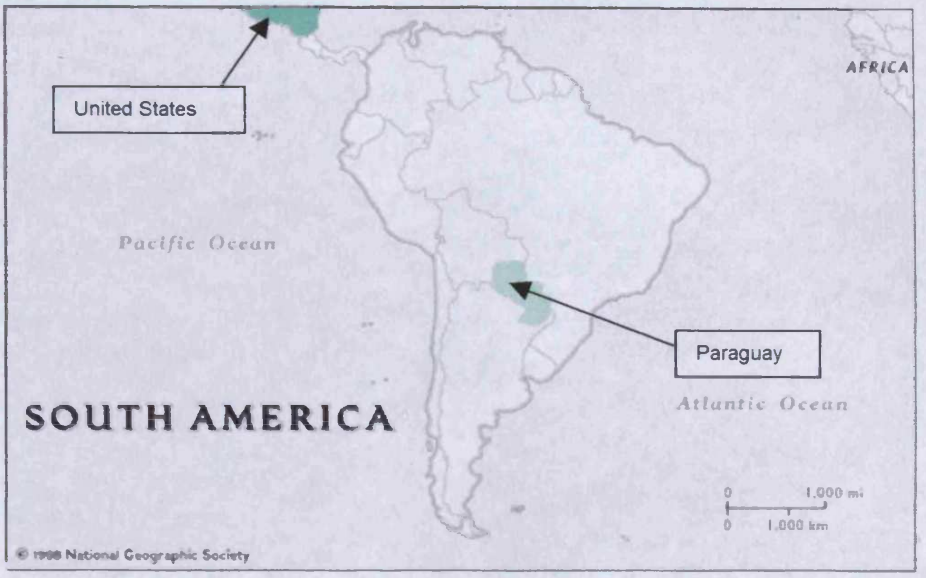


Figure 2.06: The geographical location within the Americas of the countries in which cotton lint samples were produced. Adapted with permission from the National Geographic Society, [WWW] 2004.

2.2.1 Samples from within one country

In order to investigate the differences in cotton sample contamination from within one country, four samples from Turkey were analysed from the 2002/2003 production season and ten samples from Sudan of different grades, species, years of harvest and production region were also included. The geographical position of the three production regions within Turkey are presented in Figure 2.07, and the two production regions within Sudan are highlighted in Figure 2.08. Two samples from the Xinjiang region of China were also analysed, these were both from the 2001/2002 production season and were included in the subgroup containing samples from diverse origins. One of these was referred to as 'China Xinjiang' as the specific type of this cotton was unknown, and the other as 'China 146' as this type was known.

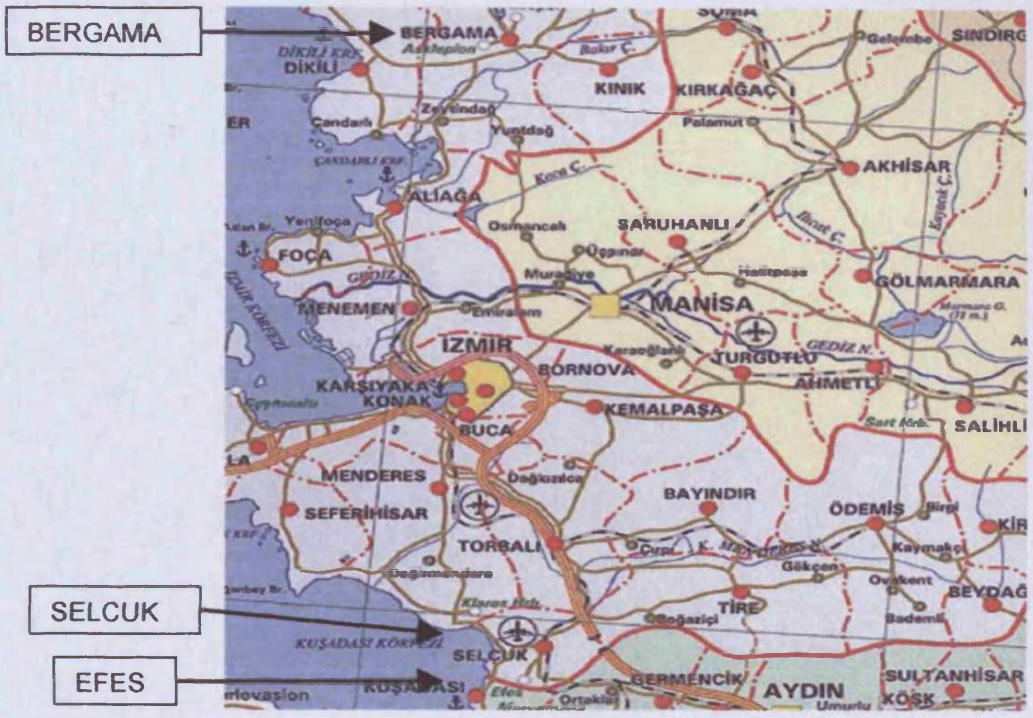


Figure 2.07: The Izmir province of the Aegean region of Western Turkey showing the three areas of cotton growth; Bergama, Efes and Selçuk. Adapted with permission from Yerelnet, [WWW] 2004.

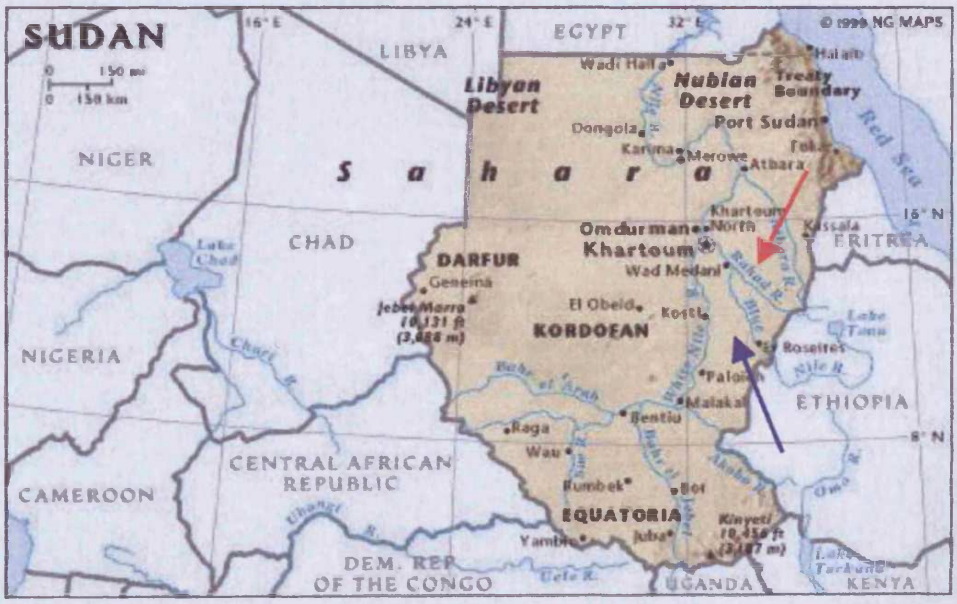


Figure 2.08: Map of Sudan with the Gezira (blue arrow) and Rahad (red arrow) cotton production regions indicated. Adapted with permission from the National Geographic Society, [WWW] 2004.

2.2.2 Production practices/conditions

Cotton production conditions vary considerably between different countries, the production season varies greatly depending on the climate of the region, and methods such as harvesting are performed by hand in many countries, although the use of mechanised picking is increasing. Table 2.02 gives details of the cotton production season and harvest method utilised in the countries from which cotton samples used in the present study originated.

Origin	Planting Period	Harvesting Period	Harvest Method
Benin	June-July	Oct.-Dec.	100% H
China	April-May	Aug.-Sept.	95% H/5% M
CIS: Turk.	April-May	Sept.-Dec	H/M
Uzbek.	March-April	Sept.-Oct.	H/M
Eritrea	NA	NA	NA
Iran	May	Sept.	100% H
Ivory Coast	June-July	Nov.-Jan.	100% H
Paraguay	Sept.-Nov.	Feb.-May	95% H/5% M
Sudan	July-Aug.	Nov.-Feb.	100% H
Syria	April-May	Sept.-Nov.	100% H
Tajikistan	April	Sept.-Nov.	100%H
Turkey	April-May	Sept.-Nov.	98%H/2% M
USA	April-June	Sept.-Dec.	100% M
Zambia	Nov.-Dec.	May-Aug.	100% H
Zimbabwe	Oct.-Dec.	April-Aug.	100% H

Table 2.02: The basic cotton production periods and methods utilised in the countries from which cotton samples originated. H = Hand harvest, M = Machine harvest, NA = data not available. Data adapted from Bremer Baumwollbörse, 2004.

Due to the long growing season involved in cotton production, the season from which a crop originates often spans more than one year. Hence, the production seasons in this investigation were presented as two years, for example 2001/2002. However, where the year in which the crop was harvested from the field is known, as with the ten samples from Sudan, the single year was given.

2.3 METHODS

The specific methods applied to each aspect of the investigation are detailed in the relevant section of each chapter. However, general overviews of the two main types of experimentation (microbiology and enzyme assay), utilised in the project are displayed in Figures 2.08 and 2.09 on the following pages.

2.4 STATISTICAL ANALYSES

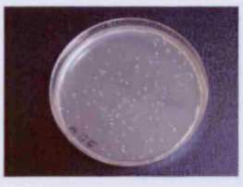
Statistical analyses were carried out using the SPSS statistical software package (Version 11 for Windows). Significance was determined by students t-test (two data sets) or ANOVA with Tukey's HSD *a posteriori* (post hoc) comparisons (three or more data sets). Data was analysed for normality and homogeneity of the variance prior to analysis, as relevant. Relationships between two data sets were analysed by Spearman's rank correlation coefficients and linear regression analysis (y=a+bx).

Overview of microbiological methods (Chapters 3,4,6 and 7)

Cotton samples
Cotton lint samples (20-30g) were received from the LCRC and stored in sealed plastic bags at room temperature

Extraction
Bacterial and fungal cells were extracted by shaking in phosphate buffered saline (PBS) at 1000rpm for 60 minutes

Culture
Supernatant containing viable cells was diluted and asceptically plated on to agar media containing appropriate antibiotics



Growth
Plates were inverted and incubated at 37°C for 24 hours (bacterial cells) or 25°C for 3 days (fungal cells) to achieve colony growth

Identification
Bacteria were identified by biochemical methods. Fungi by colony and cell morphology.

Viable counts
Viable cell counts were performed on fungal and bacterial cells to give colony-forming units (CFU) per gram of fibre

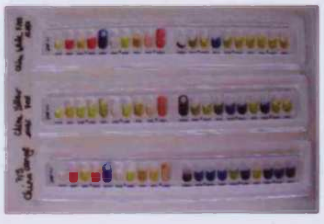


Figure 2.08: Method overview of microbiological work; viable counts and identification of Gram-negative bacteria and fungi.

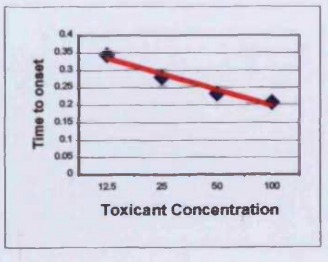
Overview of enzyme assay methods (Chapters 5 and 8)

Cotton samples
Cotton lint samples (20-30g) were received from the LCRC and stored in sealed plastic bags at room temperature

Extraction
Endotoxin and glucan were extracted by shaking in pyrogen free water (PFW) at 1500rpm for 60 minutes

Preparation
Supernatant containing toxicants was added to microtiter plate along with standards and controls. 100µl endotoxin or glucan specific lysate was added to each well

Assay
The microtiter plate was placed into spectrophotometer and time to onset OD measured kinetically for 60 minutes at 340nm (endotoxin) or 405nm (glucan)



Results
Unknown sample results were read from the standard curve simultaneously created from either endotoxin or glucan standards in units per ml

Figure 2.09: Method overview of toxicant measurement; endotoxin and glucan.

CHAPTER 3

IDENTIFICATION OF GRAM-NEGATIVE BACTERIA ON COTTON

3.1 INTRODUCTION

Developing cotton fibres grow within a leafy plant case termed the cotton boll; sunlight eventually triggers boll opening to reveal the fibres, at which point harvest begins. Prior to boll opening, as the cotton plant matures in the field, the enclosed fibres are sterile. However, within 48 hours of opening, the fibres become heavily colonised by a range of microorganisms, which remain associated with the lint throughout its subsequent processing stages. During processing, they become airborne and consequently pose a respiratory threat to cotton workers (Fisher and Domelsmith, 1997). All plant parts including the leaves, stems, and roots are contaminated in this way, but it has been reported that 67% of microflora are present on the cotton lint itself (Fisher and Foarde, 1989). A variety of Gram-negative and Gram-positive bacteria have previously been identified on cotton lint (Table 3.01).

Gram-negative bacteria	Gram-positive bacteria
Agrobacterium	*Bacillus*
Enterobacter	*Clostridium*
Escherichia	*Micrococcus*
Pseudomonas	

Table 3.01: Bacterial genera commonly reported on cotton lint. Adapted from Salvaggio, *et al.*, 1986.

Most of these organisms grow as saprophytes on plant debris or epiphytes on a number of living plants. They do not induce visible symptoms of plant disease, and form a reserve from which new cotton plants are contaminated. It is Gram-negative bacteria (GNB) exclusively that possess and release the respiratory toxicant endotoxin from their outer membrane. This membrane consists of a lipid bilayer with interspersed proteins, between an inner layer of phosholipid, with endotoxin on the exterior (Figure 3.01). In view of these facts, the work in this chapter has focussed on the Gram-negative bacterial component of cotton microflora.

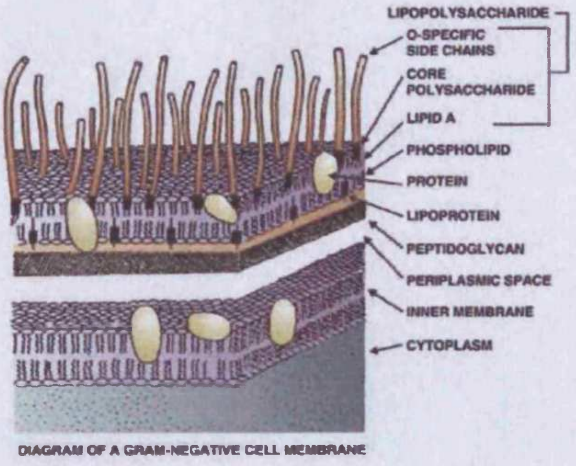


Figure 3.01: The structure of the Gram-negative bacterial membrane showing endotoxin (lipopolysaccharide) protruding outwards from the cell. Adapted with permission from ERDG, [WWW] 2003.

Understanding the GNB populations of cotton plants is important for several reasons. Firstly, knowledge relating to the source and profile of bacteria on the fibres may ultimately aid the development of techniques to reduce the bacterial load, in order to lower the risk of inhalation at a later stage of processing. Identification of particular bacterial genera and species on cotton samples is also necessary, since endotoxin from different species of bacteria

vary in their potency to evoke an inflammatory response (Baseler, *et al.*, 1983; Helander, *et al.*, 1982). More specifically, LPS molecules extracted from different cotton bacteria have exhibited this variability in inhalation studies using guinea pigs (Helander, *et al.*, 1980). Therefore, if cotton lint from different geographical regions are colonised predominantly by potent endotoxin-containing bacteria, this could be indicative of increased respiratory risk to industrial workers in those regions.

Studies involving bacterial identification from numerous cotton samples originating in diverse countries, have not been carried out up until now. Investigations of this nature have been limited to cotton samples taken from different regions within the North American cotton belt (Chun and Perkins 1997; Millner, *et al.*, 1982), or comparison of cotton GNB from a small number of countries, such as US and Indian cotton (Gokani, *et al.*, 1987). For these reasons the Gram-negative flora of cotton lint fibres from various regions were isolated and identified in this chapter.

Aims

- To identify the most common species of Gram-negative bacteria, which contaminate various cotton fibre samples.
- To compare the Gram-negative bacterial species isolated from cotton fibre samples of diverse geographic origins and within one country.
- To consider sources of bacterial contamination and any implications for occupational respiratory risk.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Equipment

All equipment was obtained from reputable sources, and was sterile wherever possible. Full details are available in Appendix I.

Cotton/trash samples

See Chapter 2 for details.

3.2.2 Methods

Methods were carried out utilising conventional aseptic techniques to maintain sterile conditions and ensure external contamination was avoided.

Microbe extraction

Microbes were extracted from 0.6000g (±0.0005g) of cotton lint, into 10 ml phosphate-buffered saline, by shaking on a vortex multi-mixer at 1000 rpm for 60 minutes in a centrifuge tube (techniques were adapted from Nicholls, *et al.*, 1991 following preliminary tests to determine the optimum weights/volumes). The cotton fibres were then physically submerged using a sterile pipette and the supernatant removed to a fresh tube. Due to the limited availability of trash material, microbes were extracted from 0.3000g (±0.0005g) into 5ml phosphate-buffered saline as described above, all other aspects of extraction and culture for trash remained the same as those applied to lint.

Agar plates

Tryptic soy agar (TSA) was made up according to manufacturers instructions (36g/litre) and autoclaved for 1 hour at 120°C. It was then allowed to cool to

approximately 40°C, at which point the antibiotics cycloheximide (50μg/ml) and vancomycin (15μg/ml) were added in order to prevent the growth of fungi and Gram-positive bacteria respectively, during the incubation period (techniques adapted from Chun and Perkins, 1996; Fisher and Sasser, 1987) (see Appendix II for antibiotic mode of action). The molten agar was mixed gently to avoid bubble formation by inverting the flask several times, before 25ml agar was poured into each petri dish under sterile conditions and allowed to solidify. Plates to be used for spread plating were 'over dried' in a laminar flow unit for 30 minutes prior to inoculation in order to remove excess water which may have caused coalescence of colonies, hindering isolation.

Spread plating

Following extraction, supernatant was vortexed for 30 seconds, a 0.1ml aliquot was inoculated onto the centre of an over dried agar plate and evenly spread with a sterile (flamed) glass spreader. All plates were left to stand at room temperature for 30 minutes prior to incubation.

Incubation

Plates were inverted and incubated at 37°C (± 2°C) for 18-24 hours in a cupboard incubator.

Colony isolation

Colony isolation was based on morphological differences such as colour, size and shape. Colonies appearing different were 'picked off' with a flamed wire loop and streaked using the conventional 'three point' technique onto a fresh agar plate (TSA with added cycloheximide and vancomycin). Subcultured plates were then incubated as before for 18 hours. This procedure was

repeated four times on separately extracted and cultivated bacteria for each sample.

Bacterial Identification

Identification procedures were carried out on subcultured isolates 18-24 hours old to ensure that all biochemical pathways were fully functional. Isolates were initially screened by Gram stain and catalase test (Bergey, 1989) (see Appendix III for the basis of the Gram stain). The cell morphology of each isolate was observed using light microscopy at x200 to x1000 magnification following Gram stain. They were then identified by the Analytical Profile Index (API) system.

API identification method

Gram-negative bacteria were identified by the API 20 E system. The kit consisted of strips containing twenty microtubes of dried reagents, upon re-hydration of these wells with bacterial suspension, twenty different chemical reactions occurred, the results of which were used to generate a reaction profile to identify the particular bacteria analysed (see Appendix IV for reactions).

Preparation of the strip

5 ml of distilled water was added to each strip tray to avoid dessication during the incubation period, and the strip placed inside.

Preparation of the inoculum

Isolated bacterial colonies were suspended in an ampule of 0.85% NaCl, using a sterile wire loop and emulsified by gentle vortex to give a homogenous bacterial suspension.

Inoculation of the strip

A sterile pipette was used to fill the tubes of the dessicated test reagents with the bacterial suspension as described by the manufacturer; sterile mineral oil was used to overlay the wells requiring anaerobiosis. Strips were then covered with the tray lid and incubated at 37°C for 18-24 hours.

Oxidase test

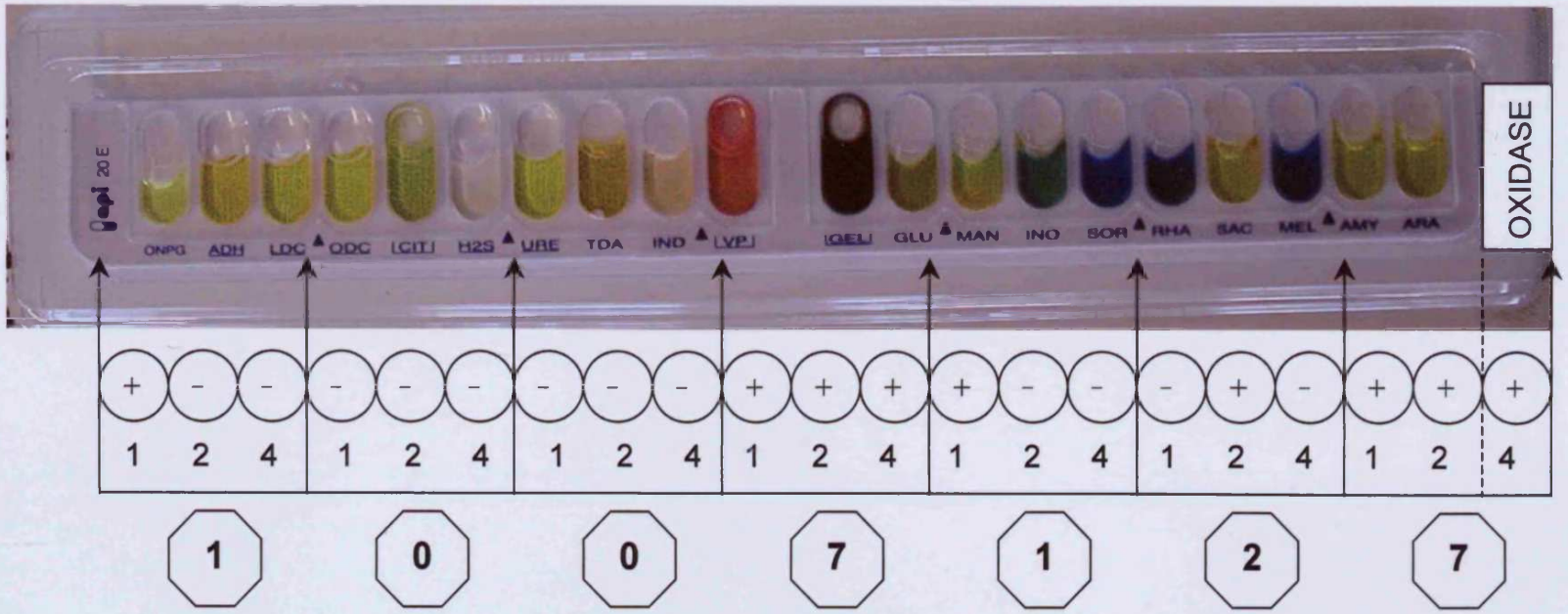
The twenty first reaction was carried out separately to the API strip; a drop of oxidase reagent was added to a filter paper disc and a colony of bacteria mixed into this with a sterile wire loop, a positive reaction resulted in the formation of a dark purple pigment.

Reading the strip

Tests were read as positive or negative by reference to the reading table (See Appendix IV).

Interpretation

Identification was obtained by numerical profile. The tests were separated into groups of 3 and a value 1, 2 or 4 designated in turn to each positive reaction. The values when added together generated a 7-digit profile number from the 21 tests (see Figures 3.02 and 3.03 for illustration). Identification was performed by looking up the numerical profile in the Analytical Profile Index reference book. Identification procedures were confirmed with reference to colony and cell morphology where required. All isolates recorded were identified to good, very good or excellent (90-99.9% probability) levels as specified by the manufacturer.



1007127: Corresponding bacteria: *Aeromonas hydrophilia* (very good identification; 99.2%)

Figure 3.02: Example of reading an API strip using positive and negative colour change reactions to generate a seven digit reference code corresponding to *Aeromonas hydrophilia*

3305573: Corresponding bacteria: *Enterobacter cloacae* (good identification; 92.5%)

Figure 3.03: Example of reading an API strip using positive and negative colour change reactions to generate a seven digit reference code corresponding to *Enterobacter cloacae*

3.3 RESULTS: GRAPHICAL/TABULAR REPRESENTATION

Inter-country Gram-negative bacterial identification

Seven species from five genera of Gram-negative bacteria were identified from thirteen cotton lint samples originating in diverse countries (from 2001/2002 production season). The number of different species identified in individual samples varied from one (in samples from CIS, China 146, Iran, Turkey and Zimbabwe), two (Benin, China Xinjiang, Ivory Coast, Paraguay, Syria, Tajikistan and USA) and three in the sample from Zambia (Table 3.02).

Sample	Bacterial Species
Benin	*Enterobacter sakazakii / Flavimonas oryzihabitans*
China 146	*Pantoea spp.*
China Xinjiang	*Enterobacter cloacae / Pantoea spp.*
CIS	*Flavimonas oryzihabitans*
Iran	*E. cloacae*
Ivory Coast	*E. cloacae / Serratia ficaria*
Paraguay	*Aeromonas hydrophila / S. ficaria*
Syria	*A. hydrophila / Pantoea spp.*
Tajikistan	*E. sakazakii / F. oryzihabitans*
Turkey	*E. cloacae*
USA	*E. cloacae / F. oryzihabitans*
Zambia	*Aeromonas salmonicida / E. cloacae / S. ficaria*
Zimbabwe	*E. cloacae*

Table 3.02: Total Gram-negative bacterial genera and species isolated in aqueous washes from thirteen cotton lint samples from 2001/2002 production season, originating in twelve different countries.

The most common genus of Gram-negative bacteria identified on thirteen cotton lint samples originating in diverse countries (from 2001/2002 production season) was *Enterobacter*, isolated from 69% of the samples tested. The other genera were *Flavimonas* found in 31% of samples, *Aeromonas* (in 23%), *Pantoea* (in 23%), and *Serratia* (also in 23%) (Figure 3.04).

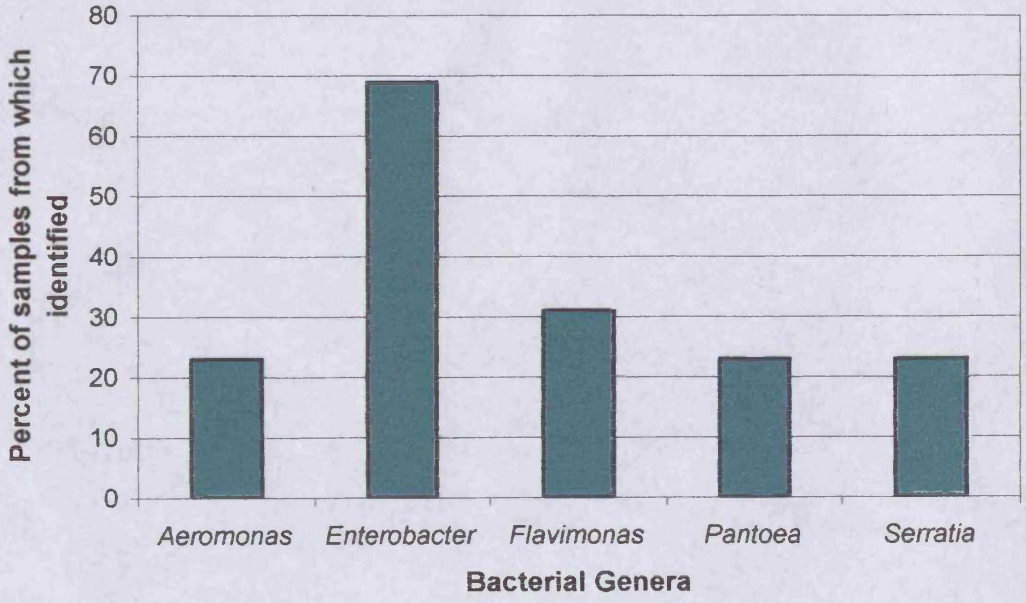


Figure 3.04: The five genera of Gram-negative bacteria, isolated in aqueous washes from thirteen cotton lint samples (2001/2002 production season), from diverse countries of origin, and the percentage of samples from which each was isolated.

Two species of *Enterobacter* were identified from thirteen cotton lint samples originating in diverse countries. *Enterobacter cloacae* was the most common species, occurring in 78% of samples, compared to *Enterobacter sakazakii,* isolated from 22% of samples (Figure 3.05).

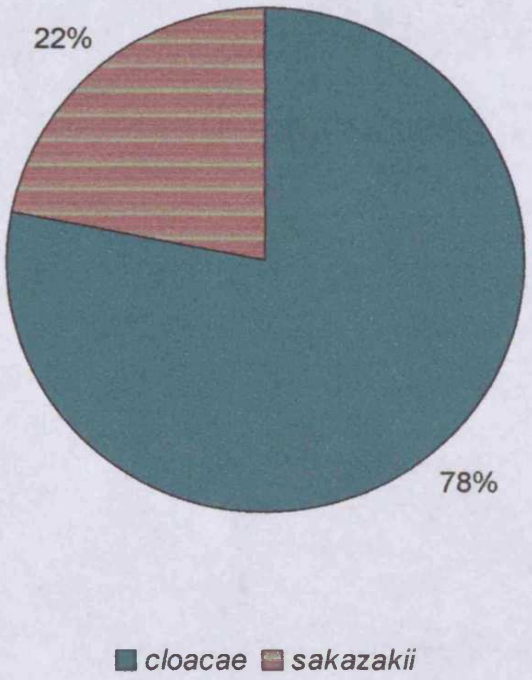


Figure 3.05: The two species of *Enterobacter* (*cloacae* and *sakazakii*) isolated in aqueous washes from thirteen different cotton lint samples from 2001/2002 production season, from diverse countries of origin. Shown as the percent of total *Enterobacter*-positive samples they constituted.

Seven species of Gram-negative bacteria were identified on cotton lint samples originating in diverse countries. *Enterobacter cloacae* was the most widely distributed, being identified in cotton from seven different countries, followed by *Flavimonas oryzihabitans*, isolated on cotton from four countries. *Pantoea spp.* and *Serratia ficaria* were found in cotton from three countries, *Aeromonas hydrophila* and *Enterobacter sakazakii,* from two countries and *Aeromonas salmonicida* was only isolated in cotton from one country of origin (Figure 3.06).

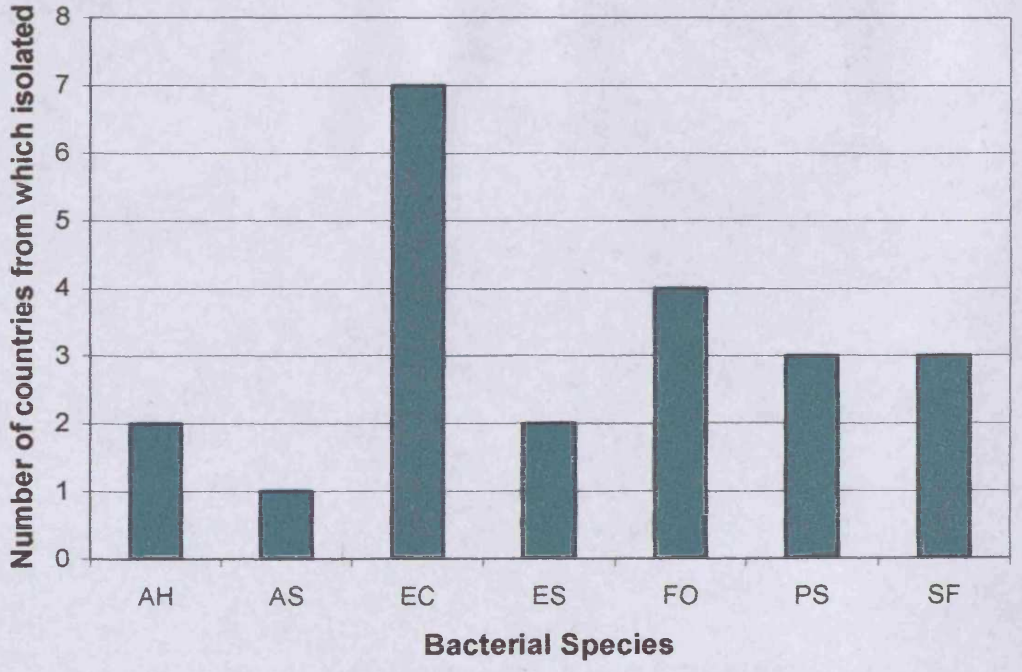


Figure 3.06: The geographic prevalence of Gram-negative bacterial species isolated from aqueous washes from thirteen cotton lint samples from diverse countries (from 2001/2002 production season). AH = *Aeromonas hydrophila,* AS = *Aeromonas salmonicida,* EC = *Enterobacter cloacae,* ES = *Enterobacter sakazakii,* FO=*Flavimonas oryzihabitans,* PS=*Pantoea spp.,* SF=*Serratia ficaria*.

Intra-country bacterial identification; Turkey

Three Gram-negative bacterial genera of different species were identified on cotton lint samples originating in three production regions within Turkey (from 2002/2003 production season); one species was identified in the samples from Bergama, Selçuk and the Efes 1 sample, and two species were identified in the Efes 2 sample (Table 3.03).

Sample (Region of Turkey)	Bacterial Species
Bergama	*Pantoea spp.*
Efes 1	*Serratia ficaria*
Efes 2	*Enterobacter sakazakii / Pantoea* spp.
Selçuk	*Serratia ficaria*

Table 3.03: The Gram-negative bacterial species identified in aqueous washes of four cotton lint samples from 2002/2003 production season, originating from different regions of Turkey.

Intra-country bacterial identification; Sudan

Ten various cotton lint samples from within Sudan yielded three different Gram-negative bacterial species; *Enterobacter sakazakii, Flavimonas oryzihabitans* and *Pantoea spp.*, (Table 3.04). These were all also identified on cotton lint samples from diverse countries of origin (2001/2002 production season) (see Table 3.01 above).

Sample	Bacterial Species
GBA/4B/98	*Pantoea spp.*
GBA/4B/00	*Pantoea spp.*
GBA/4B/03	*Enterobacter sakazakii / Pantoea spp.*
GBA/6B/98	*Pantoea spp.*
GBA/6B/00	*Pantoea spp.*
GBA/6B/03	*Flavimonas oryzihabitans / Pantoea spp.*
GAC/3G/98	*Enterobacter sakazakii / Pantoea spp.*
GAC/3SG/02	*Pantoea spp.*
GAC/4G/02	*Pantoea spp.*
RAC/3SG/02	*Enterobacter sakazakii / Pantoea spp.*

Table 3.04: The Gram-negative bacterial species identified in aqueous washes from various cotton lint samples from within Sudan. Samples vary in harvest year, production region, species and grade of cotton. Production regions are Gezira (G) and Rahad (R), cotton species are Barakat (BA), and Acala (AC), grades are 4B, 6B, 3G, 3SG and 4G. Years of harvest are 1998, 2000, 2002, 2003 (98/00/02/03 respectively).

Pantoea spp., was the most prevalent Gram-negative bacterial species isolated from ten various cotton lint samples originating in Sudan, being identified from 100% of these samples. Followed by *Enterobacter sakazakii* identified in 30% of samples and *Flavimonas oryzihabitans* was the least common, identified in 10% of samples (Figure 3.07).

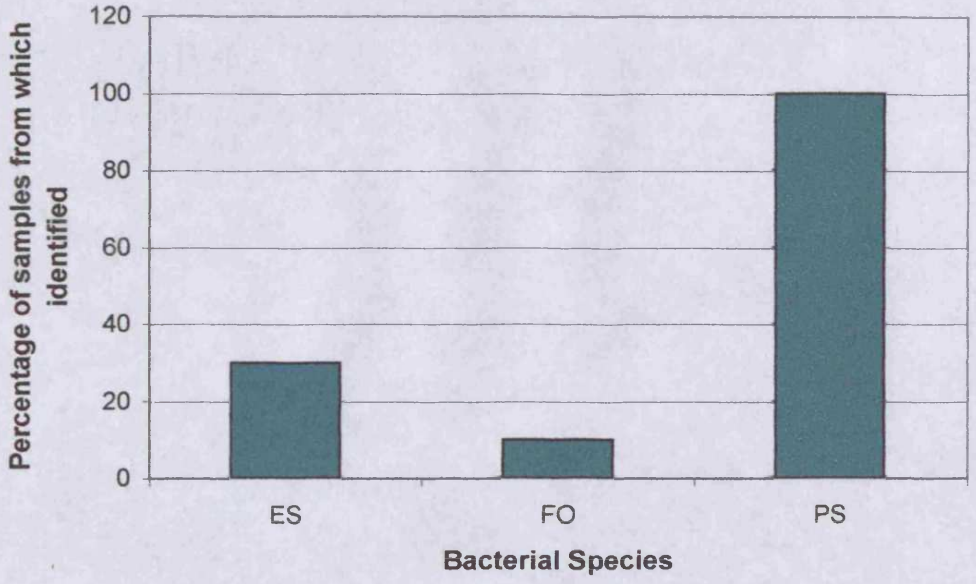


Figure 3.07: The Gram-negative bacterial species identified in aqueous washes from ten various cotton lint samples from within Sudan. Shown as the percentage of samples from which each were identified. ES = *Enterobacter sakazakii*, FO = *Flavimonas oryzihabitans*, PS = *Pantoea spp.*

Factors influencing intra-country bacterial species

Pantoea spp. was isolated from all Sudanese cotton samples; hence, from both cotton species (Barakat and Acala) and from all five different cotton grades, including 3SG (2002) from both production regions. *E. sakazakii* was isolated from both cotton species but was absent from three grades of Gezira cotton, including 3SG (2002), although it was identified in the 3SG (2002) sample from the Rahad region. *F. oryzihabitans* was only isolated from one sample (Gezira Barakat 6B/2002) (Table 3.05).

	Bacterial Species		
	E. sakazakii	*Pantoea spp.*	*F. oryzihabitans*
Cotton Species			
Barakat	✓	✓	✓
Acala	✓	✓	
Cotton Grade			
4B	✓	✓	
6B		✓	✓
3G	✓	✓	
3SG	✓**	✓	
4G		✓	
Production Region			
Gezira 3SG 2002		✓	
Rahad 3SG 2002	✓	✓	

Table 3.05: Factors influencing the distribution of Gram-negative bacterial species identified in aqueous washes from ten cotton lint samples from within Sudan, samples are grouped to show any variation in species, grade (although harvest years also vary) and production region of cotton. ✓ = positive identification, **only identified in Rahad sample.

Influence of harvest year on intra-country bacterial species

Analysis of cotton lint samples originating in Sudan of two cotton grades (4B and 6B), harvested in three different years (1998, 2000, 2003) revealed that *Pantoea spp.*, was present in both grades from all three years of harvest, and was the only species identified on samples from the 1998 and 2000 harvest. *E. sakazakii* was only identified on 4B grade cotton from 2003 and *F. oryzihabitans* on the 6B cotton from 2003, showing samples from this year had the most diverse species range (Table 3.06).

		Bacterial Species		
Sample	**Harvest**	***E. sakazakii***	***Pantoea spp.***	***F. oryzihabitans***
GBA 4B	1998		✓	
	2000		✓	
	2003	✓	✓	
GBA 6B	1998		✓	
	2000		✓	
	2003		✓	✓

Table 3.06: Distribution of Gram-negative bacterial species identified in aqueous washes from cotton lint samples from within Sudan. Showing two sample grades (Gezira Barakat (GBA) 4B and 6B) from three different years of harvest (1998, 2000, and 2003). ✓ = Positive identification.

Effect of geographic cotton production region on bacterial species

By grouping the cotton samples from all studies into general geographic regions (continents) of production, it was seen that the majority of bacterial species were present on cotton samples from across the world. However, although *Aeromonas hydrophila* was present on cotton from both Asia and the Americas, it was not present on samples from Africa, whereas *Aeromonas salmonicida* was only present on cotton from Africa (Table 3.07).

	Geographic Production Region					
	Asia		Africa		Americas	
Bacterial Species	13	TUR	13	SUD	USA	PAR
Aeromonas hydrophila	✓					✓
Aeromonas salmonicida			✓			
Enterobacter cloacae	✓		✓		✓	
Enterobacter sakazakii	✓	✓	✓	✓		
Flavimonas oryzihabitans	✓		✓	✓	✓	
Pantoea spp.	✓	✓		✓		
Serratia ficaria		✓	✓			✓

Table 3.07: The species of Gram-negative bacteria identified in aqueous washes of cotton lint samples from different production regions. Countries from within Asia were CIS, China, Iran, Syria, Tajikistan and Turkey. Those from Africa were Benin, Ivory Coast, Zambia and Zimbabwe. Those from the Americas were Paraguay (PAR) and United States (USA). ✓ = positive identification, 13 = identified in at least one of thirteen samples from 2001/2002 production season used in inter-country study, TUR = identified in at least one of four Turkish samples of 2002/2003 season from intra-country study, SUD = identified in at least one of ten samples in Sudan intra-country study.

Identification of trash bacteria

Comparison of Gram-negative bacterial species identified in cotton lint and equivalent trash samples, originating in three regions of Turkey (from 2002/2003 production season) revealed differences in species on these two materials from the same region, except in the sample from the Bergama region where the single species identified from both lint and trash was *Pantoea spp.*, and both materials from Efes 2 sample carried *Enterobacter sakazakii* (Table 3.08).

	Bacterial Genera/Species	
Sample Region	**Cotton Lint**	**Cotton Trash**
Bergama	*Pantoea spp.*	*Pantoea spp.*
Efes 1	*Serratia ficaria*	*Pantoea spp.*
Efes 2	*Enterobacter sakazakii* *Pantoea spp.*	*Enterobacter sakazakii* *Flavimonas oryzihabitans*
Selçuk	*Serratia ficaria*	*Enterobacter sakazakii* *Flavimonas oryzihabitans*

Table 3.08: The species of Gram-negative bacteria identified from aqueous washes of four cotton lint samples from 2002/2003 production season, and equivalent trash samples from different regions of Turkey.

Comparison of Gram-negative bacterial species identified in cotton lint and equivalent trash samples originating in three different countries from 2001/2002 production season (China, Iran and Turkey), revealed species variation on these different materials except in the sample from Iran where *Enterobacter cloacae* was identified on both lint and trash (Table 3.09).

	Bacterial Species	
Sample Origin	**Cotton Lint**	**Cotton Trash**
China 146	*Pantoea spp.*	*Enterobacter sakazakii*
Iran	*Enterobacter cloacae*	*Enterobacter cloacae* *Pantoea spp.*
Turkey	*Enterobacter cloacae*	*Enterobacter sakazakii*

Table 3.09: The species of Gram-negative bacteria identified from aqueous washes of 2001/2002 cotton lint samples and equivalent trash samples from different countries of origin.

Analysis of the total Gram-negative bacterial species identified on cotton lint samples compared to those isolated from equivalent cotton trash samples, showed that the majority of bacterial species involved in this study were present on both cotton lint and trash; with the exception of *Flavimonas oryzihabitans,* which was only identified on trash material and *Serratia ficaria,* only present on cotton lint (Table 3.10).

	Cotton Material	
Bacterial Species	**Lint**	**Trash**
Enterobacter cloacae	✓	✓
Enterobacter sakazakii	✓	✓
Flavimonas oryzihabitans		✓
Pantoea spp.	✓	✓
Serratia ficaria	✓	

Table 3.10: Gram-negative bacterial species identified from aqueous washes of cotton lint and trash from several different countries (China, Iran and Turkey), including four samples from different regions of Turkey. ✓ = Positive identification.

Bacterial profile of seed cotton

Two different samples of raw seed cotton from Eritrea yielded only one bacterial species each, and these varied between samples; *Enterobacter sakazakii* was isolated from Gedera 5A seed cotton and *Pantoea spp.* from SJ2G seed cotton (Table 3.11).

Cotton Variety	Bacterial Species
Eritrea Gedera 5A	*Enterobacter sakazakii*
Eritrea SJ2G	*Pantoea spp.*

Table 3.11: The Gram-negative bacterial species identified in aqueous washes from two different types of seed cotton from Eritrea (2001/2002 production season).

3.4 DISCUSSION

Method appraisal

In order to examine the range of Gram-negative bacterial species found on diverse cotton lint samples, the most prevalent species of bacteria were identified from 29 cotton samples. A non-selective medium (TSA) was used in order to grow a broad range of bacteria, whilst not providing a selective advantage to any species, which would create an appearance of false dominance. This medum, along with the incubation conditions employed would have allowed the growth of general heterotrophic bacteria, at mesophilic temperatures (25-37°C). Preliminary studies using MacConkey Agar, which is not only selective for enteric bacteria but also differentiates certain species by colour (lactose fermenters appear pink, nonfermenters of lactose appear colourless) proved too selective and resulted in very limited, irregular bacterial growth (data not shown).

It should be noted that the bacteria identified in this study were common, saline-extractable flora, and therefore may have varied from those identified using different extraction and identification methods in previous studies. Bacteria were identified by the API 20 E system, which has been employed in other cotton studies (Fischer and Foarde, 1989; Fischer and Kylberg, 1983; Heintz, 1997); this is specific for *Enterobacteriaceae* and other non-fastidious, Gram-negative rods. However, the vast majority of genera expected to be present on cotton (i.e. those which have been repeatedly identified previously) are identifiable by this method, thus it was chosen over

the more costly and complex methods utilised elsewhere, such as whole cell fatty acid analysis by gas chromatography (Chun and Perkins, 1997). In addition, it should be noted that API identification systems are designed for clinical isolates as opposed to those from the environment. Thus, some common environmental bacterial species are not included in the API reference tables; this renders identification to genus level more reliable than to individual species. However, with this in mind, particular species identified in this study are also discussed here, as they were all identified to above 90% probability.

Profile and source of cotton lint bacteria

Five genera and seven species of bacteria were isolated from the thirteen inter-country cotton samples (see Table 3.02). This included the genera *Enterobacter*, *Flavimonas*, *Pantoea* and *Serratia*, which were also the only genera identified from the intra-country studies in both Turkey and Sudan. These are common soil and plant organisms, which have all been reported previously on cotton lint (Chun and Perkins, 1997), although this does not include all the individual species found in this study. *Aeromonas* (to my knowledge) has not previously been isolated from cotton. However, in this study, it was isolated from 23% of samples from the inter-country study. This included two different species from three diverse countries and as such, it could not be considered a chance occurrence. *Aeromonads* (family *Vibrionaceae*) are ubiquitous in all aquatic environments including chlorinated, polluted, fresh and brackish waters, and survive well in soil (Bergey, 1989). Hence, it is feasible that the source of these cells arose from

contact with contaminated water. This is likely to have occurred during irrigation of cotton cropland, a procedure carried out routinely in many countries (Anthony, 1991). Indeed, *Aeromonas* contamination, (including *A. hydrophila* specifically), of a number of agricultural products (although not cotton) has been attributed to irrigation water in previous studies (Callister and Agger, 1987; Monge, *et al.*, 1998). The production regions of cotton from which *Aeromonas* were isolated in this study, (Paraguay, Syria and Zambia), all use irrigated land to some extent, although other countries investigated, from where cotton samples did not contain *Aeromonas* also use irrigated land (Worldfacts, [WWW] 2004).

Enterobacter (family *Enterobacteriaceae*) was the most common genus identified in the inter-country study occurring in 69% of samples tested from diverse origins (see Figure 3.04), and this has been isolated previously from cotton lint (Fisher and Sasser, 1987; Millner, *et al.*, 1982; Rylander and Lundholm 1978; Simpson, *et al.*, 1989), and cotton dust (Rylander, *et al.*, 1975). *Enterobacter* is widespread in nature where it occupies fresh water, plants, soil and sewage (Bergey, 1989) it has a fermentative metabolism and can thereby utilise many of the sugars available on cotton lint and flourish there. *Enterobacter agglomerans* (known previously in botanical literature as *Erwinia herbicola*), now reclassified *Pantoea agglomerans* (Gavini, *et al.*, 1989), is the species most frequently reported on cotton (Millner, *et al.*, 1984) and often at the highest rate of incidence (Simpson, *et al.*, 1989), but is not identified separately in the API 20 E reference system, so it could not be reported in this study. However, although the API system does not

differentiate particular species of *Pantoea*, it is probable that at least some of the *Pantoea* isolated in this study was *Pantoea agglomerans,* although *Pantoea ananas* has also been isolated from cotton lint previously (Chun and Perkins, 1997), and may also have been present here.

Enterobacter cloacae (Figure 3.08 below), has also been previously reported on cotton lint (Akinwunmi and Heintz, 1997; Chun and Perkins, 1997).

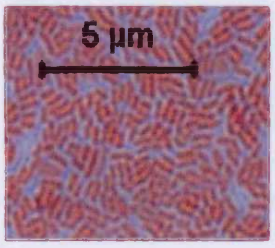


Figure 3.08: *Enterobacter cloacae* isolated from Ivory Coast cotton lint

Interestingly, one study has revealed that *E. cloacae* is present in the gut of whiteflies (common pests of cotton plants), from where it passes to the honeydew (Davidson, *et al.*, 2000), and may be ubiquitous on cotton plants via this potential source. Indeed, in this chapter, *E. cloacae* was the most widespread species since it was isolated from seven different countries (Figure 3.06). *Enterobacter sakazakii,* on the other hand is less commonly reported in cotton lint (Millner, *et al.*, 1982; Simpson, *et al.*, 1989), although it was identified in this study in several samples from different countries.

Pseudomonas is also considered a genus which is common to cotton (Fischer and Kylberg, 1983), since the *Pseudomonads* are heterotrophic bacteria commonly found in soil, with *P. syringae* being reported as one of

the most common species on cotton (Akinwunmi and Heintz 1997; Chun and Perkins, 1997; Simpson, *et al.*, 1984). Although *Pseudomonas* appears absent from the cotton samples examined in this chapter, the *Flavimonas oryzihabitans* (Figure 3.09 below) identified in 31% of the inter-country samples in this study has been recently reclassified as *Pseudomonas oryzihabitans* (Anzai, *et al.*, 1997) and these genera are closely related.

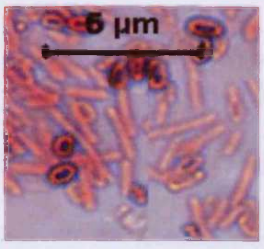


Figure 3.09: *Flavimonas* (now *Pseudomonas*) *oryzihabitans* isolated from USA cotton lint.

Pseudomonas oryzihabitans has previously been reported on cotton lint (as *Flavimonas oryzihabitans*) (Akinwunmi and Heintz, 1997; Chun and Perkins, 1997). Indeed, a range of *Pseudomonas* species have been described on all parts of the cotton plant, including the lint, roots, stems, flowers and seeds (Akinwunmi and Heintz, 1997; Simpson, *et al.*, 1989). Therefore, it was surprising that *Pseudomonas oryzihabitans* was the only species of this genus identified from the samples analysed in this chapter. However, a small number of colonies, present on samples from Benin, China, and Zambia, which gave an API reading of low discrimination, were possibly *Pseudomonas* (*fluorescens* or *putida*) according to the API reference tables (data not shown).

Serratia, also in the family *Enterobacteriaceae*, is very similar to the genera *Klebsiella* and *Enterobacter*. It is frequently isolated from food, soil, water, plants and sewage (Bergey, 1989). *Serratia ficaria* specifically has not been previously identified on cotton fibres, but *Serratia* as a genus has been reported, with *S. proteamaculans* observed on lint (Chun and Perkins, 1997) whilst *S. liquefaciens* and *S. plymuthica* have been noted on cotton plant roots (Akinwunmi and Heintz, 1997; Akinwunmi, *et al.*, 1989).

Escherichia, (specifically *E. coli*) has also been isolated from cotton in numerous studies, but surprisingly this genus was absent from the samples studied in this chapter. However, it was also absent in a previous study where other common bacteria were identified (Salkinoja-Salonen, *et al.*, 1982), suggesting that although it can be found in large numbers on many plants, *E. coli* is not consistently present throughout the phylloplane. Other bacterial genera previously identified on cotton (which can be readily identified by API 20E), but not found in this study are: *Acinetobacter*, *Citrobacter, Klebsiella,* and *Yersinia*. These species have been reported in the literature from studies on US cottons using different extraction and identification methods (Chun and Perkins, 1997; Millner, *et al.*, 1982) but they have not been widely isolated from cotton lint in the past (Fischer and Kylberg, 1983).

Distribution and diversity of bacterial species

The majority of earlier investigations identifying cotton bacteria were conducted on various US cottons, with only a relatively small number of

bacterial species being isolated. In the current investigation, only seven different bacterial species of five genera were isolated from 29 cotton samples originating from fourteen different countries, harvested over five seasons. These genera (with the exception of *Aeromonas*) are frequently reported on cotton lint, suggesting that only a restricted number of species consistently contaminate cotton fibres and these few are found all over the world. This finding concurs with the work of Fischer and Kylberg, (1983), who stated that, "cotton from many different sources contains significant numbers of only a small range of organisms". Although the species identified from trash and lint of equivalent samples varied in most individual cases, the bacteria identified in trash samples also belonged to this limited group, as did the bacteria isolated from the two samples of seed cotton (see Table 3.11). This lack of diversity, predominantly consisting of enteric bacteria, implies that the cotton plant represents a particular ecological niche, which may supply specific nutrients for these types of bacteria (Fischer and Kylberg, 1983), or provide particular growth conditions.

It is also noteworthy that only three species of bacteria were identified from the four Turkish samples and the ten cotton samples from within Sudan (see Tables 3.03 and 3.04 respectively). This may indicate that within a geographic region there is even less bacterial diversity, perhaps with one predominant type; for example, *Pantoea* was present in all samples from Sudan (see Figure 3.07). An early investigation on US cotton (Clark, *et al.*, 1947), concluded that *Enterobacter cloacae* predominated in cotton from Texas and Oklahoma, and a more detailed study found variation in

predominant bacterial species in cotton dust from fibres originating in three different areas of the US cotton belt (Table 3.12 below).

		% total isolates identified as individual species					
Area	Grade	AC	EA	EC	CF	PL	PC
Miss.	43	-	30	8	5	50	-
Miss.	41	-	8	-	-	13	-
Tx.	43	-	33	3	3	30	10
Tx.	41	-	30	-	-	26	15
Ca.	43	-	17	-	-	-	-
Ca.	41	6	22	-	-	-	6

Table 3.12: Predominance profiles of particular cotton bacterial species in air samples from different areas and grades of U.S cotton. Miss=Mississippi, Tx=Texas, Ca=California, AC=*Acinetobacter calcoaceticus*, EA=*Enterobacter agglomerans*, EC=*Enterobacter cloacae*, CF=*Citrobacter freundii*, PL=*Pseudomonas*-like sp., PC=*Pseudomonas cepacia*. Adapted from Millner, *et al.*, 1984.

These data also reveal differences in the bacterial species isolated from different grades of cotton from the same region (i.e. within Mississippi). This is also indicated in the results of the Sudan study in this chapter (see Table 3.05), since *Pantoea* was detected in all five different grades from the Gezira region, whereas *Enterobacter sakazakii* was only found in two (4B and 3G) and *Flavimonas oryzihabitans* in only one (6B).

It must be acknowledged that general analyses of data from the intra-country Sudan study (looking at grades etc.) also compared cotton from varying harvest years. When focussing on the two grades (4B and 6B) that were sampled from three different harvest years, it appears that in both cases the

most recent samples (harvested in 2003) had a more varied bacterial profile (see Table 3.06). The bacterial profile data for these samples before they were placed in storage was unavailable, so it is unknown whether this was the case at harvest and hence, if it arose from the particular field conditions for that year, or if storage time had affected the bacterial species present on the samples involved in this study. Total Gram-negative bacterial counts on cotton lint reportedly decrease with storage time (Chun and Perkins, 1996), and more specifically, "the Gram-negative bacterial population became a substantially smaller percent of the remaining viable bacterial population after the first year of storage" (Chun and Perkins, 1996). It may be that there is also a loss of bacterial diversity during storage; possibly the most common bacteria remain predominant while others diminish to insignificant levels. Notwithstanding this, comparison of the one cotton grade in the Sudan study, which had been harvested in the same year from the two different growing regions (Acala 3SG, 2002), revealed *Pantoea spp.* in samples from both Gezira and Rahad, but *E. sakazakii* was only found in the Rahad sample (see Table 3.05). This also reflects the findings shown in Table 3.12 above, where Mississippi grade 43 cotton, was contaminated by different bacteria than California grade 43 cotton.

Dominance of bacterial species has also been shown to vary between cotton plant species in a study on US cotton (Table 3.13 below), this was highlighted by focussing on the relationship between two bacterial species.

Cotton Species	EA %	PS %
G. herbaceum	90	0
G. hirsutum	80	18
G. barbadense	9	56
NX-1	7	88

Table 3.13: Bacterial species profile of different species of cotton from U.S cotton belt, presented as ratio of % *Enterobacter agglomerans*: % *Pseudomonas syringae*. EA = *Enterobacter agglomerans*, PS = *Pseudomonas syringae*, G = *Gossypium*, *G. herbaceum* = semi-closed cotton, *G. hirsutum* = Upland cotton, *G. barbadense* = long staple cotton, NX-1 = *hirsutum/barbadense* hybrid. Adapted from Fischer and Foarde, 1989.

Although, in the present study only identification of bacterial species and not enumeration of individual species was performed, it can be seen that *E. sakazakii* and *Pantoea* were present on both Sudanese Barakat (*Gossypium barbadense*) and Acala (*Gossypium hirsutum*) cotton (see Table 3.05). It would be an interesting study in future to investigate how the ratio of these two species varied between samples.

The relationship between *E. agglomerans* and *P. syringae* on cotton fibres was also examined in one of the few studies carried out previously to compare the Gram-negative bacterial profile of cotton from different countries (Fisher and Foarde, 1989) (Table 3.14). These authors analysed the relationship between these two common cotton bacteria, they did not show the range of bacteria identified in lint from these countries. However, it is interesting to note that although there were slight variations between each

region, all countries exhibited *E. agglomerans* as the predominant species of the two (in an average ratio of approximately 70:25 %), with the exception of the lint from Portugal where this situation was reversed.

Country of Origin	EA%	PS%
Chad	74	21
Mali	70	15
Portugal	34	63
Syria 1	54	36
Syria 2	66	27
Tanzania	75	15

Table 3.14: Bacterial species profile of various cotton lint samples from different countries, presented as average (n=3) ratio of % *Enterobacter agglomerans* / % *Pseudomonas syringae*. EA = *Enterobacter agglomerans*, PS = *Pseudomonas syringae*. Adapted from Fischer and Foarde, 1989.

It is frequently reported that the conditions to which the cotton lint is exposed during field weathering, significantly effects the bacterial profile. Therefore, it is surprising that the relationship between two species of bacteria would be similar in diverse cotton samples such as those from Syria, a Middle Eastern country, and Chad, a country in Central Africa. However, following more detailed analysis, it would appear that the African samples in Fisher's study are more comparable, in that they varied from 70-75% *E. agglomerans*, and 15-21% *P. syringae*, whereas, the samples from Syria had an average of 60% *E. agglomerans* and 32% *P. syringae*.

In order to evaluate the effect of geographical production conditions, the cotton samples assessed in this chapter were grouped into continental regions of origin (see Table 3.07). However, the distribution of the majority of species in this study across both Asia and Africa (there was too small a number of samples from the Americas to comment reliably), did not display a discernable pattern. It would also be interesting here to investigate the relationships of these species with respect to each other, in order to establish whether they change and if there is an overall trend in predominant species in different geographical regions.

Comparison of bacterial species identified on lint and trash derived from equivalent samples revealed a divergence in most cases (see Tables 3.08 and 3.09), suggesting that certain bacteria differentially favour these two different cotton components. In the general comparison of the two materials (see Table 3.10), *Serratia* was not isolated from the particular trash samples involved, whereas *Flavimonas* was only isolated on this material. Previous studies have also reported variations in the range of bacterial species found on different parts of the cotton plant; "GNB were reported to be found everywhere on the plant...however, each kind was not constantly present on all parts of the cotton plant" (Akinwunmi and Heintz, 1997). Table 3.15 below, presents a large scale study, where the widest range of bacterial species appears to have been isolated from the cotton lint (and roots), with a more limited variety of bacteria isolated from leaves, flowers, seeds and other parts (Akinwunmi, *et al.*, 1989).

Spec	Roots	Stems	Leaves	Flower	Lint	Bract	Seeds
EA	✓	✓	✓	✓	✓	✓	✓
EC	✓	✓			✓		
EI					✓		
SP	✓						
PS	✓	✓	✓	✓	✓	✓	
PF	✓				✓		
XC	✓	✓	✓	✓	✓	✓	✓

Table 3.15: The distribution of a range of Gram-negative bacteria on cotton plant parts. EA = *Enterobacter agglomerans*, EC = *Enterobacter cloacae*, EI = *Enterobacter intermedium*, SP = *Serratia plymuthica*, PS = *Pseudomonas syringae*, PF = *Pseudomonas fluorescens*, XC = *Xanthomonas campestris*, ✓ = positive identification. Adapted from Akinwunmi, *et al.*, 1989.

The general trash analysed in this chapter was a mixture of many plant parts such as stem, leaf, and bract. Analysis of a wider range of trash samples would be needed to further clarify whether this material carries a more limited number of different bacterial species.

Toxicity of different bacterial species

All Gram-negative bacteria possess and release endotoxin. This is an integral constituent of their outer membrane; therefore, it is likely that different bacterial species release similar quantities of endotoxin. However, LPS molecules from different bacterial genera have structural differences, which affect their toxicity, i.e. their ability to elicit an inflammatory response. For example, the ability to induce inflammatory cell migration varies amongst different cotton bacteria (Salkinoja-Salonen, *et al.*, 1982). Table 3.16 shows this variation, where some bacteria (including *Enterobacter* and

Pseudomonas) have high activity in this respect, but *Agrobacterium,* although a Gram-negative bacterium, has very little, exhibiting a low propensity to mobilise neutrophils.

Bacteria in aerosol	Macrophages	Leucocytes
Control	145	65
Enterobacter cloacae	452*	662*
Enterobacter agglomerans	402*	527*
Agrobacterium spp.	149	86
Klebsiella oxytoca	407*	663*
Pseudomonas syringae	417*	596*

Table 3.16: Number of macrophages and leucocytes in guinea pigs airways exposed to aerosols of bacteria from cotton. *Statistical difference compared to control (P< 0.001). Adapted from Salkinoja-Salonen, *et al.*, 1982.

Moreover, endotoxin purified from different bacteria isolated from cotton shows a similar variation in response to that induced by whole bacterial cells (Helander, *et al.*, 1980), and this is exemplified in Table 3.17 where again *Agrobacterium* spp. as well as *Xanthomonas* (also a Gram-negative bacteria) demonstrated less cell migratory potency than other bacteria. This disparity is thought to be due to the chemical structure of the LPS involved, more specifically to differences in the Lipid A and core segments of the molecule.

LPS	Total cells	Macrophages	Neutrophils
Control	12.6 ± 4.4	9.7 ± 4.0	0.4 ± 0.1
Enterobacter agglomerans	53.4 ± 16.0*	25.7 ± 8.1*	20.1 ± 9.0*
Pseudomonas putida	43.9 ± 19.5*	25.8 ± 11.4*	12.1 ± 5.4*
Klebsiella oxytoca	83.4 ± 22.3*	35.8 ± 12.6*	35.6 ± 20.2*
Agrobacterium sp.	15.5 ± 7.4	9.2 ± 4.3	2.9 ± 2.5
Escherichia coli	23.1 ± 7.0*	15.2 ± 4.5	4.5 ± 2.1*
Xanthomonas sinensis	17.8 ± 6.1	13.0 ± 4.1	1.5 ± 0.9

Table 3.17: Mean numbers ± SD x10^6 of free lung cells in guinea-pigs lungs 24 hrs after a 40-minute exposure to an aerosol suspension of 5μg/ml LPS in sterile water. * Significance compared to control (P< 0.001). Adapted from Helander, *et al.*, 1980.

Helander, *et al.*, (1980), examined the carbohydrate composition of the molecules used in the study shown above (Table 3.17). The less potent LPS, from *Agrobacterium* and *Xanthomonas*, were found to lack the sugar heptose and possessed less KDO (the eight-carbon sugar, which anchors the oligosaccharide section of the molecule to lipid A) than the other more toxic LPS. However, further tests involving an LPS that contained heptose but no KDO was also inactive in the same inhalation study.

The Lipid A fatty acid composition of those LPS exhibiting activity are different to those where it is absent or reduced. This is verified by experiments examining activity of LPS from bacterial species isolated from cotton. Those with similar chemical composition had similar levels of activity

i.e. *Enterobacter* and *Citrobacter*, compared to the chemically different Agrobacterial LPS (Helander, *et al.*, 1982). More specifically, the study described above (Helander, *et al.*, 1980), concluded that all LPS with high intrinsic cell migratory activity contained lauric acid, compared with non-active LPS. More recently, studies using chemically synthesised and modified lipid A molecules have revealed that endotoxicity is related to the number and arrangement of acyl chains and phosphate groups attached to this lipid component (Erridge, *et al.*, 2002). Understanding of this concept is significant due to the high prevalence of potent inflammatory response-inducing bacteria such as *Enterobacter* identified on cotton in this chapter and previous studies. This has an impact on efforts to protect cotton workers from respiratory disease, especially considering the high numbers of bacteria present on cotton fibres, which will be addressed in the next chapter.

Summary

The range of Gram-negative bacteria identified on the diverse cotton samples in this study was very limited, mainly consisting of enteric bacteria, which have been commonly identified on cotton previously. This implies that the same bacterial species consistently colonise cotton from across the world. The most geographically widespread genus was *Enterobacter*; this is significant, since *Enterobacter* possess endotoxin with highly potent inflammatory activity. *Aeromonas* colonies were identified on cotton lint for the first time, and it was hypothesised that polluted water contacting plants via irrigation of cropland was a possible source. Bacterial diversity was further limited on cotton samples from within Sudan, these samples were

universally colonised by *Pantoea*, suggesting that individual countries have predominant cotton bacterial genera. Results from the Sudan study also indicated that cotton grade and region of production had an influence on the bacterial species present. However, when samples were grouped according to geographical production region, no discernable pattern was observed. Cotton grades harvested most recently (in 2003) and hence stored for the briefest time, appeared to carry the widest range of bacterial species, leading to the conclusion that lint storage time may affect the bacterial profile of cotton lint.

CHAPTER 4

ENUMERATION OF GRAM-NEGATIVE BACTERIA ON COTTON

4.1 INTRODUCTION

In order to grow cotton commercially, a temperate climate is required, with ample water supply. Similar conditions favour the growth of the majority of bacteria; couple this with the nutrition provided by the large quantities of plant and insect sugars present on cotton fibres, and the ideal environment for the proliferation of microbial populations is created. By the time the sterile cotton fibres in the boll are revealed to the environment, the cotton plant on which the fibres have grown is already contaminated with bacteria from surrounding plants and soil. Hence, in a short time the new fibres are also colonised (Bell, 1997). Although high bacterial numbers have been measured on all cotton plant parts, the lint is reported to harbour 67% of Gram-negative bacteria with levels of approximately 10^6–10^8 cells per gram of dry fibre (Akinwunmi and Heintz, 1997).

Since cotton fibres can only be harvested after boll opening, which does not occur at a uniform time, cotton locks could be left in the field for several weeks prior to harvest (see Figure 4.01). The numbers of bacteria on cotton lint therefore, depend on the conditions to which locks are exposed during field weathering. Moisture is a key factor, and very high numbers of bacteria have been isolated from cotton lint exposed to rainfall during field weathering (Fischer and Sasser, 1987; Heintz, *et al.*, 1990), whereas areas with drier

climates yield low bacterial levels (Simpson and Marsh, 1985). Relative humidity has also been investigated as an influential factor on the level of Gram-negative bacterial cotton contamination (DeLucca, *et al.*, 1990), and studies concerning more extreme weather conditions have demonstrated increased bacterial counts on cotton bracts following frost, due to the sudden availability of nutrients released from lysed plant cells (Morey, *et al.*, 1984).

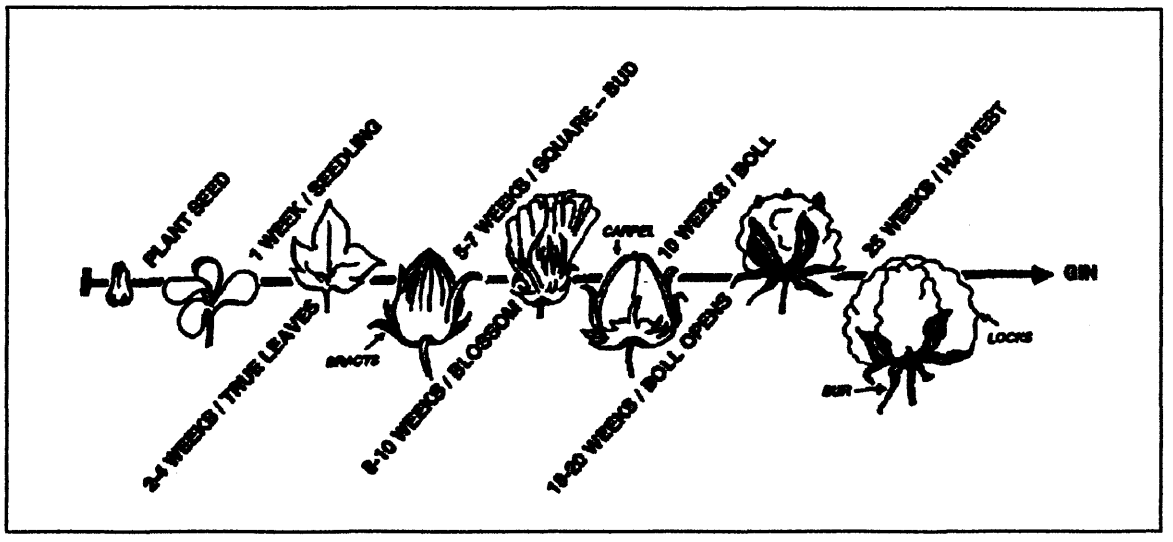


Figure 4.01: Timescale for cotton fibre development, showing potential for several weeks of field weathering prior to harvest. Adapted with permission from Cotton's Journey, [WWW] 2004.

There are no prolonged heating or washing stages applied to early cotton processing, hence high numbers of bacteria survive to become airborne with the dust produced when cotton is agitated during blowing and carding. Analysis of cotton from Lancashire spinning mills indicated a highly significant association between the concentration of airborne bacteria and the prevalence of byssinotic symptoms (of at least grade C½) (Cinkotai and Whitaker, 1978). This study also showed a poor correlation between total and

respirable dust levels and byssinotic symptoms; highlighting the importance of focussing on the measurement of components within the fibres and resulting dust. Therefore, enumeration of viable Gram-negative bacterial cells present on cotton fibres may give a better indication of the risk to respiratory health of individuals exposed to cotton dust.

Understanding the relationship between bacteria and cotton is important in furthering knowledge of the risk of exposure. There have been only limited studies comparing levels of Gram-negative contamination on cotton samples from different countries and regions; therefore, this was the focus of the work presented here. Developing reliable techniques to assess the level of bacterial contamination of cotton fibres could also help to efficiently assess procedures designed to lower the bacterial load of cotton in the field.

Aims

- To perform viable cell counts on total Gram-negative bacterial cells extracted from a range of cotton lint and trash samples.
- To compare viable counts from cotton originating in a number of different countries and within one country, in order to consider potential respiratory risk.
- To look for patterns in bacteria numbers and consider factors which may affect the levels present.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Equipment

All equipment was obtained from reputable sources, and was sterile, wherever possible. Full details are available in Appendix I.

Cotton/trash samples

See Chapter 2 for details.

4.2.2 Methods

Microbe extraction

Microbes were extracted from 0.6000g (±0.0005g) cotton lint into 10 ml phosphate-buffered saline by shaking on a vortex multi-mixer at 1000 rpm for 60 minutes in a centrifuge tube (techniques were adapted from Nicholls, *et al.*, 1991 following preliminary tests to determine the optimum weight/volume). The cotton fibres were then physically submerged using a sterile pipette and the supernatant removed to a fresh tube. Due to the limited availability of trash material, microbes were extracted from 0.3000g (±0.0005g), into 5ml phosphate-buffered saline as described above. All other aspects of extraction and culture for trash remained the same as those applied to the lint.

Dilutions

Required dilutions were prepared using phosphate buffered saline by 1 in 10 stepwise dilutions in sterile glass test tubes to 10^{-1} or 10^{-2} as required. The dilution tubes were vortexed for 30 seconds between each transfer step.

Agar plates

Tryptic soy agar (TSA) was made according to manufacturers instructions (36g/litre) and autoclaved for 1 hour at 120°C. It was then allowed to cool until at approximately 40°C, at which point the antibiotics cycloheximide (50μg/ml) and vancomycin (15μg/ml) were added in order to prevent the growth of fungi and Gram-positive bacteria respectively (techniques adapted from Fisher and Sasser, 1987) (see Appendix II for antibiotic modes of action). The molten agar was mixed gently to avoid bubble formation by inverting the flask several times, before 25ml molten agar was poured into each petri dish and allowed to solidify, this was done in a laminar flow unit. Plates were 'over dried' in a laminar flow unit for 30 minutes prior to inoculation in order to remove excess water. Inhibition controls for each new batch of plates were carried out by streaking a loopful of stock cultures of *Staphylococcus aureus* (Gram-positive) and *Pseudomonas aeruginosa* (Gram-negative) in duplicate on plates selected at random, these were incubated alongside spread plates. Visual checks for fungal cells were performed prior to counting.

Spread plating

The required supernatant dilution was vortexed for 30 seconds prior to plate inoculation. A 0.1ml aliquot of solution was inoculated onto the centre of an over dried agar plate and evenly spread across the surface with a sterile (flamed) glass spreader. Plates were prepared in triplicate and all plates were left to stand at room temperature for 30 minutes prior to incubation.

Incubation

Plates were inverted and incubated at 37°C (± 2°C) for 18-24 hours in a cupboard incubator.

Viable counts

Counts were performed after 18-24 hours. Plates with 20-200 colony-forming units (CFU) had every unit counted by eye using a pen to mark each colony in turn (see Figures 4.02A and 4.02B). Plates with 200+ colonies were divided into quarters; every colony in two opposite quarters was counted, the average calculated and multiplied by four.

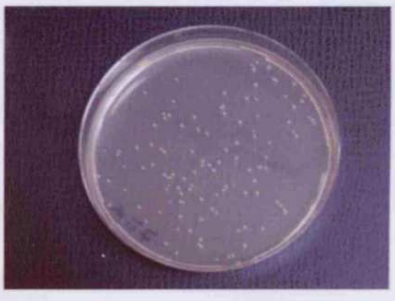
A:  B: 

Figure 4.02A and 4.02B: An agar spread plate showing individual bacterial colonies after 24 hours incubation (A), and the black pen marks made whilst counting every colony in turn (B).

4.3 RESULTS: GRAPHICAL/TABULAR REPRESENTATION

The range of inter-country Gram-negative viable cell counts

Cotton lint samples from diverse countries exhibited a wide range of bacterial counts varying from 10^2 to 10^5 CFU/g. Mean counts on samples in alphabetical order of origin were from Benin (66885 ± 10905 CFU/g), China 146 (1070 ± 360 CFU/g), China Xinjiang (1803 ± 436 CFU/g), CIS (4400 ± 793 CFU/g), Iran (3038 ± 1014 CFU/g), Ivory Coast (196300 ± 30315 CFU/g), Paraguay (1495 ± 520 CFU/g), Syria (4718 ± 1046 CFU/g), Tajikistan (713 ± 212 CFU/g), Turkey (2152 ± 1093 CFU/g), USA (43943 ± 8942 CFU/g), Zambia (88163 ± 15380 CFU/g), and Zimbabwe (216830 ± 30413 CFU/g) (Figure 4.03).

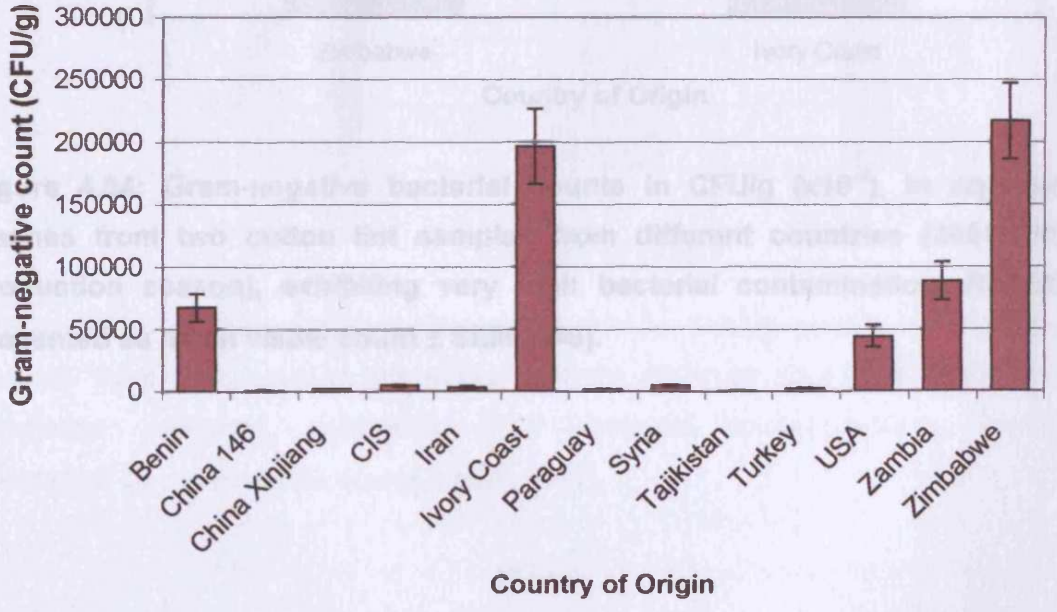


Figure 4.03: Total range of Gram-negative bacterial counts (CFU/g), in aqueous washes from thirteen cotton lint samples from diverse countries of origin (2001/2002 production season). Results presented as mean viable count ± SEM (n=6).

The division of cotton lint samples from diverse countries (from 2001/2002 production season) into groups based on bacterial numbers, revealed those with very high counts ($x10^5$) were from Africa (Ivory Coast & Zimbabwe). The cotton from Zimbabwe had the highest count ($2.2 \pm 0.3 \times 10^5$ CFU/g) compared to that from Ivory Coast ($1.9 \pm 0.3 \times 10^5$ CFU/g) and these counts were statistically similar ($P>0.05$) (Figure 4.04).

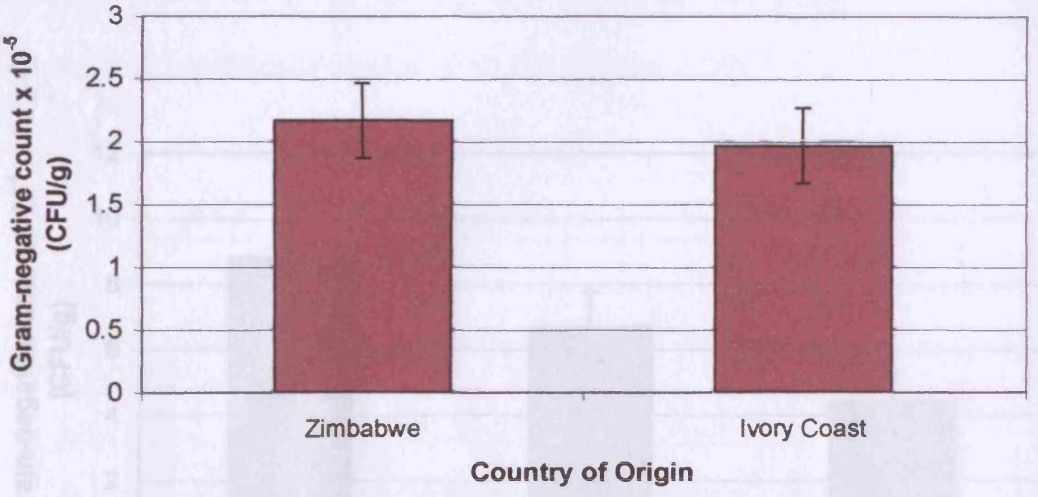


Figure 4.04: Gram-negative bacterial counts in CFU/g ($x10^{-5}$), in aqueous washes from two cotton lint samples from different countries (2001/2002 production season), exhibiting very high bacterial contamination. Results presented as mean viable count ± SEM (n=6).

The division of cotton lint samples from diverse countries (from 2001/2002 production season) into groups based on bacterial numbers, showed those with high counts ($x10^4$) included two samples from Africa (Benin & Zambia) as well as the sample from USA. The African samples showed the highest counts; cotton from Zambia had the highest of the group ($8.8 \pm 1.5 \times 10^4$ CFU/g), followed by the sample from Benin ($6.7 \pm 1.1 \times 10^4$ CFU/g) and the USA ($4.4 \pm 0.9 \times 10^4$ CFU/g), these counts were statistically similar ($P>0.05$) (Figure 4.05).

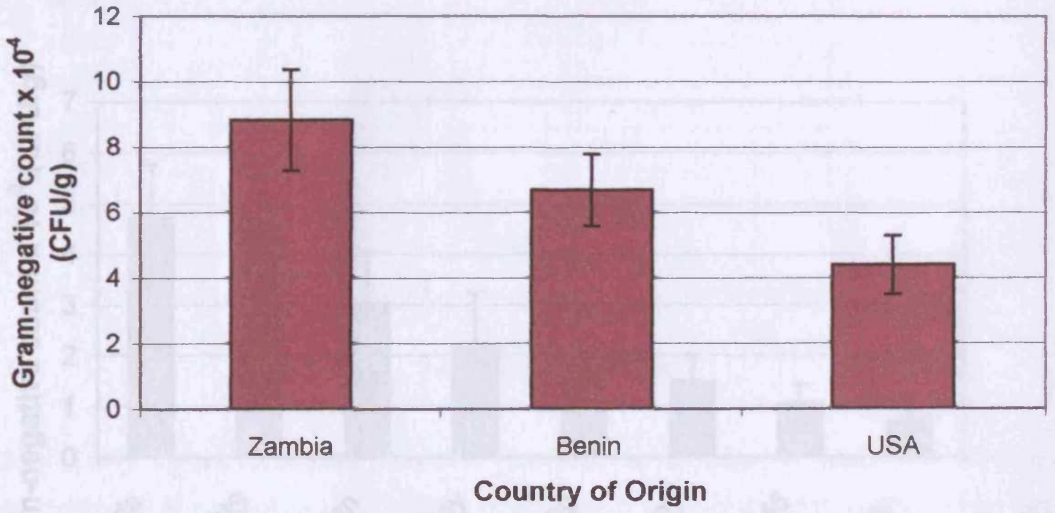


Figure 4.05: Gram-negative bacterial counts in CFU/g ($x10^4$), in aqueous washes from three cotton lint samples from different countries (2001/2002 production season), exhibiting high bacterial contamination. Results presented as mean viable count ± SEM (n=6).

The group of cotton lint samples from diverse countries (from 2001/2002 production season) with low-medium counts ($x10^2$-10^3) contained the majority of samples, which were mainly those from Asia, (with the exception of Paraguay). The highest counts were found in the sample from Syria (4.7 ± 1.1 x 10^3 CFU/g) followed in decreasing order by samples from CIS (4.4 ± 0.8 x 10^3 CFU/g), Iran (3.0 ± 1.0 x 10^3 CFU/g), Turkey (2.2 ± 1.1 x 10^3 CFU/g), China Xinjiang (1.8 ± 0.4 x 10^3), Paraguay (1.5 ± 0.5 x 10^3 CFU/g), China 146 (1.1 ± 0.4 x 10^3 CFU/g) and Tajikistan (0.7 ± 0.2 x 10^3 CFU/g) these were all statistically similar (P>0.05) (Figure 4.06).

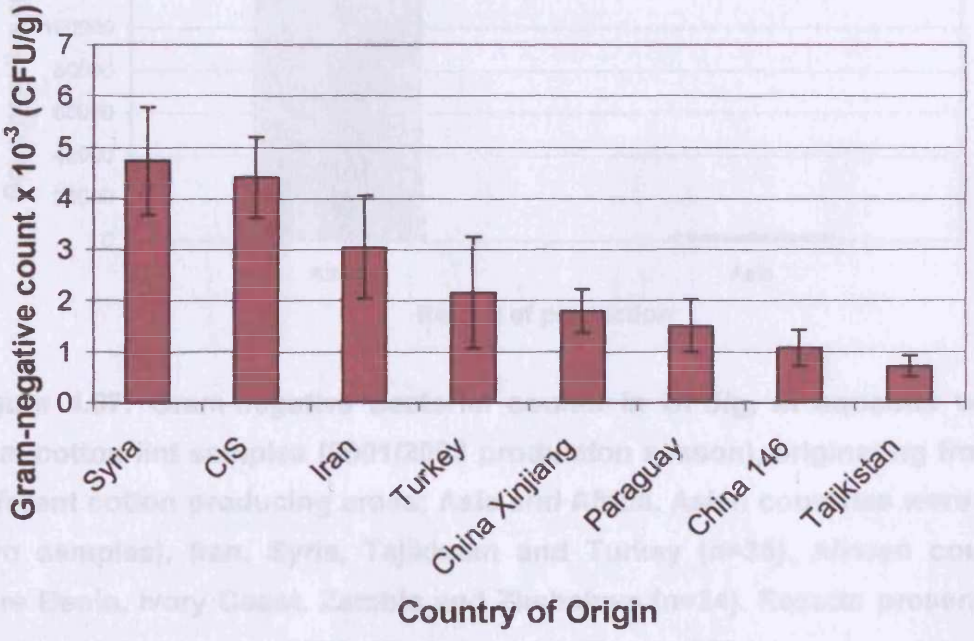


Figure 4.06: Gram-negative bacterial counts in CFU/g ($x10^{-3}$), in aqueous washes from eight cotton lint samples from different countries (2001/2002 production season), exhibiting medium-low bacterial contamination. Results presented as mean viable count ± SEM (n=6).

The influence of production region on Gram-negative viable cell counts

Comparison of mean bacterial counts on cotton samples originating from countries within Asia and within Africa, revealed mean counts from African samples (142045 ± 37737 CFU/g) were significantly higher than on those originating in Asia (2412 ± 693 CFU/g) (Figure 4.07).

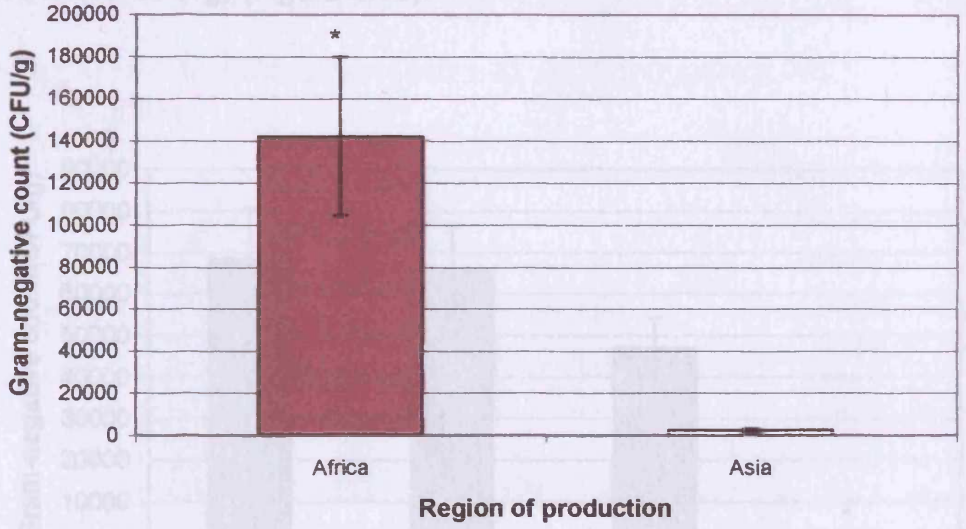


Figure 4.07: Gram-negative bacterial counts in CFU/g, in aqueous washes from cotton lint samples (2001/2002 production season), originating from two different cotton producing areas; Asia and Africa. Asian countries were China (two samples), Iran, Syria, Tajikistan and Turkey (n=36). African countries were Benin, Ivory Coast, Zambia and Zimbabwe (n=24). Results presented as mean viable count ± SEM. *Denotes significant difference in mean Gram-negative counts compared to cotton samples from Asian countries (P<0.05).

Intra-country Gram-negative viable cell counts; Turkey

The cotton lint samples from different production regions within Turkey possessed a range of bacterial counts. The cotton from the Selçuk region had the highest count (68235 ± 12510 CFU/g), followed by similar counts in the sample from Bergama (65913 ± 10638 CFU/g) and the Efes 1 sample (46997 ± 7473 CFU/g). The Efes 2 sample had a significantly lower count (980 ± 200 CFU/g) (Figure 4.08).

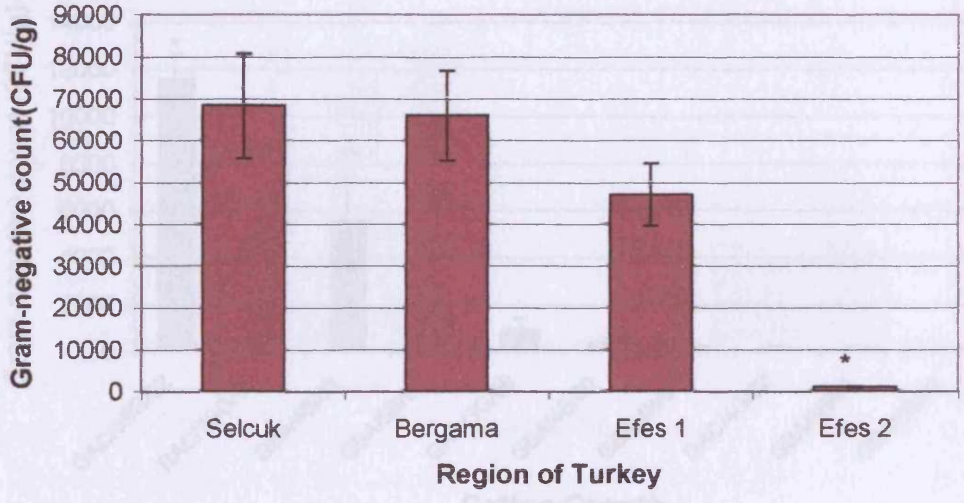


Figure 4.08: Gram-negative bacterial counts in CFU/g, in aqueous washes from cotton lint samples originating in three areas of Turkey (2002/2003 production season). Results presented as viable count ± SEM (n=6). *Denotes statistical significance compared to other samples (P<0.05).

Intra-country Gram-negative viable cell counts; Sudan

Cotton lint samples from Sudan exhibited a range of bacterial counts. Mean counts were highest on GAC/3SG/02 cotton (11542 ± 1161 CFU/g), decreasing through RAC/3SG/02 (5681 ± 831 CFU/g), GBA/4B/03 (5614 ± 1849 CFU/g), GBA/6B/00 (1736 ± 392 CFU/g), GAC/3G/98 (908 ± 522 CFU/g), GBA/4B/00 (453 ± 145 CFU/g), GBA/4B/98 (278 ± 85 CFU/g), GAC/4G/02 (194 ± 70 CFU/g), GBA/6B/98 (159 ± 37 CFU/g), to the lowest count from GBA/6B/03 cotton (119 ± 62 CFU/g) (Figure 4.09).

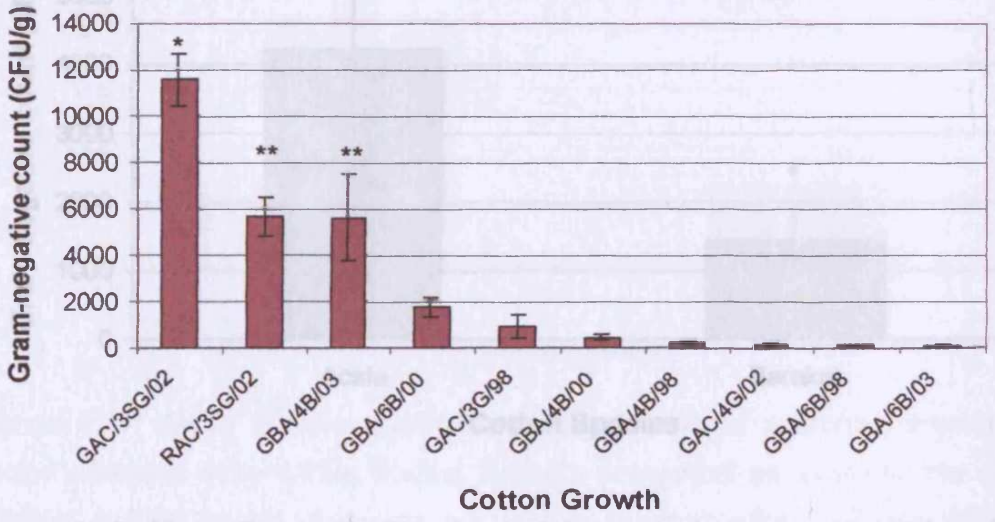


Figure 4.09: Total range of Gram-negative bacterial counts in CFU/g, in aqueous washes from ten various cotton lint samples from within Sudan. Results presented as mean viable count ± SEM (n=6). Samples vary in production region, species and grade of cotton and harvest year. Production regions were Gezira (G) and Rahad (R), cotton species were Barakat (BA) and Acala (AC), grades were 4B, 6B, 3G, 3SG and 4G. Years of harvest were 1998, 2000, 2002, and 2003 (98/00/02/03 respectively). *Denotes statistically significant difference compared to all other samples, **denotes statistical difference compared to other samples but similarity to other **.

The influence of cotton species on Gram-negative bacterial counts

Comparison of the mean Gram-negative bacterial counts on different cotton species revealed a significant difference, although samples also differed in years of harvest and cotton grades. Gezira Acala cotton (4215 ± 1320 CFU/g), had a significantly higher mean count than Gezira Barakat cotton (1433 ± 454 CFU/g) (Figure 4.10).

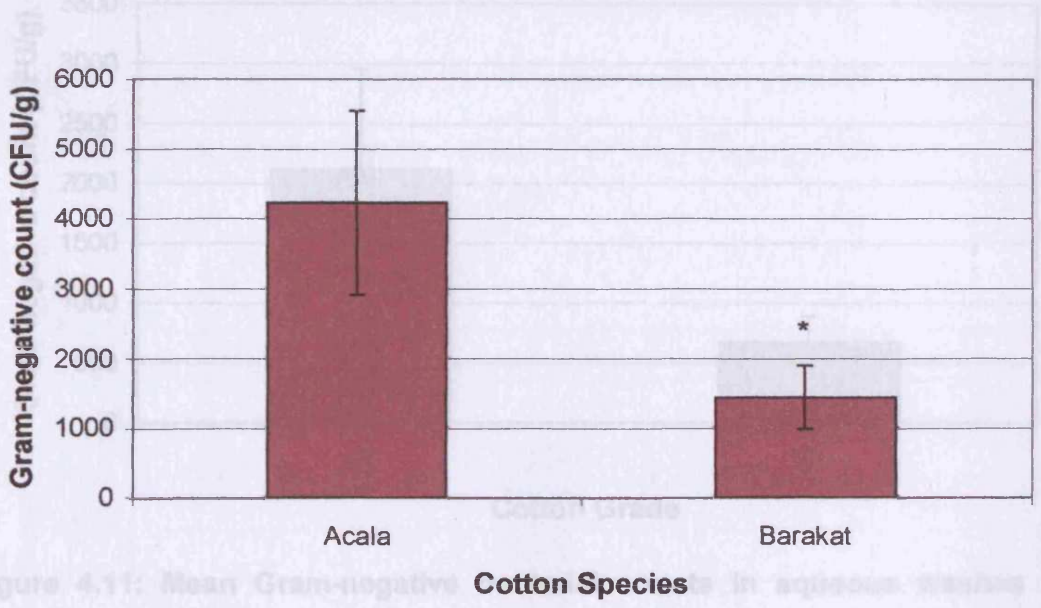


Figure 4.10: Mean Gram-negative bacterial counts in aqueous washes from cotton samples from within Sudan. Results presented as mean viable count (CFU/g) ± SEM (n=18 (Acala) and n=36 (Barakat)). Samples are Gezira Barakat or Gezira Acala cotton species of several grades from various harvest years. *Denotes statistically significant difference compared to Acala cotton (P<0.05).

The influence of cotton grade on Gram-negative bacterial counts

The mean Gram-negative bacterial count recorded on Sudan Gezira Barakat 4B grade cotton (2115 ± 835 CFU/g), was not significantly different to those on Sudan Gezira Barakat 6B grade cotton (672 ± 221 CFU/g) ($P>0.05$) (Figure 4.11).

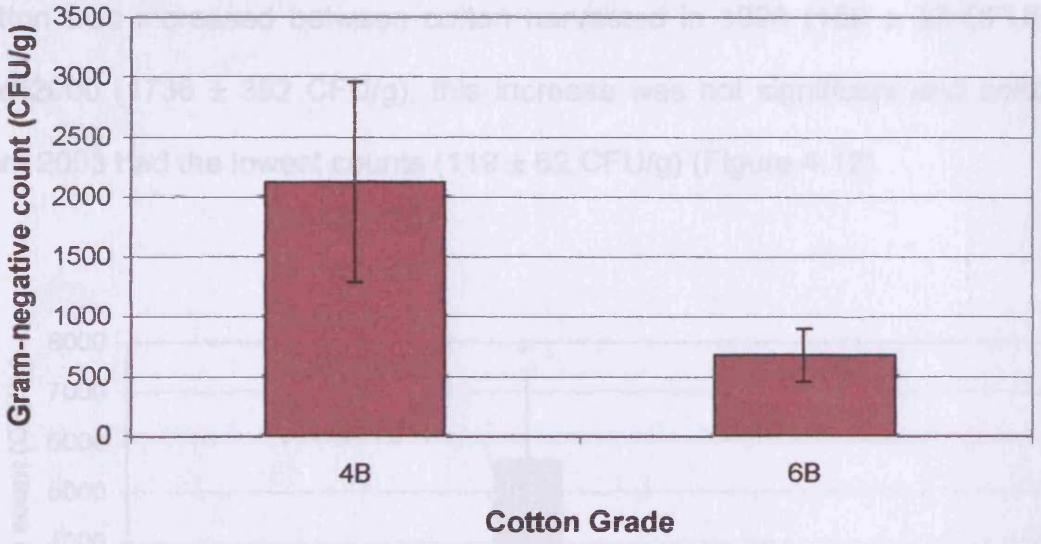


Figure 4.11: Mean Gram-negative bacterial counts in aqueous washes of cotton samples from within Sudan. Results presented as mean viable count (CFU/g) ± SEM (n=18). Samples are Gezira Barakat cotton of two different grades (4B and 6B), each harvested from three different years; 1998, 2000, and 2003.

The possible effect of storage on Gram-negative bacterial counts

Analysis of cotton samples harvested in three different years, revealed a trend between year of harvest and Gram-negative bacterial count for 4B cotton. Counts on GBA/4B cotton were lowest in cotton harvested in 1998 (278 ± 85 CFU/g); the sample from 2000 had higher counts than this (453 ± 145 CFU/g) and counts on cotton harvested in 2003 increased further and significantly (5614 ± 1849 CFU/g). However, although counts on GBA/6B cotton also increased between cotton harvested in 1998 (159 ± 37 CFU/g) and 2000 (1736 ± 392 CFU/g), this increase was not significant and cotton from 2003 had the lowest counts (119 ± 62 CFU/g) (Figure 4.12).

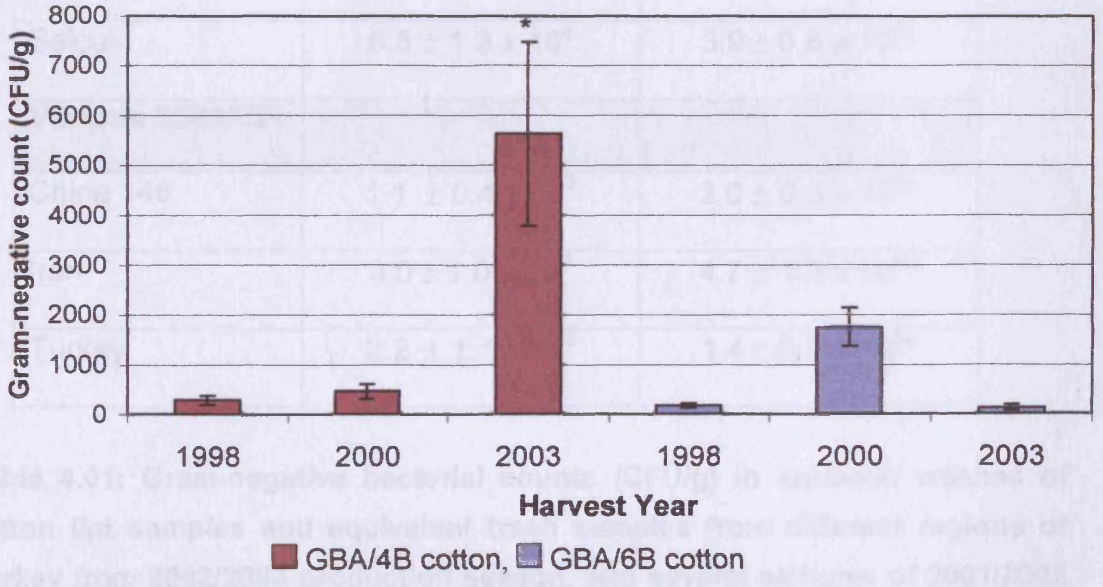


Figure 4.12: Gram-negative bacterial counts (CFU/g) in aqueous washes from Gezira Barakat (GBA) cotton samples of two different grades (4B and 6B) produced within Sudan, harvested in three different years. Presented as mean count ± SEM (n=6) *Denotes statistically significant difference compared to GBA/4B cotton from 1998 and 2000 (P<0.05).

Gram-negative counts on cotton lint and equivalent trash material

Gram-negative bacterial counts on all available trash samples revealed that cotton trash samples harboured Gram-negative bacterial counts of at least one order of magnitude higher than equivalent lint samples (Table 4.01).

	Gram-negative bacterial counts (CFU/g)	
Sample Region	**Cotton lint**	**Cotton Trash**
Turkey 2002/03		
Bergama	$6.6 \pm 1.1 \times 10^4$	$4.8 \pm 0.1 \times 10^{5*}$
Efes 1	$4.5 \pm 0.8 \times 10^4$	$3.9 \pm 0.7 \times 10^{5*}$
Efes 2	$9.8 \pm 2.0 \times 10^2$	$4.3 \pm 0.4 \times 10^{4*}$
Selçuk	$6.8 \pm 1.3 \times 10^4$	$3.9 \pm 0.8 \times 10^{6*}$
Various 2001/02		
China 146	$1.1 \pm 0.4 \times 10^3$	$2.0 \pm 0.3 \times 10^{4*}$
Iran	$3.0 \pm 1.0 \times 10^3$	$4.7 \pm 1.3 \times 10^{4*}$
Turkey	$2.2 \pm 1.1 \times 10^3$	$1.4 \pm 0.2 \times 10^{5*}$

Table 4.01: Gram-negative bacterial counts (CFU/g) in aqueous washes of cotton lint samples and equivalent trash samples from different regions of Turkey from 2002/2003 production season, and several samples of 2001/2002 production season. Results presented as mean viable count ± SEM (n=6). *Denotes statistically significant difference between bacterial counts on cotton trash, and those on cotton lint ($P<0.05$).

Gram-negative bacterial counts on seed cotton

Bacterial counts on two samples of seed cotton from Eritrea varied by 10^2, Gedera 5A cotton had significantly lower counts than the SJ2G sample ($P<0.05$), standard errors were high on this type of cotton (Table 4.02).

Variety of cotton	Gram-negative bacterial count (CFU/g)
Eritrea Gedera 5A	95 ± 33*
Eritrea SJ2G	2815 ± 1022

Table 4.02: Gram-negative bacterial counts (CFU/g) in aqueous washes from two varieties of seed cotton from Eritrea (2001/2002 production season). Results shown as mean viable count ± SEM (n=6). *Denotes statistically significant difference in bacterial counts compared to SJ2G cotton.

4.4 DISCUSSION

Method appraisal

Gram-negative specific spread plate methods were utilised in order to enumerate water-extractable bacterial numbers on a variety of cotton samples. Spread plating only enumerated total viable cells, i.e. those with the ability to replicate on solid media, hence it did not include dead cells. Despite this, the methodology utilised was useful for the largely comparative work described here. Extraction by physical agitation into buffered saline (Morey, *et al.*, 1984; Nicholls, *et al.*, 1991) or sterile water (Fisher and Sasser, 1987; Gokani, *et al.*, 1987), followed by spread plating is a technique commonly applied to enumerate cotton bacteria. Although, it should be acknowledged that some research groups perform extraction by shaking in buffer with added surfactant such as Tween 40 (Chun and Perkins, 1991) or Tween 80 (Millner, *et al.*, 1982) in order to aid the removal of bacteria from the waxy fibre surface. However, a preliminary study adding 0.5ml Tween 80 to the extraction water of fibre samples yielding very low bacterial counts, showed no increase in cell numbers following incubation (data not shown), hence this method was not retained in the current study.

Each set of spread plates was prepared in triplicate in order to calculate the mean count for each fibre subsample. As analysis was then repeated six times on different subsamples, individual counts for each cotton sample totalled eighteen. This was necessary becuase the cotton fibres within a sample bundle were not uniform; they naturally varied in maturity (thickness

and coarseness) and contained a variety of entrained contaminants, including pieces of plant leaves, stems and soil, all of which may have caused the bacteria numbers to fluctuate within the sample. The techniques used ensured that results were as representative of the original sample as possible. It was notable that although the results on cotton lint samples utilising these methods were reproducible, the analysis of the seed cotton samples from Eritrea revealed large standard errors (see Table 4.02). This may imply that these methods were not as suitable when applied to this type of cotton, perhaps due to variation originating from the added step of pulling the cotton lint away from the seeds prior to extraction.

The range of bacterial counts

All cotton samples analysed contained detectable levels of Gram-negative bacteria and counts on the different cottons varied widely overall. The cottons from diverse origins demonstrated the largest range of Gram-negative counts (10^2-10^5 CFU/g) (see Fig. 4.03), while counts on cottons from within Turkey and Sudan covered a narrower range of 10^2-10^4 CFU/g (see Figs. 4.08 and 4.09 respectively). Previous studies have also recorded a large range of Gram-negative counts in cottons from different countries (Table 4.04 below). Samples in this study (Fisher and Kylberg, 1983), had Gram-negative counts that also ranged over three orders of magnitude (from 10^3-10^6 CFU/g), although at higher numbers (a low of thousands CFU/g) than those recorded in samples from the present study (a low of hundreds CFU/g).

Origin	Thousands of CFU/g
Argentina	620
Colombia	390
Guatemala	400
Israel	57
Nicaragua	4.8
Mexico	19
Pakistan	490
Paraguay	6500
Turkey	4.5

Table 4.04: Gram-negative bacterial counts in thousands CFU/g measured in cotton fibre samples from a number of countries. Adapted from Fischer and Kylberg, 1983.

The number and variety of countries studied previously only offers a very limited opportunity to compare results recorded in the current study with those from different research groups (Table 4.05 below). This excludes the sample from the US, as a number of large scale, detailed studies have been conducted on cottons from different years and regions of the American cotton belt, and results vary considerably.

		GNB (CFU/g fibre) on different cottons		
Study	Harvest	Syria	Turkey	Paraguay
CS	2001/2002	4718 ± 1046	2152 ± 1093	1495 ± 520
	2002/2003	-	45531 ±15593[a]	-
FF	1983	91000[b]	-	-
FK	NR	-	4500	6500000

Table 4.05: Comparison of Gram-negative bacterial counts performed on cotton samples from diverse countries in the current and previous studies. CS = current study, FF = Fischer and Foarde, 1989, FK = Fischer and Kylberg, 1983, NR = not reported, [a] mean (4 samples), [b] mean (6 reported results).

These data are too few to reliably draw conclusions about overall cotton contamination levels for these countries, and the variation seen could be due to a number of factors, relating to experimental procedures or production conditions. Moreover, comparison of bacterial counts on cotton samples recorded in different studies cannot be readily carried out, when the years of harvest of the samples used are not reported, and more importantly when details of storage time and conditions are not known. The effect of storage time on cotton GNB numbers will be discussed later in this chapter.

Influence of production factors on bacterial counts

Immediately after boll opening, there is an explosive increase in bacteria numbers on cotton fibres (Zuberer and Kenerly, 1991). A number of factors may affect the bacterial numbers; variety of cotton, use of pesticides and harvesting practices have all been suggested (Bragg, *et al.*, 1983). However, the most widely demonstrated theory is that climatic conditions (especially rainfall and humidity) in the field, influence the levels of bacterial contamination on cotton fibres (Fisher and Sasser, 1987; Heintz, *et al.*, 1990; Millner, *et al.*, 1984; Simpson, *et al.*, 1983; Simpson and Marsh, 1985).

The Highest GNB counts in the inter-country study were mainly found on cottons from the African cotton belt (from Benin, Ivory Coast, Zambia, and Zimbabwe), compared to the Asian regions (China, Iran, Syria, Tajikistan and Turkey), and the mean count of samples from Africa was significantly higher (see Fig. 4.07). This may reflect general climatic differences, as cotton

production conditions in some cotton producing areas of Africa include periods of the growing season with a more humid climate compared to the drier, generally semi-arid season in many Asian countries. Although this is a generalisation, it has been previously suggested that "geographic location i.e. area of growth, especially related to weather conditions after boll opening, is associated with the relative level of bacteria on cotton fibers" (Millner, *et al.*, 1984).

In two studies by Simpson, *et al.*, (1984) and Simpson and Marsh (1985), cottons from the San Joaquin Valley in California had lower than average bacterial counts, which was attributed to extremely low rainfall and relative humidity throughout the growing season in this area. It has also been reported that periods of rainy weather prior to harvest, elevated bacteria numbers in the American mid-west, mid-south and Texas-Oklahoma regions (Fischer and Sasser, 1987; Simpson and Marsh, 1988). Almost all studies of this nature are limited to regions of the North American cotton belt. It would be interesting to carry out a more controlled investigation on cotton samples from various countries involving rainfall and humidity data from particular production areas. As previously discussed, literature comparing bacterial counts on cotton samples from a variety of different countries are limited, however those available have also found levels of contamination to vary widely. One study reported that Syrian cotton (i.e. from Asia) had lower Gram-negative counts compared to samples from Chad, Mali, and Tanzania (African countries), reflecting the findings of the current study, however, no

reference to the influence of production region was made, as it was not the focus of this paper (Fischer and Foarde, 1989).

Relating high cotton bacterial counts to climatic differences between growing regions may be appropriate on a general scale; however, the variation is likely to be multifactorial. There may also be a relationship between bacterial counts and characteristics relating to the cotton plant itself such as variety and quality (Fisher, *et al.*, 1980; Morey, *et al.*, 1981). The four cotton samples tested from different areas of Turkey did not appear to demonstrate even a loose correlation between counts and region (see Figure 4.08), as similar counts were found in cotton from Bergama and Selçuk, which are located a large distance apart (although they are both located in Western Turkey), and one sample from Efes, which is located close to Selçuk (see Figure 2.07 page 42 for map). The second sample from Efes had a significantly lower count; this sample displayed differences in structural parameters compared to the other samples. The effect of cotton structure on biological contamination will be addressed in Chapter 9.

The larger intra-country study on samples from within Sudan also revealed significant variations in GNB counts. Nine of these fibre samples originated in the Gezira region of Sudan, hence they are likely to have been grown under similar conditions, yet these also showed variation in bacterial counts (see Fig. 4.09). The samples also varied in cotton species, grade and harvest year; all variables, which may confuse comparisons that focus on only one factor. However, mean bacterial counts from Gezira Acala cotton were

significantly higher than mean bacterial numbers from samples of Gezira Barakat cotton (see Fig. 4.10). A previous study, which analysed cotton of different species from within the US, also reported a difference in the Gram-negative bacteria numbers (Table 4.06 below). However, these data demonstrated that *Gossypium hirsutum* (equivalent to Sudan Acala cotton) had lower GNB counts than that of *Gossypium barbadense* (equivalent to Sudan Barakat cotton); the converse of the results presented in the current study.

Cotton Species	GNB
Gossypium herbaceum	21
Gossypium hirsutum	0.52
Gossypium barbadense	1.5

Table 4.06: The Gram-negative bacterial counts from US cottons of different species. Presented in millions CFU/g. Adapted from Fischer and Foarde, 1989.

The difference in mean GNB counts from samples of two cotton grades from within Sudan of the same region, species and harvest years, was not significant (see Fig. 4.11). Cotton grades are determined by slight differences in the structural quality of particular cottons, for example a longer mean fibre length. Previous studies on this aspect of cotton contamination have been limited to US cottons of different grades from the same region and harvest year, and these exhibited varying levels of Gram-negative bacteria (Table 4.07 below shows some examples). These data highlight the complexities of studying cotton fibres, where several factors may underlie the overall

bacterial contamination, and the nature of cotton production makes controlled studies difficult to carry out.

	GNB in millions CFU/g for cottons of different grades					
	Cotton Grade					
Region	32	33	41	42	51	54
DT	0.33	2.3	0.079	-	2.2	0.023
LA	0.79	-	1.3	0.49	-	2.4
AO	0.0079	0.49	0.023	-	0.11	-

Table 4.07: Gram-negative bacteria (in millions CFU/g) reported on cotton fibres of different grades from three different regions of the US cotton belt from 1982. DT = Dallas Texas, LA = Little Rock Arkansas, AO = Altus Oklahoma. Adapted from Simpson, *et al.*, 1984.

Contamination of trash material

Trash material consistently exhibited significantly higher bacterial counts than those of the corresponding cotton lint samples; at least one order of magnitude in all cases (see Table 4.01). This may indicate that trash particles are more hazardous to the health of cotton workers, which is especially significant as it is these particles that are removed from the cotton fibres in the early stages of processing (i.e. blowing and carding). Workers at these processing stages will in this case be more at risk of developing respiratory diseases than those working in the later stages after these particles have been removed. This is a further indication of how monitoring dust levels alone, may not be sufficient to protect work forces, due to the fact that the composition of the dust changes, hence altering its potential risk. Trash

material is made up from pieces of stems, leaves, soil and bract (the thin brittle leaves surrounding the boll). It has been reported previously, that cotton bracts contain 10 to 100 times greater levels of bacterial contamination than fibres (Bell, 1997) and that bracts are a major constituent of the trash material (Fischer, *et al.*, 1986). This indicates that it is this component, which may be responsible for the high bacterial counts seen in the trash samples in this chapter. Trash content is often lower in cotton picked by hand compared to machines, as here only the fibre locks are pulled from the plant, whereas the less discriminatory machine also harvests surrounding plant matter. Hence, harvest practices may also have a siginificant effect on the respiratory hazards posed by particular cottons.

Bacterial counts and fibre storage

The effect of storage time on the bacterial profile of cotton lint is firstly important from a research point of view, as it is often necessary that cotton samples are stored for varying lengths of time whilst they are being analysed. Understanding bacterial changes during storage also has a more applied use, since cotton bales are often stored for anything from a few weeks to several years prior to processing (Personal communication - Dr. R. Jiang). Therefore, the numbers of bacteria released into the cotton mill atmosphere may be of a greater or lesser threat to cotton workers depending on the age of the cotton. The current study involved analysis of a number of samples from within Sudan, harvested in four different years (1998, 2000, 2002, 2003), unfortunately bacterial data from the time of harvest for these samples was not available. Therefore, it is unknown whether the counts recorded

were the result of storage time or whether these bacteria were present in similar numbers at the time of harvest and were indicative of, for example, climatic conditions during those particular growing seasons. However, it is likely that they were the product of both of these factors.

Gram-negative counts on stored cottons have been shown to peak during the first year of storage, after which they decrease rapidly (Chun and Perkins, 1996). A twelve-year study reported that, "as years of storage increased, the Gram-negative populations became a significantly and substantially smaller proportion of the viable bacterial population" (Chun and Perkins, 1996). The data presented in the current study indicated the possibility of storage time influencing the numbers of bacteria. Critically, mean counts from grade 4B cotton harvested in 2003 had significantly higher counts than that harvested in 1998 and 2000 (see Fig. 4.12). Throughout the analyses presented here, cotton samples were in subgroups according to their year of harvest for fair comparison (with the exception of samples from different harvest years within Sudan), and experiments were carried out within twelve months post harvest. A number of studies carried out previously do not appear to have accounted for sample storage time, and although in many cases (though not all) the year of cotton harvest was reported; information relating to cotton storage time prior to analysis was lacking (Fischer and Kylberg, 1983; Simpson, *et al.*, 1984; Simpson and Marsh, 1986; Akinwunmi, *et al.*, 1989). This is especially problematic when the publication date was several years after the reported cotton harvest season (e.g. Zurberer and Kenerley, 1991).

Storage time also has industrial implications, as countries where cotton is commonly stored for several years prior to processing, as well as cotton samples with low counts at harvest, may induce fewer incidences of byssinosis due to the lower concentration of Gram-negative cells released into the air. It may then follow that release of the toxicant endotoxin into the mill atmosphere is also lower during the processing of these fibres. Although enumeration of viable bacterial cells does not represent bacteria that may have died during harvesting and storage periods, Gram-negative bacterial counts on cotton samples may be an indication of the levels of endotoxin present, and hence reflect the potential respiratory health risk from cotton. In this respect, it may be possible to use measurement of GNB as a biomarker for endotoxin levels; this possibility will be examined in the next chapter.

Summary

The enumeration of Gram-negative bacteria from cotton samples of diverse origins, revealed a large variation in counts; this range was slightly narrower in the samples from within Turkey and Sudan. The numbers were generally comparable to those previously recorded in cottons from diverse countries, although these were largely from different countries than those involved in the current study. Cotton samples from within Africa had higher bacterial counts than those from within Asia, perhaps partly due to the climate during field weathering or other production factors. Different cotton species from Sudan had significantly different bacterial contamination levels, with Acala having higher counts than Barakat cotton, but counts on different cotton grades did not vary significantly. Trash material had bacterial counts at least

one order of magnitude higher than those from equivalent lint samples, and some were two orders higher, implying that cotton with higher trash may be more hazardous to occupational health than cleaner cotton. The complications caused by storing cotton prior to analysing the bacteria levels were also discussed and it seemed that cell numbers may decline progressively during several years storage, although controls for this aspect of the investigation were not available.

CHAPTER 5

MEASUREMENT OF ENDOTOXIN ON COTTON LINT

5.1 INTRODUCTION

Endotoxin has been identified as a causative agent for byssinosis and is considered a more culpable factor than textile dust alone (Rylander, *et al.*, 1985; Rylander, 1990). Because of this conclusion, measurement techniques have been developed to assess endotoxin levels in cotton fibres and resulting dust. Endotoxin measurement in these materials is based upon an enzyme assay used to screen for endotoxin in the clinical setting. Endotoxin infection is extremely dangerous clinically, where entry of the toxin into the blood stream causes severe septicaemia, potentially resulting in multiple organ failure and ultimately death (Davey and Nicholls, 1994). Thus, products such as intravenous fluids and invasive devices are screened for endotoxin, to prevent its direct introduction into the human circulatory system.

The endotoxin assay was discovered in marine research, when it was observed that the American Horseshoe Crab *Limulus polyphemus* died following contact with bacterial endotoxin, due to intravascular blood coagulation (Levin and Bang, 1964). The harvesting and subsequent lysis of the crab's circulating blood cells (amoebocytes) produces the enzymes mediating this coagulation. The *Limulus* amoebocyte lysate (LAL) assay relies on monitoring the reaction between a sample potentially containing endotoxin, and the enzymes in the lysate. Ensuing coagulation can be

evaluated by an increase in optical density measured spectrophotometerically, and corresponding values are read from a standard endotoxin calibration curve (Cooper, 2001).

Measurement of endotoxin in cotton research is commonly performed by aqueous extraction from fibres or dust, followed by analysis with commercially available LAL assay reagents. However, there is currently a lack of standards in many aspects of the measurement protocol, and sample collection, storage, extraction procedure and analysis method can all vary between research groups (Heederik and Douwes, 1997). If the ideal of implementing endotoxin safety limits in the occupational environment is to be realised, it is imperative that a uniform analysis protocol is designed and accepted. This step would also facilitate the collaboration of research from different groups, allowing fair comparison of results. Currently, even the units in which different studies report endotoxin levels can vary, since standard enzyme assay gives results in endotoxin units (EU), which are converted to units of weight based on the particular standard endotoxin used in the assay. Table 5.01 summarises previous endotoxin measurement studies carried out in cotton processing environments and highlights the differences in measurement protocol utilised in this one environment.

Area of mill	Endotoxin	
	Dust	Airborne
Opening (bales opened)	[a]4213 EU/mg [b]# [c]# [e]198 & 84.2 ng/mg	3138 EU/m^3 0.24 μg/m^3 0.33 & 0.22 μg/m^3 232.4 & 108 ng/$m^{3(OC*)}$
Blowing (impurities removed with air currents)	[b]# [c]# [e]340.5 ng/mg	0.50 & 0.11 μg/$m^{3(OB*)}$ 0.36 & 0.58 μg/m^3 (C) 498.1 ng/m^3 (C)
Carding (fibres pulled parallel)	[a]1659 EU/mg [b]# [c]# [d]# [e]448 & 163 ng/mg	1836 EU/m^3 0.53 & 0.22 μg/m^3 0.48 & 0.35 μg/m^3 283.1//840//217.3 ng/m^{3*} 535.8 & 220.8 ng/m^3
Drawing (fibres drawn into strands)	[a]731 EU/mg [b]# [c]# [e]165.7 ng/mg	1028 EU/m^3 0.10 μg/m^3 0.43 & 0.75 μg/m^3 103.2 ng/m^3
Combing (strands combed smooth)	[a]129 EU/mg [b]# [c]# [e]109.4 ng/mg	890 EU/m^3 0.07 μg/m^3 0.64 & 0.08 μg/m^3 71.1 ng/m^3
Roving (strands wound on bobbins)	[a]196 EU/mg [b]# [c]# [e]459.2 ng/mg	957 EU/m^3 (RW) 0.23 μg/m^3 0.99 & 0.04 μg/m^3 288 ng/m^3
Spinning (strands spun into yarn)	[a]33 EU/mg [b]# [c]# [e]4.3 ng/mg	231 EU/m^3 0.002 μg/m^3 (FS) 0.004 μg/m^3 2.1 ng/m^3 (FS)
Weaving (woven to cloth)	[a]30 EU/mg	18 EU/m^3

Table 5.01: Endotoxin concentrations measured in specific production areas of different cotton mills by LAL assay. C=cleaning, FS=fine spinning, OB=opening + blowing, OC=opening + cleaning, RW=roving + winding, &=results from different mills, //=same plant, different study years, #=dust measurement not reported, *Excluded from meta analysis (Figure 5.01). [a]Christiani, *et al.*, 1993 (dust < 5μm aerodynamic diameter), [b]Kennedy, *et al.*, 1987 (dust <15 μm), [c]Christiani, *et al.*, 1994 (dust < 15μm), [d]Su, *et al.*, 2002, [e]Olenchock, *et al.*, 1983 (dust <5μm). Adapted from Lane, *et al.*, 2004.

Meta analysis carried out on these data (Figure 5.01), required units to be standardised, using the convention of 1 EU=10ng, although this can again

vary according to the specific batch of control standard endotoxin used. Figure 5.01 reveals a peak of endotoxin release at the blowing stage of cotton processing, where there is intense agitation of fibres. This is followed by a sequential decrease in the subsequent, less vigorous manufacturing stages, indicating a connection between the degree of cotton fibre agitation and the levels of airborne endotoxin. These findings reflect the outcome of a single study by Christiani, *et al.*, (1993).

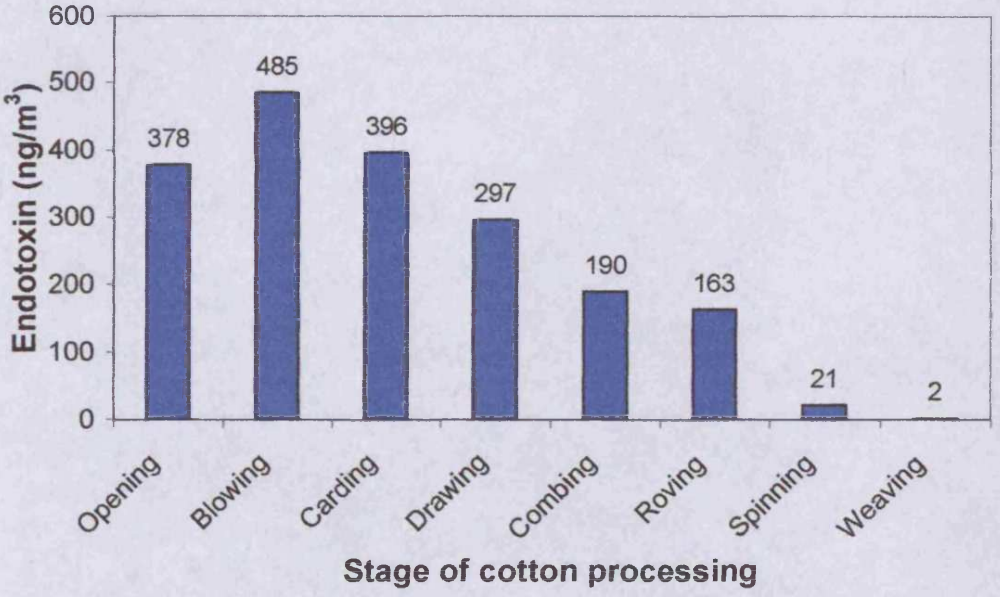


Figure 5.01: Meta analysis showing mean endotoxin level measured by different research groups, using the LAL assay on aqueous extracts from cotton dust samples collected from different production stages within various cotton mills. Adapted from Lane, *et al.*, 2004.

It is the early stages of processing which appear to hold the highest risk for cotton workers. These are the stages where raw, untreated cotton are handled and because of this, analysis of endotoxin on cotton fibres instead of

the resulting dust is also important, and there has currently been less research activity in this area.

Due to endotoxin originating in the cell membranes of cotton bacteria, a correlation between endotoxin and Gram-negative bacteria levels on the cotton is predictable. This relationship would enable the measurement of only one of these parameters to be sufficient to assess the overall contamination of cotton samples. A single analysis would decrease the cost of examining the potential health risk of cotton, especially as the methods for bacterial enumeration are significantly more cost effective than for endotoxin assessment, particularly on a large number of samples.

Aims

- To investigate extraction protocols and apply the LAL test to cotton samples from diverse sources, in order to assess whether the endotoxin content varies.
- To consider reasons for any differences in endotoxin concentration in various samples, and consider factors which may favour higher levels of endotoxin contamination.
- To investigate whether there is a relationship between endotoxin concentrations and viable Gram-negative bacterial counts, to assess the possibility of using one of these as a biomarker for contamination.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Equipment

All equipment was obtained from reputable sources, and was guaranteed pyrogen free to at least <0.005 EU/ml, full details are available in Appendix I.

Cotton/trash samples

See Chapter 2 for details of test samples. Five cotton samples were provided by the LCRC for preliminary experiments, these were selected at random and no background information regarding them was available. These samples were assigned arbitrary alphabetical characters.

Control standard endotoxin

Endosafe® control standard endotoxin was used, this was prepared from *E.coli* strain 055:B5. Each vial contained 10ng of purified LPS, freeze dried in a stabilised matrix. Potency of this standard was determined as 19 EU/ng. Lot code EX13172 was used throughout the study.

LAL reagent

Lyophilised Endosafe® KTA^2 Limulus Amebocyte Lysate (LAL) reagent, Lot code S2632L was used throughout the study.

Endotoxin-specific buffer

Endosafe® Endotoxin-specific buffer solution consisted of carboxymethylated curdlan in a pH-buffering solution containing Tris(hydroxymethyl)amino-methane at pH 7.4. The buffer was terminally sterilised and endotoxin-free.

5.2.2 Methods

Template protocol

Templates were programmed using Dynex Revelation version 4.22 software on an MRX Revelation microplate reader.

Endotoxin extraction

Endotoxin was extracted from 0.2000g (±0.0005g) cotton lint into 4 ml pyrogen free water (PFW) by shaking on a vortex multi-mixer at 1500 rpm for 60 minutes in a centrifuge tube (techniques adapted from Duke and Jacobs, 1998). The cotton fibres were then physically submerged with a sterile pipette and supernatant removed to a fresh tube.

Standard curve preparation

Control standard endotoxin was reconstituted according to lot specific Certificate of Analysis (3.8 ml LAL reagent water added to 10ng vial to obtain 50 EU/ml) and vortexed vigorously for 5 minutes. Four concentrations were used for the standard curve; 50.0, 5.0, 0.5, and 0.05 EU/ml, prepared by performing stepwise dilutions using PFW in depyrogenated borosilicate glass dilution tubes. Tubes were vortexed for 30 seconds between each transfer step.

Lysate reagent

Each vial of KTA^2 reagent powder was rehydrated immediately prior to use with 5.2ml PFW and gently swirled until dissolved into a colourless liquid.

Negative controls

Pyrogen free water was used as the negative control in each assay.

Positive controls

Positive controls were test samples spiked with 10µl of the 5.0EU/ml dilution to test for interference (inhibition/enhancement).

Preparation for analysis

100µl of negative and positive controls, standards, and samples were added to wells of a 96 well microtiter plate guided by the software template, which assigns each component. Each well was run in duplicate.

Kinetic-turbidimetric test procedure

100µl of reconstituted lysate was added quickly to each well using a repeating pipettor and syringe. The plate was then placed without lid into the pre-heated reader, and shaken for 10 seconds to mix reagents. The assay was run according to manufacturers instructions at 37°C and test wavelength 340nm for 60 minutes with automated readings taken every 30 seconds. The time taken for each well to reach the onset density of 0.03 OD was recorded and this value was automatically read off the standard curve to give the equivalent endotoxin concentration (reported in EU/ml). Standard curves were only accepted as valid when the absolute value of the correlation coefficient, r, was ≥ 0.980, and other internal validation criteria were fulfilled.

Method Validation

A freeze-dried vial containing an unknown concentration of endotoxin was provided by Charles River Endosafe (L'Arbresle, France). This was reconstituted according to supplier's instructions with 5ml Pyrogen-free water and vortexed vigorously for 5 minutes. The sample was diluted by 1 in 10 in pyrogen free water and the original and diluted samples were measured for their endotoxin concentration following the LAL assay protocol described

above. The results print out was then sent to the Charles River Endosafe laboratory to be assessed.

Preliminary studies

Tween-20; 0.5ml Tween-20 was added to 3.5ml PFW and extraction carried out as described above. 100µl undiluted tween-20 and 100µl PFW was added to a 96 well plate and analysed for endotoxin as described above.

Fibre cutting; After being weighed out, sub-samples were held by sterile tweezers and cut using sterile scissors into short lengths (approximately 2cm long) prior to extraction.

Fibre soaking; sub-samples were soaked in the PFW used for extraction at room temperature for 0, 2, 4, and 6 hours prior to shaking.

Preliminary studies; fibre cutting

All Cotton fibre sub-samples subjected to cutting prior to extraction, exhibited slightly lower endotoxin levels than uncut fibres, uncut fibre results were; sample A (46.69 ± 6.37 EU/ml), sample B (36.05 ± 5.01 EU/ml), sample C (53.91 ± 10.39 EU/ml), sample D (32.24 ± 6.89 EU/ml), and sample E (30.32 ± 4.02 EU/ml). Cut fibre results were; sample A (37.26 ± 5.09 EU/ml), sample B (29.20 ± 3.36 EU/ml), sample C (51.327 ± 11.21 EU/ml), sample D (28.34 ± 4.81 EU/ml), and sample E (26.38 ± 5.44 EU/ml). The mean result of uncut fibres (39.84 ± 4.52 EU/ml) was not significantly different to that of cut fibres (34.50 ± 4.60 EU/ml) ($P>0.05$) (Figure 5.03).

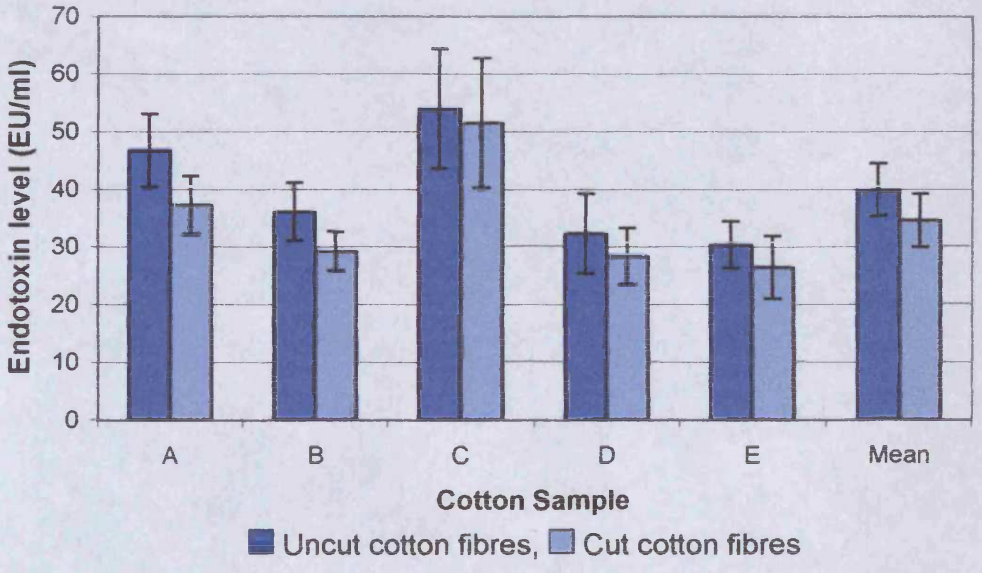


Figure 5.03: The endotoxin levels in EU/ml measured in aqueous washes from five cotton fibre samples, with and without pre-extraction fibre cutting. Results presented as mean ± SEM (n=4); the mean of all samples is also displayed ± SEM (n=20).

5.3 RESULTS: GRAPHICAL/TABULAR REPRESENTATION

Preliminary studies; Tween-20

The Tween-20 proposed to be used to aid endotoxin extraction contained significant amounts of endotoxin (259.57 ± 28.19 EU/ml) compared to pyrogen-free water (0.001 ± 0.001 EU/ml) (Figure 5.02).

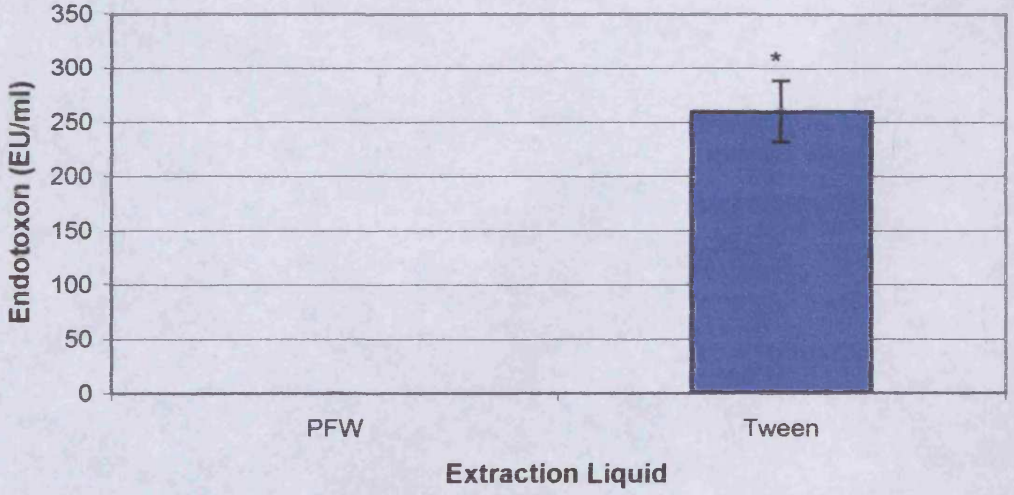


Figure 5.02: The endotoxin content of pyrogen-free water (PFW) and Tween-20 (polyoxyethylene-sorbitan monolaurate), in endotoxin units (EU)/ml. Results presented as mean ± SEM (n=6). *Denotes statistically significant difference compared to pyrogen-free water (P<0.01).

Preliminary studies; fibre soaking

Unsoaked cotton sub-samples demonstrated the lowest endotoxin level (62.00 ± 13.25 EU/ml), and levels increased with soaking times of 2 hours (93.74 ± 16.23 EU/ml), 4 hours (118.06 ± 25.56 EU/ml) and 6 hours (124.44 ± 26.51 EU/ml) although these increases were not significant ($P>0.05$) (Figure 5.04).

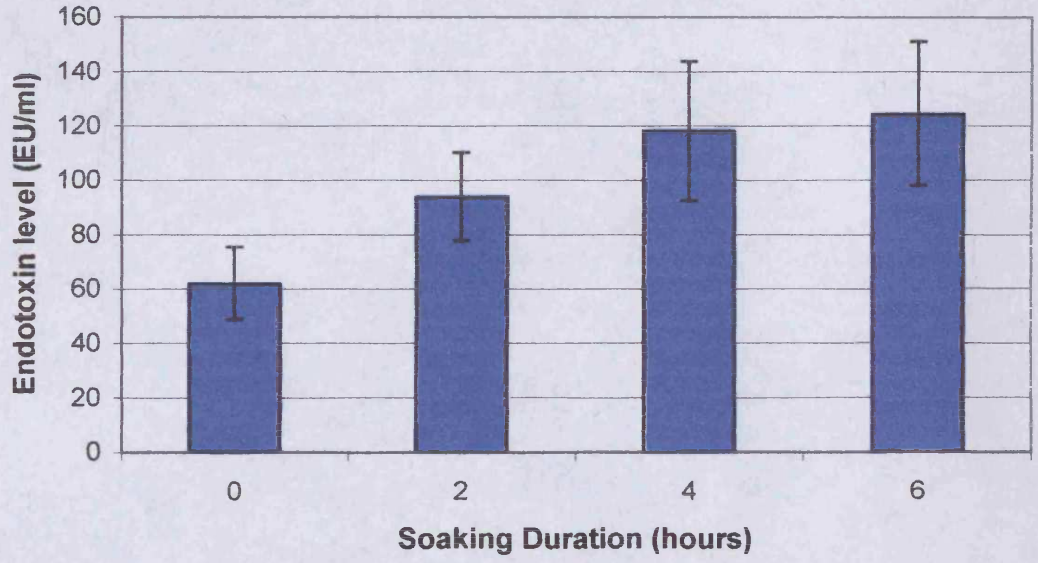


Figure 5.04: The endotoxin levels measured in aqueous washes from cotton fibre sub-samples soaked in extraction water for varying duration in hours. Samples displayed as mean ± SEM (n=6).

Inter-country comparison of endotoxin concentrations

A range of endotoxin concentrations were measured in cotton lint samples originating from diverse countries (2001/2002 harvest season). Levels were highest in the sample from Paraguay (137.89 ± 21.55 ng/g) decreasing through those from Iran (101.14 ± 10.43 ng/g), Benin (97.60 ± 9.42 ng/g), Ivory Coast (94.20 ± 12.71 ng/g), Zimbabwe (83.82 ± 6.45 ng/g), USA (57.83 ± 4.84 ng/g), Syria (57.54 ± 7.07 ng/g), CIS (39.11 ± 6.57 ng/g), Zambia (37.07 ± 7.69 ng/g), China Xinjiang (25.03 ± 3.27 ng/g), Tajikistan (15.98 ± 2.04 ng/g), and China 146 (10.93 ± 1.52 ng/g), with the lowest concentration in the sample from Turkey (8.30 ± 0.89 ng/g) (Figure 5.05).

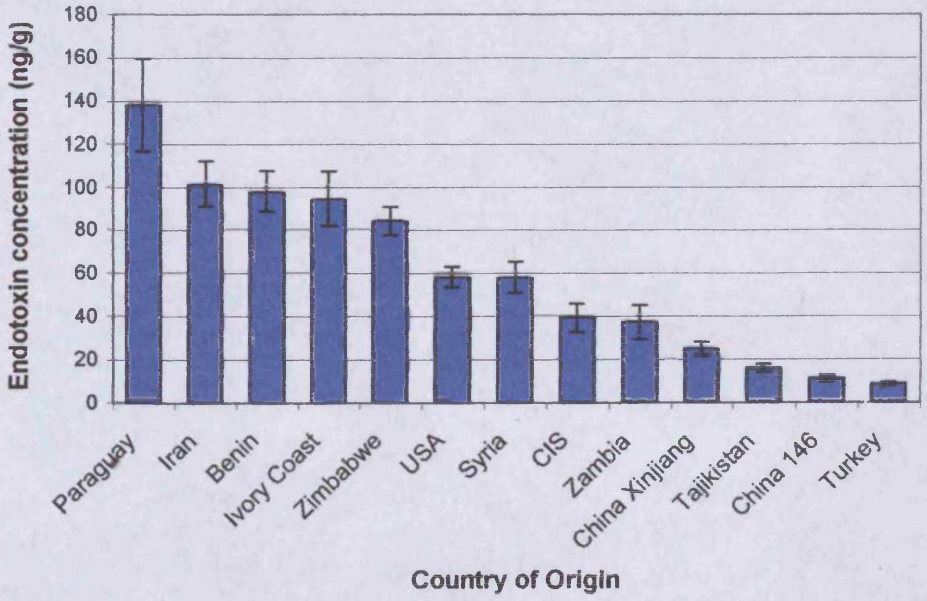


Figure 5.05: Endotoxin concentrations (ng/g) measured in aqueous washes of cotton lint samples from diverse countries of origin, (2001/2002 production season). Results presented as mean ± SEM (n=6).

The effect of production region on endotoxin concentration

Comparison of mean endotoxin concentrations on cotton samples from countries within Africa and Asia (from 2001/2002 production season) revealed a significant difference. The mean endotoxin concentration (78.20 ± 14.01 ng/g) of the four samples from African countries (Benin, Ivory Coast, Zambia and Zimbabwe) was significantly higher than the mean level (34.4 ± 12.56 ng/g) from the seven samples originating from countries within Asia (CIS, China, Iran, Syria, Tajikistan, and Turkey) (Figure 5.06).

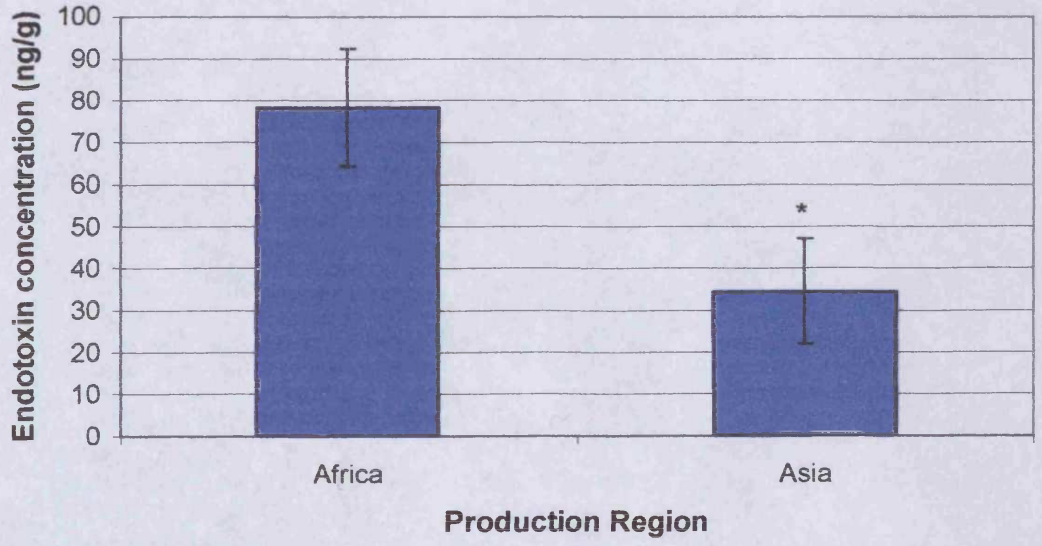


Figure 5.06: Mean endotoxin concentrations (ng/g) measured in aqueous washes of cotton lint samples originating from two different regions, Africa and Asia. African samples were from Benin, Ivory Coast, Zambia and Zimbabwe (n=24), Asian countries of origin were CIS, China, Iran, Syria, Tajikistan and Turkey (n=42). Results presented as mean ± SEM. *Denotes statistically significant difference compared to African cotton samples (P<0.05).

Intra-country analysis of endotoxin concentrations; Turkey

Endotoxin concentrations measured on cotton samples from the Bergama (104.97 ± 9.18 ng/g) and Selçuk (123.88 ± 6.48 ng/g) regions of Turkey were statistically similar to each other (P>0.05), however, the Efes 1 (71.49 ± 9.88 ng/g) and Efes 2 (10.60 ± 1.09 ng/g) samples were significantly different from all three other samples (P<0.05) (Figure 5.07).

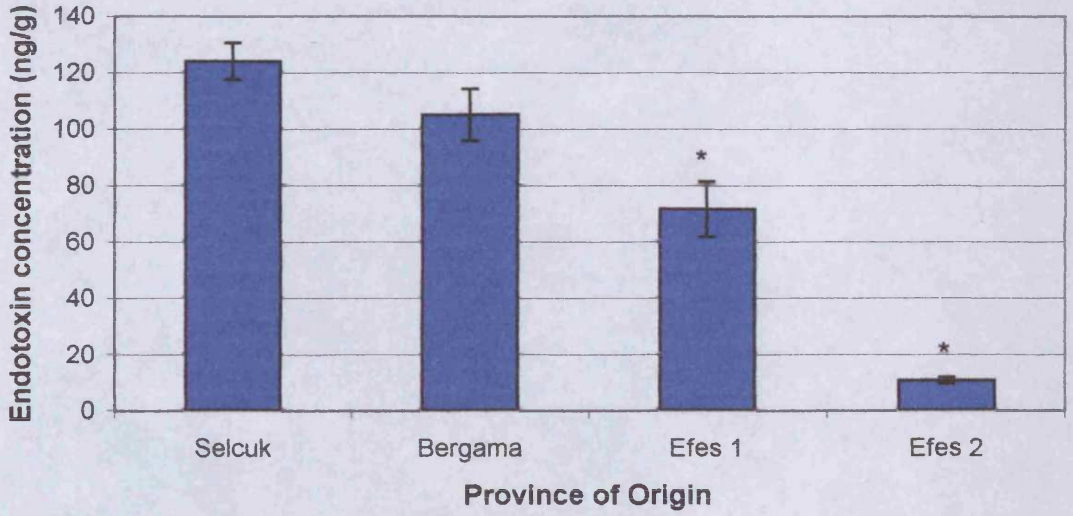


Figure 5.07: Endotoxin concentrations (ng/g) measured in aqueous washes of cotton fibres from four regions of Turkey (2002/2003 production season). Results presented as mean ± SEM (n=6). *Denotes statistically significant difference in endotoxin concentration compared to all other samples (P<0.05).

Intra-country analysis of endotoxin concentrations; Sudan

Endotoxin concentrations on various cotton lint samples from Sudan ranged from the highest in the RAC/3SG/02 sample (99.33 ± 13.02 ng/g), decreasing through samples GAC/4G/02 (72.71 ± 11.32 ng/g), GAC/3SG/02 (55.00 ± 2.81 ng/g), GBA/6B/03 (48.74 ± 2.6 ng/g), GAC/3G/98 (47.55 ± 3.18 ng/g), GBA/4B/03 (40.79 ± 4.46 ng/g), GBA/6B/00 (35.30 ± 5.63 ng/g), GBA/4B/98 (34.16 ± 3.63 ng/g), GBA/4B/00 (33.39 ± 6.66 ng/g) and GBA/6B/98 (25.22 ± 1.67 ng/g) (Figure 5.08).

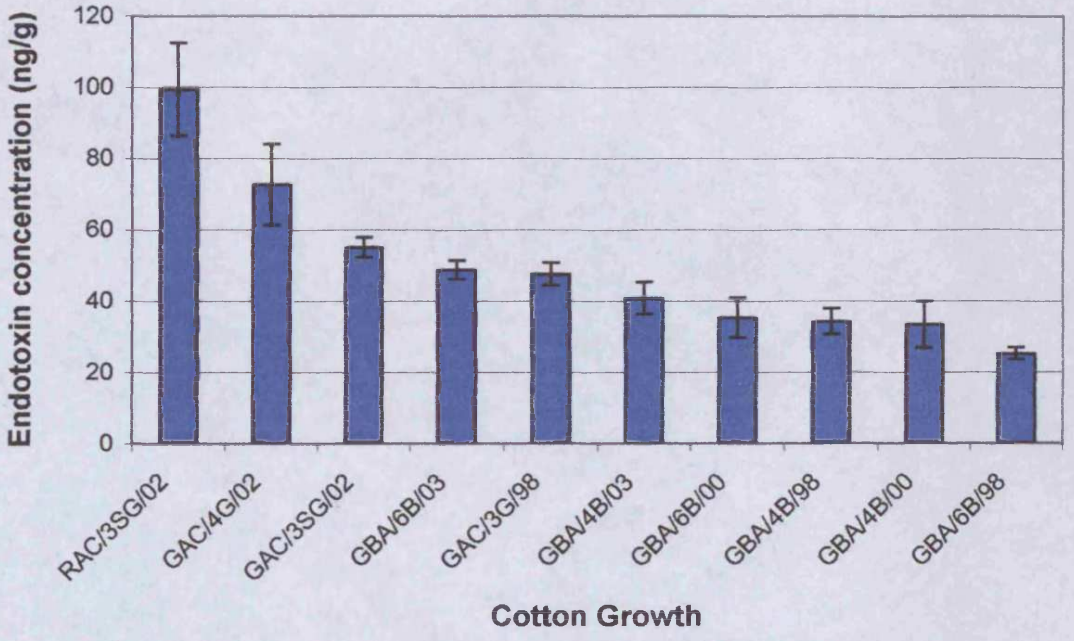


Figure 5.08: Endotoxin concentrations (ng/g) measured in aqueous washes of ten cotton lint samples from Sudan. Samples vary in cotton variety and year of harvest. Results presented as mean ± SEM (n=6). Cotton varieties are Gezira Barakat (GBA) 4B, and 6B, Gezira Acala (GAC) 3G, 3SG and 4G, and Rahad Acala (RAC). Years of harvest are 1998, 2000, 2002, 2003 (98/00/02/03 respectively).

Statistical analysis of inter-country cotton endotoxin concentrations

Statistical *a posteriori* pair-wise analysis (Tukey's HSD) performed on the mean endotoxin concentrations measured on cotton lint samples from diverse countries (2001/2002 production season), demonstrated that more statistical differences existed between samples from different geographical regions, than those from within the same region and particularly from the more diverse continents such as Asia and Africa (Table 5.02).

		Africa				Asia			Middle East				Americas	
		BEN	IVC	ZAM	ZIM	CHX	CHI	CIS	IRA	SYR	TAJ	TUR	PAR	USA
Africa	BEN		1.000	.009	1.000	.001	.000	.014	1.000	.314	.000	.000	.305	.325
	IVC	1.000		.018	1.000	.001	.000	.027	1.000	.458	.000	.000	.194	.471
	ZAM	.009	.018		.122	1.000	.893	1.000	.004	.983	.978	.809	.000	.981
	ZIM	1.000	1.000	.122		.013	.000	.167	.996	.889	.002	.000	.033	.897
Asia	CHX	.001	.001	1.000	.013		1.000	1.000	.000	.651	1.000	.997	.000	.638
	CHI	.000	.000	.893	.000	1.000		.830	.000	.125	1.000	1.000	.000	.119
	CIS	.014	.027	1.000	.167	1.000	.830		.006	.993	.955	.728	.000	.992
Middle East	IRA	1.000	1.000	.004	.996	.000	.000	.006		.196	.000	.000	.454	.205
	SYR	.314	.458	.983	.889	.651	.125	.993	.196		.260	.080	.000	1.000
	TAJ	.000	.000	.978	.002	1.000	1.000	.955	.000	.260		1.000	.000	.250
	TUR	.000	.000	.809	.000	.997	1.000	.728	.000	.080	1.000		.000	.076
Am	PAR	.305	.194	.000	.033	.000	.000	.000	.454	.000	.000	.000		.000
	USA	.325	.471	.981	.897	.638	.119	.992	.205	1.000	.250	.076	.000	

□, □, □, □ Intra-region statistical similarity, □ Inter-region statistical similarity, □ Statistically significant difference

Table 5.02: Results of post hoc tests showing multiple mean comparisons according to Tukey HSD analysis. Comparing endotoxin concentrations measured in aqueous washes from cotton lint samples from different countries, grouped into geographic regions. Presented as significance of pair-wise comparisons at P=0.05.

Statistical analysis of intra-country cotton endotoxin concentrations

Statistical *a posteriori* pair-wise analysis (Tukey's HSD) performed on the mean endotoxin concentrations measured on cotton lint samples from within Sudan, revealed statistical differences existed exclusively between samples of different cotton varieties. Cotton samples of the same species (Barakat or Acala) were all statistically similar despite variation in grade and harvest year (Table 5.03).

		Gezira Barakat						Gezira Acala			Rahad
		4B/98	4B/00	4B/03	6B/98	6B/00	6B/03	3G/98	3SG/02	4G/02	3SG/02
Gezira Barakat	4B/98		1.000	1.000	.998	1.000	.948	.969	.691	.035	.000
	4B/00	1.000		1.000	.999	1.000	.930	.956	.647	.029	.000
	4B/03	1.000	1.000		.924	1.000	.999	1.000	.955	.146	.000
	6B/98	.998	.999	.924		.996	.533	.605	.216	.003	.000
	6B/00	1.000	1.000	1.000	.996		.969	.983	.754	.046	.000
	6B/03	.948	.930	.999	.533	.969		1.000	1.000	.507	.001
Gezira Acala	3G/98	.969	.956	1.000	.605	.983	1.000		1.000	.438	.001
	3SG/02	.691	.647	.955	.216	.754	1.000	1.000		.850	.008
	4G/02	.035	.029	.146	.003	.046	.507	.438	.850		.358
Rahad	3SG/02	.000	.000	.000	.000	.000	.001	.001	.008	.358	

□, ■, Intra-region statistical similarity, □ Inter-region statistical similarity, ■ Statistically significant difference

Table 5.03: Results of post hoc tests showing multiple mean comparisons according to Tukey HSD analysis. Comparing endotoxin concentrations measured in aqueous washes from cotton lint samples within one country (Sudan), grouped into variety. Presented as significance of pair-wise comparisons at P=0.05.

The influence of cotton species on endotoxin concentrations

Comparison of the mean endotoxin concentration on different cotton species revealed a significant difference. Gezira Acala cotton had a significantly higher mean concentration (58.42 ± 7.46 ng/g), than Gezira Barakat cotton (36.27 ± 3.22 ng/g) (Figure 5.09).

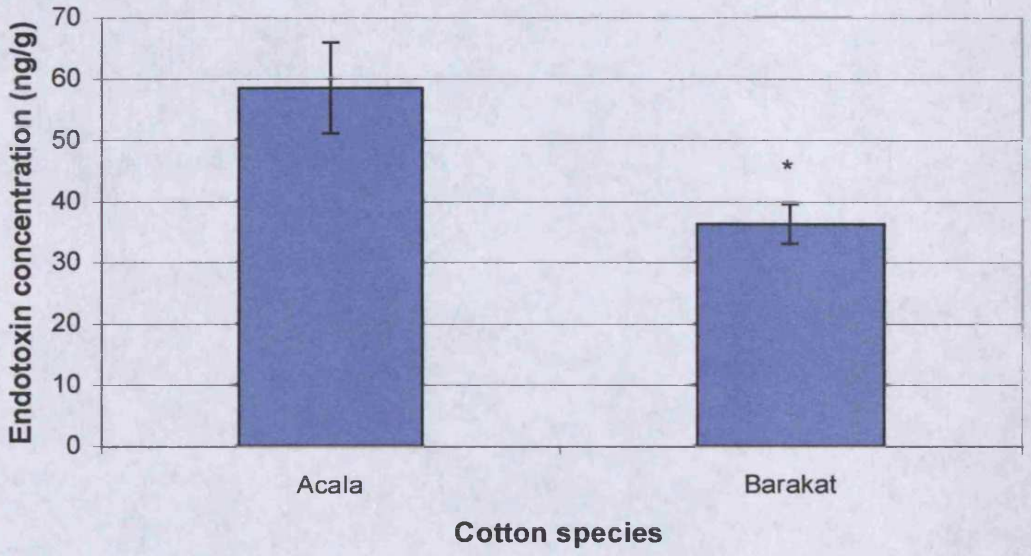


Figure 5.09: Mean endotoxin concentrations in aqueous washes from various Sudanese cotton samples of two different species: Acala and Barakat from the Gezira region of Sudan, samples are of various cotton grades and harvest years. Results presented as mean endotoxin concentration (ng/g) ± SEM (n=18 (Acala) and n=36 (Barakat)). *Denotes statistically significant difference compared to Acala cotton (P<0.05).

The influence of cotton grade on endotoxin concentration

The mean endotoxin concentration recorded on samples of Sudan Barakat 4B grade cotton (36.11 ± 2.35 ng/g) was not significantly different from Barakat 6B grade cotton (36.42 ± 6.81 ng/g) ($P>0.05$) (Figure 5.10).

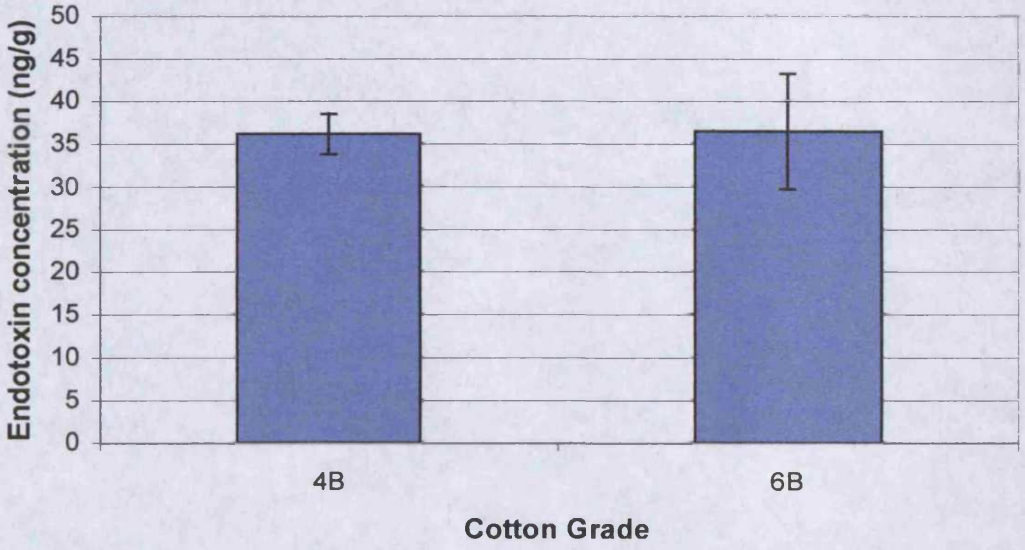


Figure 5.10: Mean endotoxin concentrations in aqueous washes of cotton lint samples from within Sudan. Results presented as mean concentration (ng/g) ± SEM (n=18). Samples are Gezira Barakat cotton of two different grades (4B and 6B), each harvested from three different years; 1998, 2000 and 2003.

The possible effect of storage on endotoxin concentrations

Analysis of cotton samples harvested in three different years, did not reveal a trend between year of harvest and endotoxin concentration. Levels on GBA/4B cotton were statistically similar in cotton harvested in 1998 (34.16 ± 3.63 ng/g), 2000 (33.39 ± 6.66 ng/g) and 2003 (40.79 ± 4.46 ng/g). Counts on GBA/6B cotton were also statistically similar on cotton harvested in 1998 (25.22 ± 1.67 ng/g), 2000 (35.30 ± 5.63 ng/g), and 2003 (48.74 ± 2.60 ng/g) (Figure 5.11).

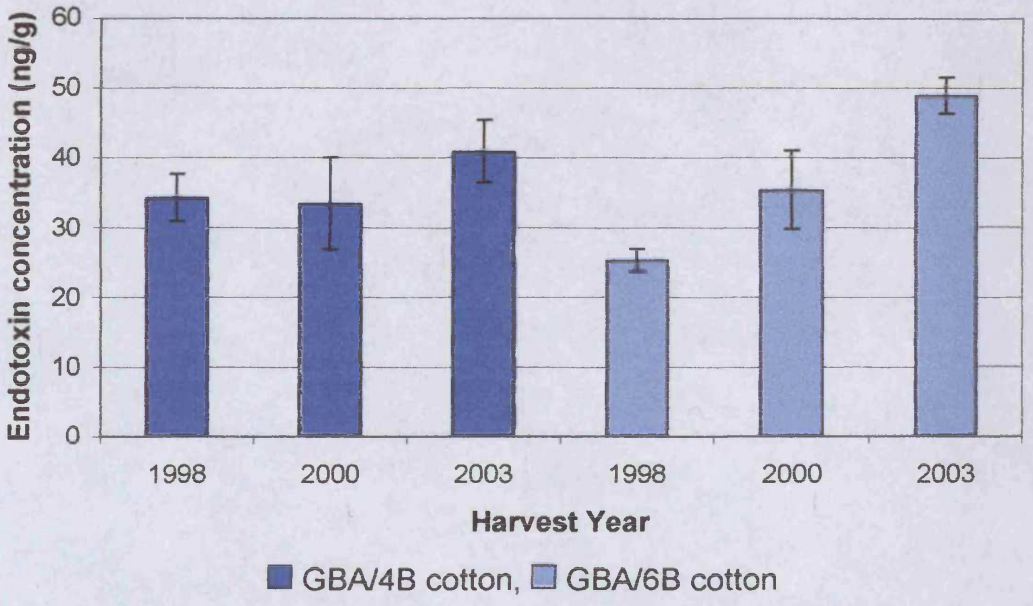


Figure 5.11: Endotoxin concentrations (ng/g) in aqueous washes from Gezira Barakat (GBA) cotton samples of two different grades (4B and 6B) produced within Sudan, harvested in three different years. Results presented as mean ± SEM (n=6).

The endotoxin concentrations of cotton lint and equivalent trash samples

Endotoxin concentrations on all analysed trash samples were significantly higher than those on corresponding lint samples (Table 5.04).

	Endotoxin concentration (ng/g)	
Sample Region	**Cotton lint**	**Cotton Trash**
Turkey 2002/03		
Efes 1	71.49 ± 9.88	1280.29 ± 401.99**
Efes 2	10.60 ± 1.09	806.97 ± 23.06*
Various 2001/02		
Iran	101.14 ± 10.43	1455.61 ± 184.71*
Turkey	8.30 ± 0.89	277.38 ± 79.88**

Table 5.04: Endotoxin concentrations (ng/g) in aqueous washes from cotton lint samples and equivalent trash samples in two samples from Turkey (2002/2003 production season) and two samples from 2001/2002 production season, from Iran and Turkey. Results presented as mean concentration ± SEM (n=6). *Denotes statistically significant difference of trash endotoxin levels compared to those on lint (P<0.05), **denotes statistically significant difference of trash endotoxin levels compared to those on lint (P<0.01).

Endotoxin concentrations on seed cotton

Endotoxin concentrations measured on two samples of seed cotton did not differ significantly, though that of the SJ2G sample (12.02 ± 5.66 ng/g) was slightly higher than that of the Gedera 5A sample (7.93 ± 1.78 ng/g) (Figure 5.12).

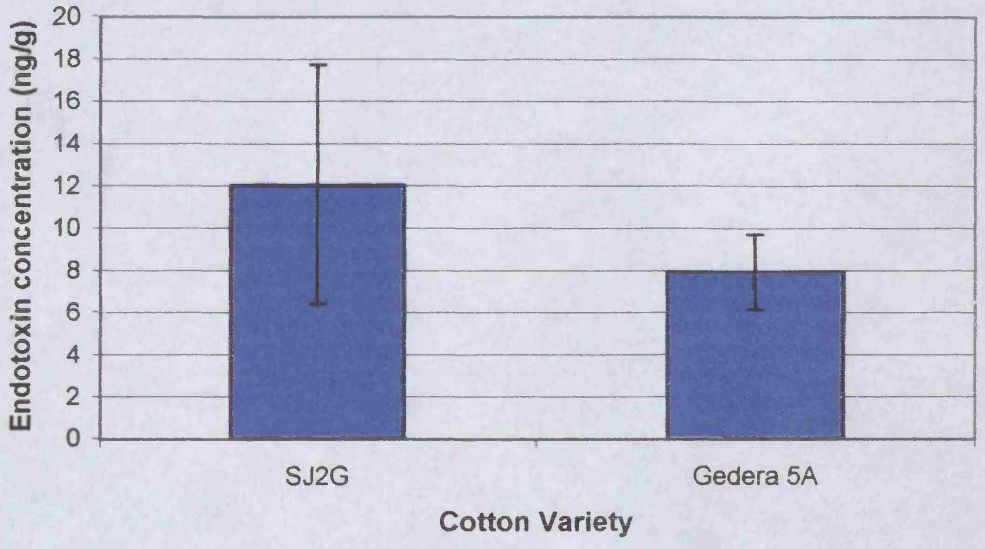


Figure 5.12: Endotoxin concentrations (ng/g) in aqueous washes from two varieties of seed cotton from Eritrea (2001/2002 production season). Results shown as mean concentration ± SEM (n=6).

Correlation of endotoxin concentration with GNB counts

Spearman's rank correlations showed a significant positive correlation between the endotoxin concentrations and Gram-negative bacterial counts on all samples involved in the study (P<0.01). Positive trends, but not significant correlations were displayed when samples from diverse countries and from Sudan were analysed separately (Table 5.05).

Cottons	N	Correlation coefficient	Sig.
Diverse samples (2001/2002)	13	0.407	0.168
Turkish samples (2002/2003)	4[a]	1.00	-
Sudan samples (various)	10	0.358	0.310
All samples in study	27	0.52**	0.005

Table 5.05: Spearman's rank correlation coefficients of endotoxin concentration and Gram-negative bacterial counts measured in aqueous washes from various cotton lint samples. [a]too few samples for reliable statistical result. **Denotes statistically significant correlation (P<0.01).

The linear relationship between Gram-negative bacterial counts and endotoxin concentration

Linear regression analysis revealed a significant correlation coefficient of 0.50, following removal of an outlier (the cotton sample from Paraguay) ($P<0.05$) (Figure 5.13).

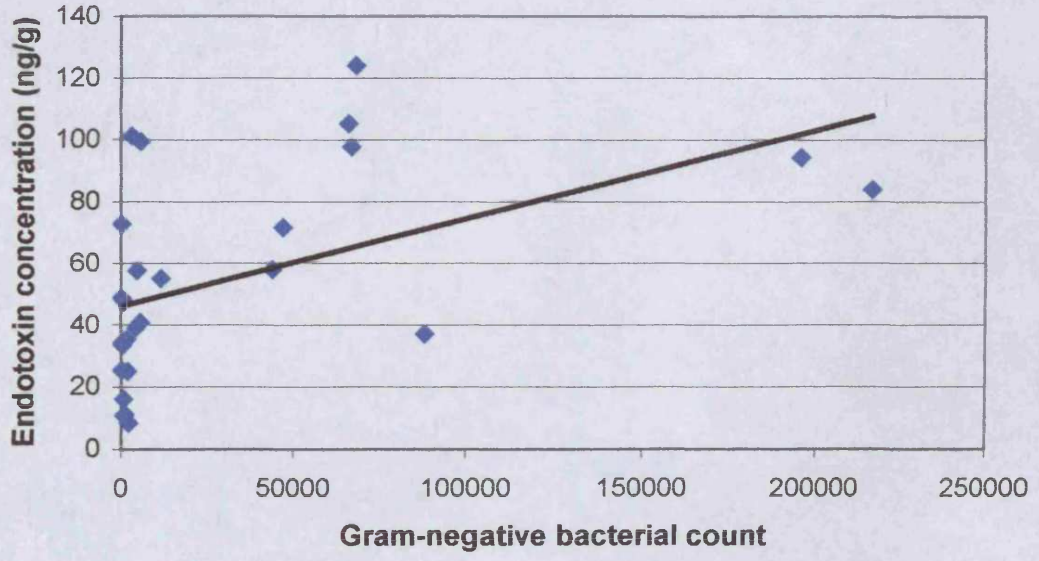


Figure 5.13: The linear relationship (y=a+bx, where a=45.87 and b=0.002), demonstrated between Gram-negative bacterial cell counts and endotoxin concentrations, measured in aqueous washes from cotton lint samples from diverse origins (n=26).

5.4 DISCUSSION

Method appraisal: endotoxin extraction

There is currently no standardised procedure for the extraction of endotoxin from cotton fibres or dust, and different research groups utilise slightly different methods, although the majority follow guidelines based upon previous research (Chun, 1999). Analysis in the current study was carried out on the same day as extraction to eliminate possible loss of endotoxin through freezing and thawing of extracted samples, which has been reported to cause the loss of 25% of endotoxin in a sample (Douwes, *et al.*, 1995). It has also been reported that endotoxin adheres to certain vessel surfaces such as polypropylene (Novitsky, *et al.*, 1986; Roslansky, *et al.*, 1991) again resulting in loss of the compound at the analysis stage yielding falsely low results. To avoid this problem, PET (tempered polystyrene) centrifuge tubes were utilised for all extraction procedures in this study.

The extraction medium successfully utilised in several research papers is pyrogen-free water (Douwes, *et al.*, 1995; Jacobs, 1997b; Olenchock, 1983), however, due to the waxy nature of the cotton fibres and the lipid component of the LPS molecule, other groups have utilised extraction media containing 0.05% Tween-20 (polyoxyethylene-sorbitan monolaurate) as a surfactant to increase the solubility of endotoxin (Domelsmith, 1992; Heederick and Douwes, 1997; Wood and Jacobs, 1997). However, preliminary studies on Tween-20 carried out for this chapter found it to contain significant levels of endotoxin (see Figure 5.02), it also caused foaming and discolouration of the extraction water, which appeared to interfere with the LAL assay (data not

shown). Although it may have been possible to render the Tween pyrogen-free, in light of its drawbacks, investigation into this method was not continued.

In order to enhance the extraction of endotoxin from the cotton fibres, the effect of increasing the surface area of the cotton by cutting it into short lengths was attempted. However, this did not result in a significant increase in the concentration measured in the supernatant after extraction, and even a slight decrease was seen in the cut samples (see Figure 5.03). Soaking the cotton fibres for several hours prior to extraction also resulted in no significant increase in endotoxin extracted from the fibres (see Figure 5.04), and any slight increase seen may be due to bacterial replication. Certainly, extending the soaking time further would have increased the risk of bacterial multiplication, which may mask the true endotoxin concentrations already present on the fibres. As a result of these experiments, it was decided to use pyrogen-free water as the extraction fluid and hence to measure only endotoxin soluble in this medium, as this was a simple, reproducible method adequate for the comparative work carried out in this instance.

Precise extraction techniques also vary between different studies. A study has examined heat extraction (68°C) of endotoxin in PFW from cotton, but this showed no benefit when compared to room temperature extraction (Chun and Perkins, 1994). Centrifugation is often included following extraction from cotton dust, to remove particles from the supernatant, however the fibres in the current study did not render the extraction medium

in need of clarification and the fibres were simply submerged with a sterile pipette to reveal the supernatant. Centrifugation has also been shown to decrease endotoxin levels in samples by removing any of the compound that remains attached to fragments and whole bacteria (Chun and Perkins, 1994). It is also noteworthy that sonication has also been utilised as an endotoxin extraction method (e.g. Walters, *et al.*, 1994), though its use has not been as widespread as physical agitation of cotton samples, and it was recommended not to pursue this technique further in the current study, due to the number of samples/sub-samples involved (Dr. D. T. Chun – personal communication). Vigorous shaking of samples for 60 minutes in pyrogen-free water is a straightforward, widely used method for extracting endotoxin from cotton dust (Chun, *et al.*, 1999; Duke and Jacobs, 1998; Jacobs, 1997; Wood and Jacobs, 1997) and was adapted for use on fibres for the current study.

Method appraisal; endotoxin analysis

The LAL assay was applied to aqueous extracts from cotton fibres to measure endotoxin contamination. The LAL assay has been adapted to assess endotoxin in a number of situations including agricultural, industrial and waste processing environments (Jacobs, 1997a) and widespread acceptance of the test lead to its adoption as the standard assay by the American Food and Drug Administration (FDA) in 1980. Although it is also possible to use gas chromatography mass spectrometry (GC-MS), which uses specific 3-hydroxy fatty acids as chemical markers of endotoxin, this method is more complex and time consuming especially for a large number of samples due to the extraction steps involved. GC-MS also measures total

endotoxin levels and not just the biologically active component, hence it is less relevant when assessing respiratory risk (Duke and Jacobs, 1998). The kinetic turbidimetric LAL assay utilised in this study is the method of choice due to its sensitivity; the detection limit of measurement is below 0.01 EU/ml (Heederik and Douwes, 1997). To ensure the specificity of the assay, an endotoxin-specific buffer was used to reconstitute the lysate, to prevent any interference from glucans also extracted from the fibres.

A drawback of the LAL assay is that qualities such as stability, and sensitivity of the lysate reagents depend on the individual manufacturers formulation. To minimise any variation, lysate reagent from a single batch was reserved and utilised throughout this study. Control standard endotoxin (CSE), also from a single production batch was used as an internal reference standard, and only results from assays which fulfilled the quality control criteria as laid out in the LAL regulatory guidelines (Charles River Endosafe, 2001; U.S Pharmacopeia, 1995) were accepted. The LAL assay criteria are that, the absolute value of the standard curve correlation coefficient, r, must be greater than or equal to 0.980, the negative controls (pyrogen-free water) are significantly lower than the lowest point of the standard curve (i.e. 0.05EU/ml) and the recovery of product spikes (positive controls) should be within the range of 50%-200% of the expected value, to ensure that there is negligible interference (enhancement or inhibition). The assay was utilised successfully on both cotton lint, seed cotton (Figure 5.12) and trash material. Further to these internal quality controls, in order to fully assess the standard of the LAL assay protocol as performed throughout the study; an external proficiency

test was performed. This quality assurance service, was provided by Charles River Endosafe (L'Arbresle, France), who supplied a blind sample of freeze-dried endotoxin. The concentration of this sample was measured using the in-house protocol and results were sent to the Charles River laboratory for validation. The results and subsequent quality report from this analysis confirmed that the concentration of endotoxin measured was correct, and the LAL assay was performed according to European Pharmacopoeia regulations.

Correlation of endotoxin concentrations with Gram-negative bacteria

A significant positive rank correlation was demonstrated between Gram-negative bacterial counts performed in Chapter 4 and the endotoxin concentrations recorded in the current Chapter (Table 5.05). The significance of this relationship was also highlighted by linear regression analysis (Figure 5.13). However, although statistically significant, this correlation is not as strong as might be expected given that GNB are the direct source of endotoxin. Highly significant correlations between endotoxin and GNB have been demonstrated in other studies on cotton fibres, although again the coefficients were relatively low at 0.50 and 0.46 (Delucca and Shaffer, 1989; Su, *et al.*, 2002 respectively). This weak relationship can be explained by the fact that endotoxin is a stable compound, and remains active in the environment after the death of the bacterial cells from which it originates (Chun and Perkins, 1991). The Gram-negative counts carried out in Chapter 4 only represented viable cells capable of replication.

The stability of cotton endotoxin has also been demonstrated in stored cotton, where numbers of GNB on fibres declined after one year in storage, but endotoxin levels reached a ceiling in the field and then remained statistically unchanged after being stored for up to 9 years (Chun and Perkins, 1996) (see Table 5.06 below). This sentiment is reflected in the results of this chapter where cottons stored since 1998, 2000 and 2003, all contained statistically similar levels of endotoxin (Figure 5.11), although this is not a definitive study on storage time as no controls were available for these samples.

Storage (years)	Endotoxin log(EU/g)
1	5.00
2	5.11
3	4.42
4	5.26
5	5.15
6	5.14
7	5.07
8	5.41
9	5.12

Table 5.06: Endotoxin levels recorded on cotton lint stored for an increasing number of years. There is no significant difference between the levels. Adapted from Chun and Perkins, 1996.

In light of this, endotoxin measurement appears to be a more reliable indicator of the respiratory risk to exposed workers, and it has been suggested that it should replace bacterial measurement wherever possible (Chun and Perkins, 1991).

Potential influence of production region

Environmental variables during the growing season have been shown to influence the numbers of GNB on cotton, as discussed in Chapter 4 of this thesis. However, given the increased stability of endotoxin compared to GNB, levels of this compound may better reflect the influence of cotton production conditions than viable bacterial counts. Mean endotoxin concentrations on cotton samples from African countries, exhibited higher contamination levels than those from Asian countries (Figure 5.06). This implies that the endotoxin content of the cotton is affected by differences in production conditions in the growing regions of theses two continents. Those conditions prevailing in Africa favoured Gram-negative bacterial growth and led to increased endotoxin levels. The range of endotoxin concentrations also appeared more controlled in the samples from within Sudan (Figure 5.08), compared to those from diverse countries (Figure 5.05), possibly due to samples from within one country being exposed to similar production conditions.

This hypothesis is further extended to the results of *a posteriori* statistical analyses on cotton samples grouped into general production region. A higher number of statistical differences existed between the mean endotoxin concentrations of cotton samples from different geographic regions than those from within the same region (Table 5.02). Notably, the highest numbers of differences were displayed when cotton samples from very diverse regions were compared (i.e. Africa and Asia, compared to Asia and the Middle East). In addition, similar *a posteriori* analysis of mean endotoxin concentrations on

samples from within Sudan showed very limited statistical differences (Table 5.03), with the majority occurring when samples from the Gezira region were compared to the sample from the Rahad region, a geographically distinct area of the country (see Figure 2.08). Other differences appeared to be due to the variety of Sudanese cotton analysed, and this will be discussed later.

Geographical production region has been reported to influence cotton endotoxin concentrations specifically, as opposed to the GNB from which they originate, in a number of studies (Delucca and Schaffer, 1989; Olenchock, *et al.*, 1984; Fischer and Foarde, 1991; Olenchock, *et al.*, 1985). One study analysing cotton dusts generated from the same variety of cotton, grown in several locations of the US cotton belt stated that "endotoxin contamination of the dusts varied markedly as a result of the geographical area in which the cottons were grown" (Olenchock, *et al.*, 1984). Table 5.07 below highlights this.

Growth area	Endotoxin (ng/m^3)
Mississippi	164.89 ± 10.41
California	16.37 ± 2.48
Texas	10.63 ± 0.72

Table 5.07: The endotoxin levels measured in cotton dusts produced from cotton grown in different areas of North America. Adapted from Olenchock, *et al.*, 1984.

An experiment conducted in Texas, in which a portion of growing cotton was sheltered from rain, demonstrated that sheltered cotton resulted in LPS measurements that were significantly lower than unprotected cotton (Fisher

and Foarde, 1991). Ideally, local climatic/environmental data would be collected during cotton growth, in order to assess their effect on endotoxin levels, although the logistical problems of this would be difficult to surmount, especially with a worldwide project. One study involving an experimental cotton production plot in North America examined endotoxin levels in conjunction with local environmental data. Delucca and Shaffer (1989), obtained ambient air temperature and relative humidity data (taken 10 km from the production site) from the National Weather Service, and measured precipitation levels on site. Although of these parameters, only daily high temperature was significantly correlated with endotoxin levels.

Specific climatic data for the regions involved in the current study was not readily available. However, mean data regarding environmental parameters within the locale of horticultural research stations, run by the International Society for Horticultural Science in the Gezira and Rahad regions of Sudan have been published on the internet (Table 5.08 below).

Parameter	Gezira	Rahad
Elevation (metres)	405	600
Annual rainfall (millimetres)	306.4	403
Hours of Sunshine (hrs/day)	9.7	9.2
Humidity (%)	58 (summer) 32 (winter)	65 (summer) 35 (winter)

Table 5.08: Environmental parameters measured in the Gezira and Rahad regions of Sudan. Adapted from Horticultural Research International, [WWW] 2005.

Although these data are very general, they do highlight slight differences between the two regions, which may have an influence on the Gram-negative bacterial and hence endotoxin contamination on cotton grown there. The cotton sample from the Rahad region of Sudan analysed in the current study possessed significantly higher levels of endotoxin, compared to the majority of samples from the Gezira region (Table 5.03). This may be due to differences in environmental conditions in the Rahad region, such as the higher rainfall and humidity demonstrated in Table 5.08, both parameters that have been shown to promote bacterial growth, as described in Chapter 4 of this thesis.

A further factor, which may influence bacterial contamination and hence endotoxin level, on cotton from these two areas, is insect infestation. Not only do whitefly and aphids play a role in transferring GNB between cotton plants, they are also responsible for many of the sugars, through honeydew production, which lead to increased cotton stickiness (Hend, *et al.*, 2003; Slosser, *et al.*, 2001). Honeydew sugars are an ideal substrate for bacteria, and higher levels could support a larger microbial population. The Rahad region of Sudan is known to have higher whitefly infestation than that of the Gezira region, resulting in increased honeydew on Rahad cotton (Khalifa, 2001), and a study on cotton lint from regions of Turkey has shown that samples from a honeydew-rich region had higher levels of endotoxin (Hend, *et al.*, 2003). These studies provide circumstantial evidence to support this theory, and suggest that a means of controlling whitefly infestation on cotton, such as use of insecticides may also have a role in reducing byssinosis

prevalence. However, despite this evidence for the role of geographic region in endotoxin contamination, analysis of cotton samples originating within Turkey from the same production season, displayed a significant difference between endotoxin concentrations measured on two samples from the same region (Figure 5.07). This indicates that the factors influencing bacterial counts are multifactoral, and highlights the complexities of analysing naturally grown cotton samples.

Other factors effecting endotoxin concentration

Studies analysing the effect of geographical growth region on cotton endotoxin concentrations, which have involved different types of cotton, have noted that geographic region has an effect on this contamination, regardless of cotton variety (Olenchock, *et al.*, 1985; Fischer and Foarde, 1991). One study stated, "endotoxin levels associated with cotton lints ... are relatively constant throughout the growing season and are determined more by the area of growth than by the type of cultivar planted" (Fischer and Foarde, 1991). In the current study, significant differences in endotoxin concentration were revealed by *a posteriori* analysis between one Gezira Barakat cotton sample (4G), and the two Gezira Acala cotton varieties (4B and 6B). However, no differences existed between cottons of the same species (Table 5.03). The possible influence of cotton species on endotoxin was also highlighted when mean endotoxin concentrations for all Gezira Barakat and Acala samples involved in the study were compared (i.e. there was a significantly higher level on Acala cotton) (Figure 5.09). Whereas, the grade of the cotton did not appear to influence the endotoxin concentration, as no

significant difference was seen between the mean levels on 4B and 6B cotton (Figure 5.10).

The higher contamination level on Acala cotton was also reflected in the GNB counts displayed in Chapter 4 (Figure 4.10), and may be due to differences in structure of the cotton fibres produced by these two different plant species. Acala (*Gossypium hirsutum*) cotton fibres are bushier than those of Barakat fibres (*G. Barbadense*) (Khalifa, 2001) and hence are more likely to entrap bacteria. *G. hirsutum* has also been shown to be more susceptible to whitefly infestation (Khalifa, 2001), and hence this may lead to higher levels of endotoxin on Acala cotton, via the honeydew theory described above.

Endotoxin contamination of cotton trash material

As with Gram-negative bacterial counts, contamination levels of endotoxin were significantly (at least one order of magnitude) higher on trash material than on equivalent samples of cotton lint. A previous study also reported high levels of LPS on cotton bracts (Fischer and Foarde, 1991), the main constituent of cotton trash. This may also be related to increased nutrient availability on these parts of the cotton plant. The high endotoxin concentration on trash material has implications for the health of cotton workers in the early stages of cotton production, where the removal of these particles by physical agitation methods results in large clouds of airborne trash particles.

Summary

After examining several methods to enhance the extraction of endotoxin from cotton fibres, a simple method utilising vigorous shaking in pyrogen-free water was adopted followed by analysis using a kinetic turbidimetric LAL assay. A significant correlation was revealed between cotton endotoxin concentrations and Gram-negative bacterial counts. Endotoxin appeared more stable over time than GNB, and hence was considered a better marker for industrial exposure. Evidence for the role of production region in determining levels of endotoxin was discussed; cotton species and whitefly infestation may also contribute. Cotton trash material was found to have increased endotoxin contamination compared to equivalent lint samples, suggesting that workers at the earliest stages of production are most at risk of developing respiratory illness.

CHAPTER 6

IDENTIFICATION OF FUNGI ON COTTON

6.1 INTRODUCTION

Fungi are composed of thread-like tubular cells called hyphae, these cells are eukaryotic, and hence contain nuclei, mitochondria, ribosomes, golgi bodies and lysosomes. The organelles are contained within cytoplasm bound by a plasma membrane. The membrane in turn is surrounded by a rigid cell wall containing chitin, a polymer of the glucose derivative N-acetylglucosamine. Fungal cell walls superficially resemble those of plant cells, but differ significantly in chemical structure as they often lack cellulose and are 80-90% polysaccharide, with various proteins, lipids and polyphosphates making up the rest of the wall matrix (Brock, 2000). Fungal hyphae form extensive branching systems termed mycelia, which form the largely undifferentiated somatic body of the fungus (Griffin, 1994). From the mycelium, further hyphae may branch upwards into the air and upon these, aerial spores called conidia are formed. Conidia are asexual spores, which are relatively resistant to changes in temperature and moisture; hence, they aid the dispersal of fungi. Conidia are also a useful tool for the recognition of particular fungal colonies; they are often pigmented, displaying a range of colours, and their arrangement along the hyphae to which they are attached reveals many different patterns (conidial ontogeny), when examined under a microscope.

Fungal classification is based upon morphology, life cycle and reproductive characteristics; different groups of fungi produce specific sexual spores (e.g. ascospores, basidiospores, and zygospores). The common groups of fungi based on sexual spore formation are displayed in Table 6.01, although it should be noted that fungal classification is constantly evolving and can be confused by varying terminology.

Group	Common name	Genera e.g.	Diseases
Ascomycetes	Sac fungi	*Neurospora, Saccharomyces*	Dutch elm, chestnut blight, ergot, rots
Basidiomycetes	Mushrooms	*Agaricus* (edible mushroom)	Black stem, wheat rust, corn smut
Deuteromycetes	Fungi imperfecti	*Aspergillus, Penicillium*	Plant wilt, animal infections (ringworm)
Oomycetes	Water moulds	*Allomyces*	Potato blight, certain fish diseases
Zygomycetes	Bread moulds	*Mucor, Rhizopus*	Food spoilage

Table 6.01: The classification and major properties of fungi. Adapted from Brock, 2000.

Cotton fibres are naturally colonised by a range of fungi and these can be roughly divided into two groups; field fungi, which colonise fibres in the field prior to harvest (also known as outdoor fungi), and the storage fungi, which are more tolerant to drying and lack of oxygen and can be commonly isolated after cotton has been harvested and stored (indoor fungi) (Dutkiewicz, 1997). Fungi previously identified in occupational dust, including that from cotton,

are largely ascomycetes, deuteromycetes and zygomycetes; examples of common genera are shown in Table 6.02.

Genera	Group	Source
Alternaria	Deuteromycetes	Field
Aspergillus	Deuteromycetes	Storage
Cladosporium	Ascomycetes	Field
Eurotium	Ascomycetes	Storage
Fusarium	Ascomycetes	Field
Penicillium	Deuteromycetes	Storage
Rhizopus	Zygomycetes	Storage

Table 6.02: Fungal genera commonly identified in organic fibres and dusts and whether they are generally found in cotton prior to or after harvest. Compiled from Dutkiewicz, 1997; Kaese, *et al.*, [WWW] 2003; Brock, 2000.

The presence of fungal contamination in the occupational setting has health implications; not only can inhalation of spores elicit allergic and inflammatory responses, but fungi also release various compounds which are toxic to humans. Mycotoxins, such as aflatoxin from *Aspergillus*, can cause a range of conditions including acute kidney failure, damage to the central nervous system and some are known carcinogens (Fischer and Dott, 2003; Lugauskas, *et al.*, 2004). *Aspergillus*, *Fusarium* and *Penicillium*, all of which have been identified on cotton fibres previously (Kaese, *et al.*, [WWW] 2003), are known to produce mycotoxins (Dutkiewicz, 1997).

The identification of fungal species on cotton fibres has been previously carried out on cotton from a number of countries (Kaese, *et al.*, [WWW] 2003; Piecková and Jesenská, 1996). Although, these studies did not include

detailed evaluation of the health effects on industrial workers. Extending cotton fungi identification to a further range of production regions will reveal whether (as in the case of bacteria) the same fungal genera are widely spread across the globe and can be repeatedly isolated from diverse cottons. Analysing cotton samples stored for differing amounts of time may also reveal information about the prevalence of the so-called field and storage fungi.

Aims

- To identify the major species of fungi contaminating various cotton samples.
- To compare fungal genera isolated from cotton fibres of diverse geographic origins and different cotton types.
- To assess sources of contamination, i.e. whether there are field or storage fungi.
- To consider implications for respiratory risk with respect to different fungal types.

6.2 MATERIALS AND METHODS

6.2.1 Materials

Equipment

All equipment was obtained from reputable sources, and was, where possible sterile, full details are available in Appendix I.

Cotton/trash samples

See Chapter 2 for details.

6.2.2 Methods

Methods were carried out utilising conventional aseptic techniques to maintain sterile conditions and ensure external contamination was avoided.

Fungal cell extraction

Fungal cells were extracted from 0.6000g (±0.0005g) cotton lint into 10 ml phosphate buffered saline, by shaking on a vortex multi-mixer at 1000 rpm for 60 minutes in a centrifuge tube (techniques were adapted from Chun and Perkins, 1991; Kaese, *et al.*, [WWW] 2003). The cotton fibres were then physically submerged using a sterile pipette and the supernatant removed to a fresh tube. Due to the limited availability of cotton trash, 0.3000g (±0.0005g) material was shaken, as before in 5ml phosphate buffered saline in the extraction procedure, all other aspects of extraction and culture remained the same as those applied to the lint.

Agar plates

Malt extract agar (MEA) was made up following manufacturers instructions (33.6g/litre) and autoclaved for 1 hour at 120°C. It was then allowed to cool to approximately 40°C, at which point the antibiotic chloramphenicol (30μg/ml)

was added in order to prevent the growth of both Gram-positive and Gram-negative bacteria (techniques adapted from Chun and Perkins Jr., 1991; Fisher and Sasser, 1987) (see Appendix II for antibiotic action). The agar was then mixed gently by inverting the bottle several times, before 25ml molten agar was poured into each petri dish and allowed to solidify. Plates to be used for spread plating were 'over dried' in a laminar flow unit for 30 minutes prior to inoculation to remove excess water, in order to ensure discrete colonies.

Spread plating

0.1ml aliquots of supernatant were inoculated onto the centre of an over dried agar plate and spread with a sterile (flamed) glass spreader.

Incubation

All plates were inverted and incubated at 25°C (±2°C) for 4 days.

Colony isolation

Colony isolation was based upon morphological differences such as colour, size and shape. Colonies appearing different were 'picked off' with a sterile cotton swab and streaked onto a fresh agar plate. Subcultured plates were then incubated as before for 4 days. Colonies were isolated from four separate sub-samples of each cotton sample in order to confirm the presence of every fungal species. The most commonly occurring species (i.e. those with the highest number of colonies) from each sample was also recorded.

Fungal Identification

Identification procedures were carried out on subcultured isolates, based on macroscopic and microscopic morphology using taxonomic reference

material (Funder, 1961; Malloch, [WWW] 1997). Colour, shape and size of the colony were recorded, along with the macroscopic appearance of individual structures such as spores. Further to this, all isolates were stained using lactophenol blue solution and examined under light microscope at x200 to x1000 magnification using tape lift techniques (Forbes, *et al.*, 2002) (see below) to observe hyphae and spore structure arrangement (conidial ontogeny). For delicate colonies, which disintegrated upon standard slide preparation, slide cultures were prepared (see below).

Tape lift procedure

Fungal spores are often on raised hyphae in order for efficient dispersal, in order to 'lift off' these structures for microscopic examination, the sticky side of a piece of cellophane tape was gently pressed against a 3-5 day old subcultured colony using sterile tweezers, so that some of the spores and hyphae stuck to it. It was then mounted on a microscope slide with lactophenol blue solution for examination.

Slide cultures

To overcome the identification problems faced with delicate fungal spore arrangements, which collapse during usual slide mounting techniques (such as tape lifts), slide cultures were prepared. A $1cm^2$ block of MEA seeded with chloramphenicol was placed on top of a sterile microscope slide. The slide was then placed in a petri dish containing damp filter paper to maintain humidity. A few fungal spores were inoculated along the edges of the agar block using a sterile cotton swab, and a cover slip added. After 3-5 days incubation at 25°C the slide was removed from the dish and placed with the agar block under the microscope with the fungal spores undisturbed.

6.3 RESULTS: GRAPHICAL/TABULAR REPRESENTATION

Inter-country fungal identification

At least five different fungal genera were identified from thirteen cotton lint samples from diverse countries (2001/2002 production season). The number of different genera identified in individual samples varied from one (in the sample from Iran), two (in CIS cotton), three (cotton from China 146, Ivory Coast, Syria, Tajikistan, Turkey, and USA), four (Benin, China Xinjiang and Paraguay) and two samples had five different fungal genera (from Zambia and Zimbabwe) (Table 6.03).

Sample	Fungal Genera/Species
Benin	*Aspergillus niger* / AO / ***Fusarium*** / WC
CIS	***Aspergillus niger*** / *Cladosporium*
China 146	***Cladosporium*** / *Penicillium* / WC
China Xinjiang	AO / ***Cladosporium*** / *Penicillium* / WC
Iran	***Alternaria*** / *Aspergillus niger*
Ivory Coast	*Aspergillus flavus* / *Cladosporium* / ***Fusarium***
Paraguay	***Aspergillus flavus*** / *Aspergillus niger* / *Rhizopus* / WW
Syria	*Aspergillus niger* / ***Fusarium*** / ***Rhizopus***
Tajikistan	*Aspergillus flavus* / *Cladosporium* / ***Fusarium***
Turkey	***Aspergillus niger*** / *Aspergillus flavus* / *Rhizopus*
USA	*Aspergillus niger* / ***Cladosporium*** / *Rhizopus*
Zambia	*A. niger* / *Cladosporium* / ***Fusarium*** / *Rhizopus* / *Penicillium*
Zimbabwe	*A. flavus* / *A. niger* / *Cladosporium* / ***Fusarium*** / *Penicillium*

Table 6.03: Total fungal genera and species isolated in aqueous washes from thirteen cotton lint samples from 2001/2002 production season, originating in twelve different countries. AO = unidentified orange *Aspergillus* colony, WC = unidentified white cone-shaped colony, WW = unidentified white woolly colony, the most prevalent colony is displayed in bold font.

The most commonly isolated fungal genus identified from cotton samples originating in diverse countries was *Aspergillus* (isolated from 92.3% of samples), followed by *Cladosporium* (from 61.5%), *Fusarium* (46.2%), *Rhizopus* (38.5%), *Penicillium* (30.8%), unidentified genera (30.8%) and the least common genus was *Alternaria* (only identified from 7.7% of samples analysed) (Figure 6.01).

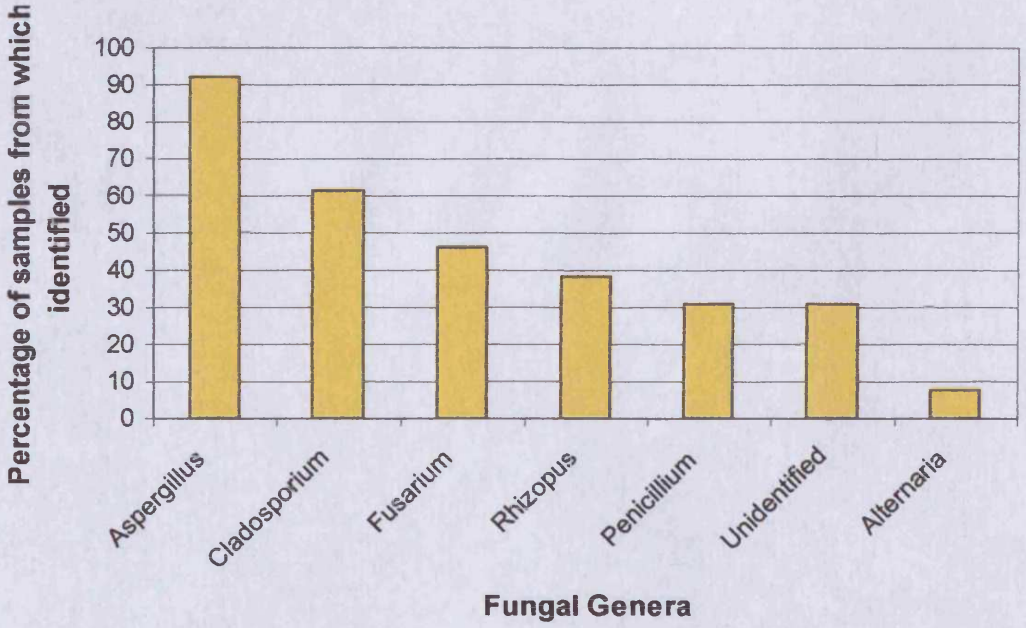


Figure 6.01: The fungal genera identified in aqueous washes from thirteen cotton lint samples from diverse countries of origin (2001/2002 production season). Results presented as the percentage of samples from which each was isolated.

Three species of *Aspergillus* were isolated from cotton samples originating in diverse countries, the most prevalent of these was *Aspergillus niger* (which made up 56% of *Aspergillus* isolates), followed by *Aspergillus flavus* (31% of isolates) and an unidentified orange *Aspergillus* colony was the least prevalent (constituting 13% isolates) (Figure 6.02).

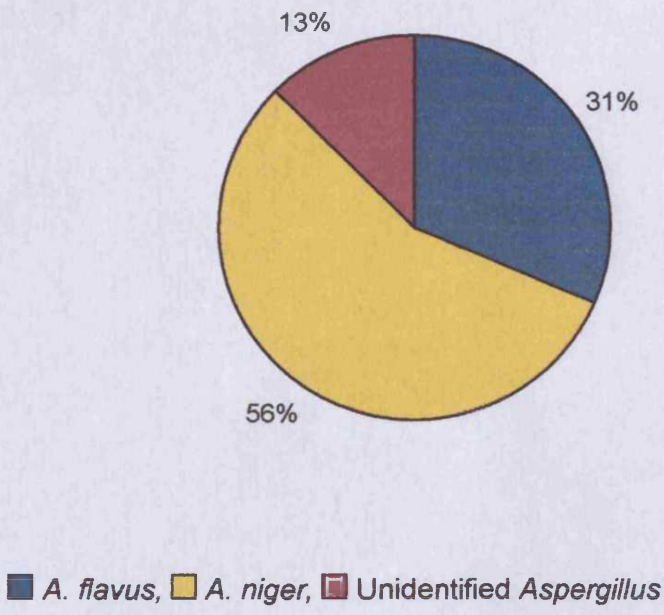


Figure 6.02: The three species of *Aspergillus* (*niger*, *flavus* and other unidentified species), isolated in aqueous washes from thirteen different cotton lint samples (2001/2002 production season), from diverse countries of origin. Results displayed as percentage of total *Aspergillus* isolates.

Intra-country fungal identification; Turkey

Aspergillus was the most common fungal genus identified in cotton lint samples from different regions of Turkey, being isolated from all four samples, with *Aspergillus niger* being the most prevalent species. *Fusarium* was isolated from three samples and other fungi were identified in only one sample each. The fungi isolated from these samples varied considerably, even between the two samples from the Efes region (Table 6.04).

Sample (Region of Turkey)	Fungal Genera/Species
Bergama	***Aspergillus niger*** */ Cladosporium / Rhizopus*
Efes 1	*Aspergillus flavus /* ***Aspergillus niger*** */ Fusarium*
Efes 2	*Alternaria /* ***Aspergillus niger*** */ Fusarium /* WW
Selçuk	*Aspergillus flavus /* ***Aspergillus niger*** */ Fusarium*

Table 6.04: The fungi identified in aqueous washes from four cotton lint samples from 2002/2003 production season, originating from different regions of Turkey, WW = unidentified white woolly colony, the most prevalent colony is displayed in bold font.

Intra-country fungal identification; Sudan

The variety of cotton lint samples from within Sudan yielded similar fungal species to those isolated in cotton samples from diverse origins, though *Alternaria* was absent. The number of isolates from each sample varied from two (GBA/4B/98) three (GBA/4B/00 and GBA/6B/03), four (GBA/4B/03, GBA/6B/98, GBA/6B/00, GAC/3SG/02 and GAC/4G/02) and the highest number of isolates from one sample was five (GAC/3G/98 and RAC/3SG/02) (Table 6.05).

Sample	Fungal Genera/Species
GBA/4B/98	***Aspergillus niger*** / *Cladosporium*
GBA/4B/00	***Aspergillus niger*** / *Penicillium* / *Rhizopus*
GBA/4B/03	*Aspergillus flavus* / ***Aspergillus niger*** / *Cladosporium* / *Rhizopus*
GBA/6B/98	*Aspergillus flavus* / ***Aspergillus niger*** / *Cladosporium* / WW
GBA/6B/00	*Aspergillus flavus* / ***Aspergillus niger*** / *Rhizopus* / WC
GBA/6B/03	***Aspergillus niger*** / *Cladosporium* / *Rhizopus*
GAC/3G/98	*Aspergillus flavus* / ***Aspergillus niger*** / *Cladosporium* / *Rhizopus* / WC
GAC/3SG/02	***Aspergillus niger*** / *Cladosporium* / *Fusarium* / *Rhizopus*
GAC/4G/02	*Aspergillus flavus* / ***Aspergillus niger*** / *Cladosporium* / WC
RAC/3SG/02	*Aspergillus flavus* / *Aspergillus niger* / ***Cladosporium*** / *Fusarium* / *Rhizopus*

Table 6.05: Fungi isolated in aqueous washes from various Sudanese cotton samples. Samples varied in production region; Gezira (G) and Rahad (R), cotton species; barakat (BA) and acala (AC), cotton grade; 4B, 6B, 3G, 3SG and 4G, and harvest year; 1998, 2000, 2002, 2003. WC = unidentified white cone-shaped colony, WW = unidentified woolly colony, the most prevalent colony is displayed in bold font.

Aspergillus was the most prevalent fungal genus isolated from the ten various cotton lint samples from Sudan (identified in 100% of samples), *Cladosporium* was the second most commonly isolated (80% samples), followed by *Rhizopus* (70% samples), unidentified isolates (40%), *Fusarium* (20%) and the least prevalent genus was *Penicillium* (isolated from 10% cotton samples) (Figure 6.03).

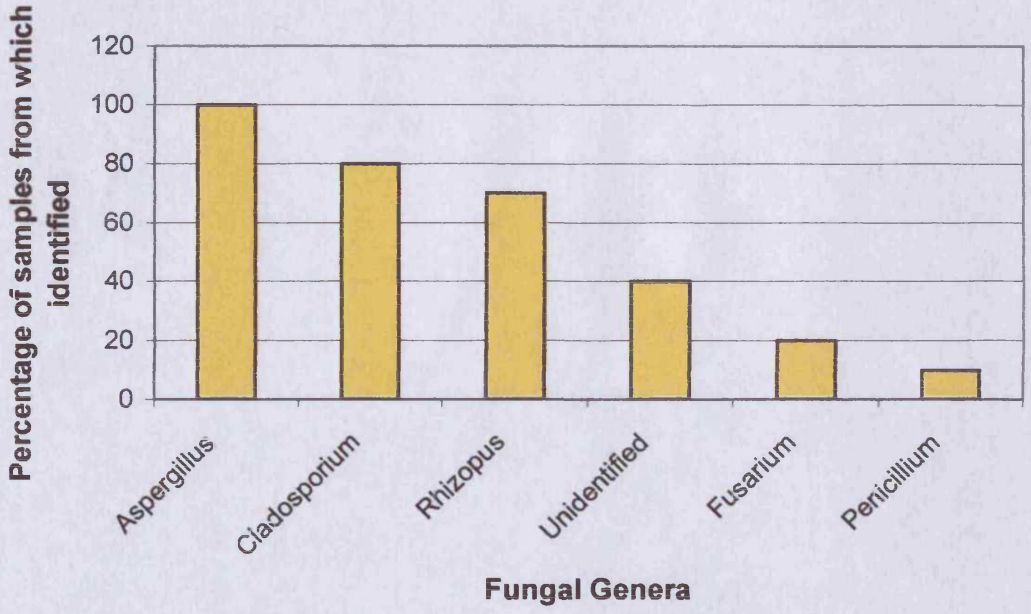


Figure 6.03: The fungal genera identified in aqueous washes from ten various cotton lint samples from within Sudan. Results presented as the percentage of samples from which each was isolated.

Two species of *Aspergillus* were isolated from the cotton samples originating from Sudan. The most prevalent of these was *Aspergillus niger* (which made up 62% of *Aspergillus* isolates), followed by *Aspergillus flavus* (38% of isolates) (Figure 6.04), these figures are comparable to those of cotton samples from diverse countries for these species (see Figure 6.02 above).

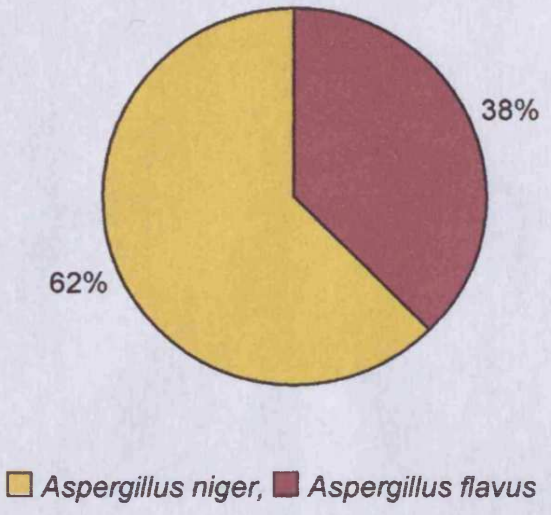


Figure 6.04: The two species of *Aspergillus* (*niger* and *flavus)* isolated in aqueous washes from ten cotton lint samples from within Sudan. Results displayed as percentage of total *Aspergillus* isolates.

Factors influencing the fungal profile of Sudanese cotton

The two different Sudanese cotton species showed a slight variation in the fungal genera isolated, and there were also differences in the fungi isolated from varying cotton grades (these also vary in harvest year). There was no difference in the fungal genera isolated from equivalent cotton samples grown in different production regions (Table 6.06).

	Fungal Genera						
	Asp.	**Clad.**	**Fus.**	**Pen.**	**Rhiz.**	**WC**	**WW**
Cotton species							
Barakat	✓	✓		✓	✓	✓	✓
Acala	✓	✓	✓		✓	✓	
Cotton grade							
4B	✓	✓		✓	✓		
6B	✓	✓			✓	✓	✓
3G	✓	✓			✓	✓	
3SG	✓	✓	✓		✓		
4G	✓	✓				✓	
Production region							
Gezira 3SG/02	✓	✓	✓		✓		
Rahad 3SG/02	✓	✓	✓		✓		

Table 6.06: Factors influencing the distribution of fungal genera identified in aqueous washes from ten cotton lint samples from Sudan, samples are divided into groups to display variation in cotton species, grade (though harvest years also vary), and production region. Fungi are *Aspergillus* (Asp.), *Cladosporium* (Clad.), *Fusarium* (Fus.), *Penicillium* (Pen.), and *Rhizopus* (Rhiz.), WC = unidentified white cone-shaped colony, WW = unidentified white woolly colony, ✓ = positive identification.

Influence of harvest year/storage time on fungal profile

The fungal profile of Barakat cotton lint samples of two different grades from the Gezira region of Sudan varied with year of harvest. Although all contained *Aspergillus, Cladosporium* was not present in either grade from the year 2000 harvest and *Rhizopus* was not present in either sample from the 1998 harvest. *Penicillium*, WC and WW were only present in one sample each (Table 6.07).

		Fungal Genera					
Sample	**Harvest**	**Asp.**	**Clad.**	**Pen.**	**Rhiz.**	**WC**	**WW**
GBA 4B	1998	✓	✓				
	2000	✓		✓	✓		
	2003	✓	✓		✓		
GBA 6B	1998	✓	✓				✓
	2000	✓			✓	✓	
	2003	✓	✓		✓		

Table 6.07: Distribution of fungal genera identified in aqueous washes from cotton lint samples from Sudan. Displaying two sample grades (4B and 6B) of Gezira Barakat cotton (GBA) from three harvest years (1998, 2000 and 2003). Fungi are *Aspergillus* (Asp.), *Cladosporium* (Clad.), *Penicillium* (Pen.), and *Rhizopus* (Rhiz.), WC = unidentified white cone-shaped colony, WW = unidentified white woolly colony, ✓ = positive identification.

Geographic distribution of cotton fungi

The fungal genera *Aspergillus, Cladosporium, Rhizopus* and WW were present in samples spanning all three production regions. *Fusarium, Penicillium* and WC were absent from the samples grouped into Americas but present in African and Asian cotton, *Alternaria* had the most limited distribution as it was only isolated from Asian cotton samples (Table 6.08).

	Geographic Production Region					
	Asia		Africa		Americas	
Fungal genera	**13**	**TUR**	**13**	**SUD**	**USA**	**PAR**
Alternaria	✓	✓				
Aspergillus	✓[FNO]	✓[FN]	✓[FNO]	✓[FN]	✓[N]	✓[FN]
Cladosporium	✓	✓	✓	✓	✓	
Fusarium	✓	✓	✓	✓		
Penicillium	✓		✓	✓		
Rhizopus	✓	✓	✓	✓	✓	✓
WC	✓		✓	✓		
WW		✓		✓		✓

Table 6.08: The fungal genera identified in aqueous washes of cotton lint samples from different geographic production regions. Countries within Asia were CIS, China, Iran, Syria, Tajikistan and Turkey. Those from Africa were Benin, Ivory Coast, Zambia, Zimbabwe and Sudan and samples from the Americas were from the United States (USA) and Paraguay (PAR). ✓= positive identification, 13 = identified in at least one of the thirteen diverse samples from 2001/2002 production season, TUR = identified in at least one of the four Turkish samples from 2002/2003 production season, SUD = identified in at least one of the ten samples from Sudan. WC = unidentified white cone-shaped colony, WW = unidentified white woolly colony. [F]denotes *A. flavus*, [N]denotes *A. niger*, [O]denotes unidentified *Aspergillus* species.

Identification of fungal cells on cotton lint and equivalent trash material

Comparison of fungi isolated from cotton lint and equivalent trash samples revealed that trash material contains higher numbers of isolates (an average of five compared to three on cotton lint). The most prevalent isolate also varied in a number of cases (Table 6.09).

	Fungal Genera/Species	
Sample	**Cotton lint**	**Cotton trash**
Bergama	***Aspergillus niger*** / *Cladosporium* / *Rhizopus*	*A. niger* / *Cladosporium* *Penicillium* / ***Rhizopus***
Efes 1	*Aspergillus flavus* ***A. niger*** / *Fusarium*	*A. flavus* / ***A. niger*** / *Cladosporium* *Fusarium* / *Penicillium* / *Rhizopus* / WC
Efes 2	*Alternaria* / ***A. niger*** *Fusarium* / WW	*Alternaria* / *A. flavus* / *A. niger* ***Cladosporium*** / WW
Selçuk	*A. flavus* / ***A. niger*** *Fusarium*	*Alternaria* / ***A. flavus*** / *A. niger* *Cladosporium* / *Fusarium* / *Rhizopus*
China 146	***Cladosporium*** *Penicillium* / WC	***A. niger*** / *Cladosporium* *Rhizopus* / WC
Iran	*Alternaria* / ***A. niger***	*Alternaria* / *A. flavus* / ***A. niger*** *Cladosporium* / *Rhizopus*
Turkey	*A. flavus* / ***A. niger*** *Rhizopus*	*Alternaria* / ***A. niger*** *Penicillium* / *Rhizopus* / WC
Mean isolates	3	5

Table 6.09: The fungal taxa identified in aqueous washes of cotton lint and equivalent trash samples from four samples from regions of Turkey (2002/2003 production season) and from three other samples (2001/2002 production season), WC = unidentified white cone-shaped colony, WW = unidentified woolly colony, most prevalent displayed in bold font. Mean number of isolates from each material is also displayed.

Analysis of the total fungi identified on the seven cotton lint samples from which trash was available, compared to those identified on the corresponding trash, revealed that all the fungi identified were present on both lint and trash (Table 6.10).

	Cotton Material	
Fungal genera	**Lint**	**Trash**
Alternaria	✓	✓
Aspergillus	✓ FN	✓ FN
Cladosporium	✓	✓
Fusarium	✓	✓
Penicillium	✓	✓
Rhizopus	✓	✓
WC	✓	✓
WW	✓	✓

Table 6.10: Fungal genera identified in aqueous washes from cotton lint from the seven cotton lint samples from which trash was available (samples were China 146, Iran and Turkey from 2001/2002 production season and four samples from different regions of Turkey from 2002/2003 production season), ✓=positive identification, WC = unidentified white cone-shaped colony, WW = unidentified white woolly colony, [F]denotes *Aspergillus flavus*, [N]denotes *Aspergillus niger*.

Fungal profile of seed cotton

Two different samples of raw seed cotton from Eritrea were analysed. The Gedera 5A sample yielded two fungal isolates and the SJ2G sample contained three isolates. Although the most prevalent species on both samples was *Cladosporium*, the other genera varied between samples (Table 6.11).

Cotton Variety	Fungal Genera/Species
Eritrea Gedera 5A	***Cladosporium*** */ Penicillium*
Eritrea SJ2G	*Aspergillus niger* / ***Cladosporium*** */ Fusarium*

Table 6.11: The fungal species identified in aqueous washes of two different types of seed cotton from Eritrea (2001/2002 production season), most prevalent colony displayed in bold font.

6.4 DISCUSSION

Method appraisal

In order to identify the main fungal species contaminating various cotton samples, traditional microbiological techniques were employed. These are convenient and inexpensive and were used here with success. Fungal species are naturally very distinctive, even at the macroscopic level, exhibiting a wide range of colony shapes, sizes and colours (Figure 6.04).

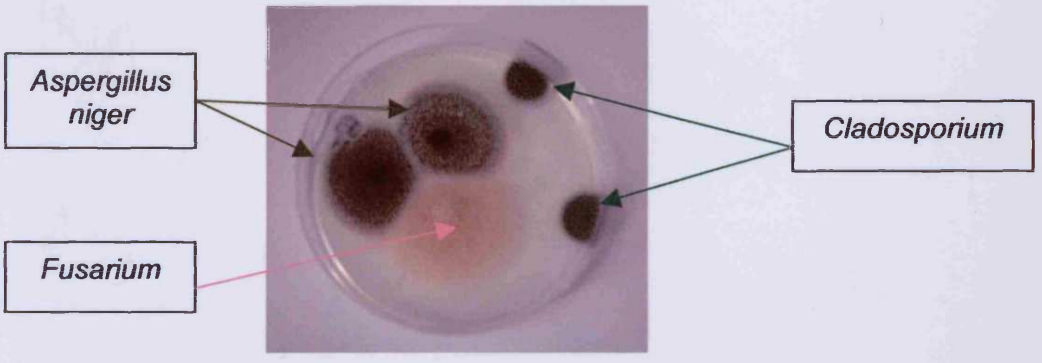


Figure 6.04: An agar plate exhibiting growth of three different fungal genera, *Aspergillus*, *Cladosporium* and *Fusarium*, following four days incubation.

Furthermore, under the microscope, the conidial ontogeny was distinctive, at least to genus level and these cells were reliably identified. In cases such as *Cladosporium*, where spores were connected by fragile chains, tape lifts only revealed indistinguishable loose spores and a network of hyphae. Hence, slide cultures were used to observe the undisturbed structures while they were growing; this proved very successful. A further minor problem encountered was the prolific growth of *Rhizopus*, which overgrew and inhibited other species, often filling the petri dish after only a few days

incubation, making the identification of other colonies difficult (although rendering the identification of *Rhizopus* very simple) (Figure 6. 05 below). To overcome this problem, samples containing *Rhizopus* were diluted by 10^{-2} to retard growth, and agar plates were then checked every day, colonies were 'picked off', then subcultured as soon as growth appeared, before the *Rhizopus* could become established.

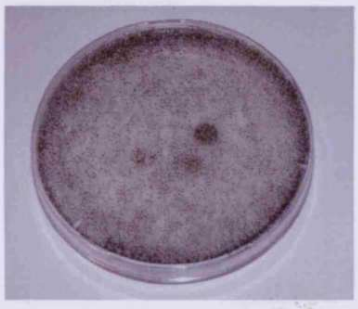


Figure 6.05: *Rhizopus spp.*, after four days incubation, illustrating prolific growth, which has led to dominance of the petri dish obscuring other colonies and hindering identification.

The aim of this study was not to identify every fungal genera/species present on cotton fibres; this would have required the use of a variety of nutrient media and different growth temperatures, as well as examination of cultures at different stages of their life cycle. The purpose of the study was to investigate the fungi, which could be simply isolated from a range of cotton samples for comparison. Identification procedures were based on relation of isolates to taxonomic reference material initially regarding 12 common cotton fungal genera, although this was extended in an attempt to identify any further colonies that developed. Two isolates were not successfully identified; these were a white cone-shaped colony and a white colony with a woolly appearance. Since microscopic examination of these colonies did not reveal

distinctive spores or other cells, it is possible these were asporous lifecycles of particular fungi and identification would only be possible under different growth conditions.

Although identification to genus level was straightforward in most cases, the more subtle variations of species were harder to observe reliably; hence, the majority of isolates in the study were only identified to genus level. One exception was *Aspergillus*. In this case, species often exhibit different spore pigments and hence colony colour. Coupled with distinctive microscopic spore arrangements, two species of *Aspergillus* were identified, namely *A. niger* (black colonies) and *A. flavus* (green colonies) (see Figure 6.06). At least one further species with an orange colony was also isolated though it could not be identified reliably (several similar species can exhibit this colouring). This could have been *A. ochraceus* or *A. terreus*, both of which have been previously identified on cotton fibres (Kaese, *et al*, [WWW] 2003).

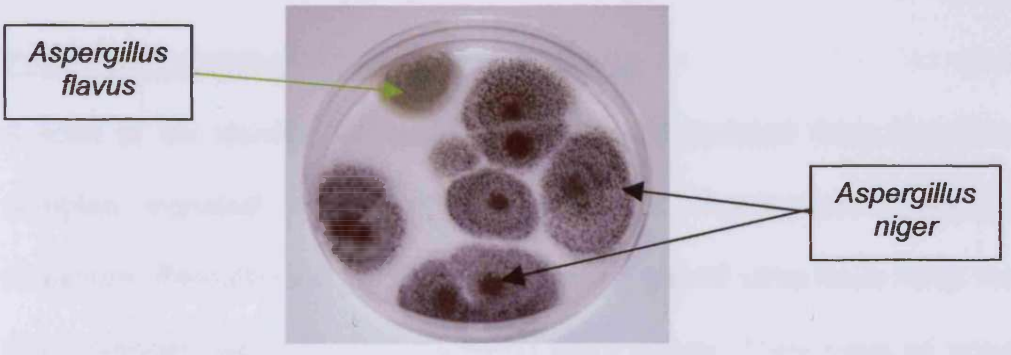


Figure 6.06: An agar plate exhibiting *Aspergillus niger* and *Aspergillus flavus* colony growth following 4 days incubation

Figure 6.06 also highlights how the most prevalent isolate was found; in this example it is clearly *Aspergillus niger* that was present in the highest

numbers. Supernatant from four separate cotton sub-samples were incubated and the isolate consistently constituting the highest number of colonies was deemed the most prevalent.

It is also possible to speculate on the species of other genera identified in the study based on their appearance, the likelihood of their growth under the conditions used and whether they have been reported on cotton previously (for example *Alternaria alternata*, *Rhizopus stolonifer*, *Cladosporium sporioides* and *Cladosporium herbarum* are all contenders for the colonies isolated in this study). However, these could not be identified with confidence and other genera such as *Fusarium* are difficult to identify to species level, due to variability between isolates and the poor development of features required for identification. In light of this, the following discussion will focus on the fungal genera involved in the study, but will also include reference to particular species where relevant.

Profile and distribution of cotton lint fungi

A total of six identifiable fungal genera were isolated from the 29 cotton samples included in the study (*Alternaria, Aspergillus, Cladosporium, Fusarium, Penicillium* and *Rhizopus*). These are all ubiquitous fungi found in soil, compost, leaf litter and decaying plant debris. They have all previously been isolated from cotton fibres (Kaese, *et al.*, [WWW] 2003; Piecková and Jesenská, 1996) and with the exception of *Alternaria* have been found to dominate airborne dust inside textile plants processing cotton (Su, *et al.*, 2002). These particular fungi have also been identified in the air of various

other occupational environments, including rice and grain mills, the wood processing industry and swine containment units (Desai and Ghosh, 2003a; Desai and Ghosh, 2003b; Fischer and Dott, 2003; Lugauskas, 2004). In the current study, *Aspergillus* was the most common genus, isolated from over 90% of cotton samples from diverse countries and 100% of Turkish and Sudanese samples (see Figures 6.01, 6.03 and Table 6.04), with *Aspergillus niger* the most frequently identified species (see Figures 6.02 and 6.04).

However, although *Aspergillus* was consistently the most prevalent genus (i.e. was found in the highest numbers) in the majority of samples from Turkey and Sudan (Tables 6.04 and 6.05), *Cladosporium* and *Fusarium* were the most prevalent species in a number of samples from other countries (Table 6.03), indicating that the dominant fungus varies with production region. This idea extends further on closer examination, as the dominant isolate from the four African cotton samples was *Fusarium* and that from the two Chinese samples was *Cladosporium*. Whereas, *Aspergillus flavus* was only present in the highest numbers on the cotton sample from Paraguay, the lone sample from South America. Continuing this theme, *Aspergillus niger* was cultured in the highest numbers from all four Turkish cotton samples, and all the Sudanese samples grown in the Gezira region, whereas, *Cladosporium* was the dominant genus on the sample from the Rahad region (Tables 6.03, 6.04 and 6.05).

Detailed investigations which enumerate specific cotton fibre fungi from particular regions have not previously been carried out, however, *Aspergillus*

and *Cladosporium* have been shown to be present in high numbers in the air of textile mills processing cotton in Taiwan, especially at the blowing and carding stages, which process raw cotton lint (Su, *et al.*, 2002). A detailed study analysing fungi present on various North American cotton plant tissues such as roots and bolls (but not cotton fibre) at various stages of plant growth recorded *Alternaria alternata* as the most common species present (Palmateer, *et al.*, 2004). However, *Alternaria* was only isolated from a small percentage of samples from the current study, not including the sample from USA, which demonstrated *Cladosporium* as the most prevalent genus.

Aspergillus, *Cladosporium* and *Rhizopus* were the most widely distributed genera in the current study, since these were identified in cotton samples from across the world (see Table 6.08). A previous report (Kaese, *et al.*, [WWW] 2003), which identified fungi from cotton fibres of different origins, also found *Aspergillus* to be widely distributed geographically (Table 6.12 below), with *A. niger* the most widely distributed species. Although results from Kaese, *et al.*, involved different cotton sample origins, a further similarity was that *Alternaria* was present in a low number of samples. However, *Cladosporium* and *Rhizopus* were not as widely distributed as in the current study and no other parallels can be drawn between the results of the two investigations. It is noteworthy that Kaese, *et al.*, isolated several fungal genera not identified in the current study, including *Eurotium* species which were present in a number of samples, as well as *Chrysosporium, Microascus, Phoma* and *Scopulariopsis*, which were only isolated from a single sample in each case (Kaese, *et al.*, [WWW] 2003).

	AUS	USA	C. America		Eur	Asia		Africa	
Fungi	**AUS**	**USA**	**MEX**	**NIC**	**GRE**	**PAK**	**IND**	**BF**	**SEN**
Alt. Macrospora				✓					
Aspergillus flavus	✓					✓	✓		✓
Aspergillus niger	✓			✓		✓	✓	✓	
Aspergillus spp.	✓	✓		✓	✓	✓	✓	✓	✓
Cl. cladosporioides								✓	✓
Fusarium spp.	✓						✓		
Penicillium spp.		✓	✓						
Rhizopus stolonifer					✓			✓	

Table 6.12: The geographic distribution of fungal taxa from cotton fibre samples grown in different countries. AUS = Australia, MEX = Mexico, GRE = Greece, PAK = Pakistan, IND = India, BF = Burkina Faso, NIC = Nicaragua, SEN = Senegal, Eur = Europe, C. America = Central America. Adapted from Kaese, *et al.*, [WWW] 2003.

Another study, which analysed fungi on cotton mainly from Uzbekistan but also involved a small number of samples from USA, China, Egypt and Russia, (but did not report findings on the cotton from different origins separately), found *Penicillium* to be the most common genus (Piecková and Jesenská, 1996). *Aspergillus*, and *Cladosporium* were also present in a high percentage of samples in this study (Table 6.13 below). Samples in this report also isolated a number of other *Aspergillus* species, *Mucor* spp., and small numbers of *Nigrospora* spp., these fungi were not identified in the samples analysed in the current study.

Fungal Taxa	% Positive samples
Penicillium	84.4
Aspergillus flavus	71.1
Cladosporium	71.1
Aspergillus niger	68.9
Alternaria	46.7
Rhizopus	26.7
Fusarium	11.1

Table 6.13: The fungi isolated from cotton fibre samples from a number of countries and the percentage of samples from which they were identified. Adpted from Piecková and Jesenská, 1996).

The number of fungal genera isolated in this thesis was limited; this could be a reflection of the methods used to isolate the fungi, which may have lead to dominance of certain fungi that inhibit the growth of other genera. An exhaustive two-year study, on American Upland cotton fungi associated with all cotton plant materials such as roots, leaves, stems, and bolls (Palmateer, *et al.*, 2004), found over 30 fungal genera. It also reported that 67% of the fungi identified in this study was made up of only eight different species, including those from the genera, *Alternaria, Fusarium,* and *Rhizopus*. Hence, it would seem that, similar to Gram-negative cotton bacteria, there are large numbers of a restricted variety of fungal taxa present on cotton from all over the world.

Health implications of cotton fungi

The presence of fungal growth in indoor environments may affect human health in several ways. 1). A small number of fungi are capable of causing human infection, although these are mainly opportunistic pathogens. 2). The

spores of many filamentous fungi are capable of initiating allergic responses upon inhalation, causing conditions such as allergic rhinitis. 3). Some fungi or fungal particles cause inflammatory (immunotoxic) reactions upon inhalation, leading to conditions such as organic dust toxic syndrome and byssinosis. 4). Many fungi produce toxic compounds called mycotoxins, which have potentially severe effects. Table 6.14 below summarises the allergic, inflammatory effects and toxin production of fungi isolated in this study.

Genus	Pathogenicity	Allergies	Toxins
Alt.	PO, HO Nail infections, nasal lesions	Type I: i.e. hay fever & asthma, Type III: HP e.g. wood worker's lung	Alternariol, altenuene, tenuazonic acid
Asp.	HO; invasive lung disease, ear & corneal infections	Type I. Type III: HP e.g. malt worker's and farmer's lung. Allergic bronchopulmonary aspergillosis (APBA)	*A. flavus*; aflatoxin B1 & B2, kojic acid. *A. niger*, malformin C & oxalic acid
Clad.	PO, rare human pathogen	Type I: i.e. hay fever & asthma. Type III: HP e.g. mouldy wall hypersensitivity	Cladosporin, emodin (neither highly toxic)
Fus.	PP or PS, HO; keratitis,	Type I: Hay fever & asthma	Trichothecenes, zearalenone, fumonisin
Pen.	Very rarely pathogenic	Type I. Type III: HP e.g. cheese washer's lung, and Suberosis	Numerous e.g. citrinin, patulin penicillic acid, ochratoxin, VOCs
Rhiz.	PP, HO; Zygomycosis	Type I: Hay fever & asthma. Type III: HP e.g sawmill lung	None known

Table 6.14: Fungal genera identified in the study, their general effects and potential toxicity. Genera are *Alternaria*, *Aspergillus*, *Cladosporium*, *Fusarium*, *Pencillium*, and *Rhizopus*, HO = human opportunist. PO = plant opportunist, PP = plant parasite, PS = plant saprophyte, HP = Hypersensitivity pneumonitis. Derived from Environmental Microbiology Laboratory, [WWW] 2004.

Fungal Infections

Few filamentous fungi are human pathogens because they grow poorly at temperatures as high as 37°C. Those that are capable of infecting people are opportunistic pathogens and only attack those individuals with severely weakened immune systems. For example, *Rhizopus* is the principle cause of zygomycosis, which occurs in patients suffering from diabetic ketoacidosis, malnutrition, severe burns, or those who are otherwise immunocompromised. Invasive aspergillosis also occurs in individuals with low immunity, for example following bone marrow transplants, or in AIDS patients (Environmental Microbiology Laboratory, [WWW] 2004). It has recently been stated that "there are no reports in the literature indicating that high fungal spore counts of pathogens increase the risk for infections of workers with normal immune status" (Fischer and Dott, 2003). As the current study is primarily concerned with general working populations, it is assumed that these are people with normally functioning immune systems and such infections will not be further discussed here.

Allergy/inflammation

The spores of fungi are often less than 5 µm aerodynamic diameter, and are hence able to enter the lungs via inhalation (Sorenson, 1999). Inhalation of spores from the fungi isolated in this study are able to elicit type I allergic responses, such as hay fever and symptoms of asthma. Furthermore, these fungal cells or fragments thereof, are causative factors in a number of occupational lung conditions, including allergic alveolitis (HP) diseases such

as farmer's lung, mushroom picker's lung and sawmill lung, and have been implicated in organic dust toxic syndrome and byssinosis (Krysińska-Traczyk, *et al.*, 2001; Lugauskas, *et al.*, 2004; Nordness, *et al.*, 2003; Sorenson, 1999). One recent report suggested that *Aspergillus* was the most likely cause of hypersensitivity pneumonitis in corn mill workers (Moreno-Ancillol, *et al.*, 2004), which is notable because this was the most prevalent genus found in the current work. Inhalation of *Aspergillus* (mainly *Aspergillus fumigatus*) spores can also lead to specific lung diseases such as allergic bronchopulmonary aspergillosis (ABPA). This is common in asthmatics and is characterised by malaise, coughing and wheezing. If untreated, this disease can lead to permanent lung damage. A similar condition can arise in the sinuses called allergic fungal sinusitis (AFS), which also occurs in those with underlying allergic disease (ACOEM, 2003). A separate disease known as aspergilloma or chronic pulmonary aspergillosis may also ensue when the fungus grows within the lung cavity, usually following damage caused during an episode of tuberculosis. Incidences of these conditions, specifically relating to the presence of fungi in the cotton production environment have not been widely reported due to the lack of focus in this area. However, since fungal spores are capable of causing lung diseases in farmers, malt workers, saw mill workers, mushroom pickers and a plethora of other industries (Fischer and Dott, 2003), the risk to cotton workers is likely to be significant.

(1-3)-β-D-glucans, polyglucose compounds with a structural role in the fungal cell wall have also been linked with chronic pulmonary disease (Piecková and Jesenská, 1999; Rylander and Lin, 2000; Young, *et al.*, 1998). It has

been suggested that levels of indoor airborne glucan may vary with fungal species, for example 10^6 spores of *Stachybotrys* (a toxic mould associated with damp buildings), has significantly higher mean levels of glucan (approximately 13,000ng/10^6 spores) than *Penicillium* and *Aspergillus* (around 350ng/10^6 spores each) (Fogelmark and Rylander, 1997). Hence, the presence of a range of fungal cells on cotton fibres has serious implications for the occupational health of industrial workers.

Mycotoxins

The highest potential respiratory threat in terms of chemical potency is posed by mycotoxins, secondary fungal metabolites of low molecular weight. These are not volatile and respiratory exposure takes place via inhalation of mould spores, fragments and contaminated dusts (Bennett and Klich, 2003). Upon inhalation, spores are lysed, releasing primary and secondary metabolites (Fischer and Dott, 2003). However, these toxins are only produced under very specific conditions, which are influenced by the age of the colony, growth substrate, moisture levels, temperature and competition from other micro-organisms (ACOEM, 2003), therefore, it is possible either that these toxins are rarely released in the occupational setting, or that they are liberated in such small quantities that health risks are minimal. Although there have been numerous studies on the effects of ingestion of fungal metabolites, little is currently known regarding the effects of these compounds upon inhalation (Desai and Ghosh, 2003a). However, it is known that mycotoxins have increased potency when delivered via the respiratory

tract compared to the alimentary canal, the dose required to trigger toxic effects being ten times less when inhaled (Hendry and Cole, 1993).

In light of this and due to their possible presence in the cotton production environment, it is worth considering the dangers posed by toxins produced by the fungi isolated in this study. The general effects of the most common mycotoxins produced by *Aspergillus*, *Fusarium* and *Penicillium* are shown in Table 6.15 below.

Fungus	Mycotoxin	Common sources	Chronic effects
A. flavus	Aflatoxin B1 & B2	Cereals, figs, nuts, oilseed, tobacco, other commodities	Liver cancer, immune suppression (HC) lung neoplasms (HI)
A. niger	Malformin C	Wheat, rice, grain	Changes in liver and kidney (AC)
	Ochratoxin A (also produced by *Penicillium*)	Barley, oats, wheat, coffee, rye, beans, plant products	Nephrotoxin, carcinogen (A/HC), liver toxin, immune suppressant, teratogen (AC)
Fusarium	Fumonisins	Corn	Oesophageal cancer (HC) Neural tube defects (AC)
	Trichothecenes	Food crops, mouldy hay, safflower seeds, mixed feeds	Inhibit protein synthesis, range of gastrointestinal & neurological symptoms (A/HC)
	Zearalenone	Cereal crops	A mycoestrogen, causes reproductive problems (A/HC), low toxicity
Penicillium	Citrinin (also produced by *Aspergillus*)	Wheat, oats, rye, corn, barley and rice	Acts as a nephrotoxin, also causes vasodilation and bronchiol constriction (A/HC)

Table 6.15: The common mycotoxins produced from cotton fungi, examples of sources and recorded effects, A = animals, C = upon consumption, H = humans, I = upon inhalation. Derived from Bennett and Klich, 2003.

Although the effects of many mycotoxins have only been examined following consumption, a study evaluating fungi on cotton from several countries reported 73.2% of *Aspergillus flavus* strains were aflatoxin producing (Piecková and Jesenská, 1996). This is a much larger percentage than that reported in Indian rice mills (8%) and maize processing buildings (10%) (Desai and Ghosh, 2003a and 2003b respectively). Hence, it is possible that at least a small number of fungal isolates in the present study were aflatoxin postive, especially as the cotton sample from Paraguay had high levels of *A. flavus* colonies. In addition to this, a variety of other mycotoxins produced by *Aspergillus, Fusarium* and *Penicillium* have been measured in occupational grain dusts (Krysińska-Traczyk, *et al.*, 2001) and a significant correlation has been shown between the occurrence of *Fusarium* species and the concentration of mycotoxins in such dusts (Krysińska-Traczyk, *et al.*, 2003). There are more than 300 known mycotoxins, nearly all of which are cytotoxic and interfere with vital processes such as DNA, RNA and protein synthesis (Bünger, *et al.*, 2004). There is virtually no treatment for mycotoxin exposure, and although threshold levels in food products have been introduced for several of these compounds to limit intake via this route (Bennett and Klich, 2003), further investigations are urgently required to assess the risk posed by occupational exposures.

Volatile Organic Compounds (VOCs)

A number of fungi also produce volatile organic compounds, such as alcohols, aldehydes, esthers and ketones. These are responsible for the

musty odours associated with fungal colonies. VOCs have been shown to produce irritation to the eyes and respiratory tract as well as inflammatory reactions in humans (Fischer and Dott, 2003). Although usually present in low concentrations in many environments, it is likely that levels are significantly higher in the industrial environment. Indeed, strong musty odours were detected from a number of cotton samples during the project.

Factors influencing the fungal profile of cotton lint

This is the first time fungal identification on a number of fibre samples from within one country has been performed. Analysis of the fungal colonies isolated from ten Sudanese cotton samples (Table 6.06), revealed that although the majority of genera were present on both Barakat and Acala cotton, *Fusarium* was not isolated from Barakat samples and *Penicillium*, as well as the unidentified white woolly colony were absent from Acala cotton, perhaps indicating that these different cotton species support slightly different fungi, possibly due to their different physical structures. Acala cotton (*Gossypium hirsutum*) consists of shorter, bushy fibres compared to the long smooth fibres of Barakat cotton (*G. barbadense*) (Khalifa, 2001). Different cotton grades also revealed a number of differences in the fungi isolated. Although *Aspergillus* and *Cladosporium* were found on all five grades, *Fusarium* and *Penicillium* were only isolated from one grade each. However, since these samples also varied in harvest year, it is more difficult to identify specific patterns. The two 3SG samples from 2002, originated from different production regions and did not exhibit any difference in contaminating fungal genera, although the dominant genus was different in the cotton sample from the Rahad region as discussed previously.

The fungal profile of the two Gezira Barakat cotton grades harvested in three different years also varied (Table 6.07), *Aspergillus* was again present in all samples, this is known as a storage fungi. *Rhizopus*, another storage fungi was surprisingly not present on either sample stored since 1998 (i.e. the longest period in storage), but was found on both the more recent samples from 2000 and 2003. In comparison, the field fungus *Cladosporium* was present on both samples from 1998 and 2003 but absent in both cases from the year 2000 samples. The storage fungus *Penicillium* was only present on one sample (from 2000). It is noteworthy that two further common field fungi, *Alternaria* and *Fusarium*, isolated elsewhere in the study, were not present on any of these samples. Storage fungi are more tolerant to higher temperatures (30°C) and decompose stored organic products under specific conditions such as 20% water content and water activity of above 0.90 a_w, they can be isolated anytime after harvest (Dutkiewicz, 1997). Although three of the genera isolated on these samples were storage fungi, compared to only one classed as a field fungus, the years in storage did not seem to define the variety of fungi present. As *Cladosporium* was isolated in samples stored since 1998, it also seems resistant to the conditions that occur during storage. Importantly, cotton stored for several years as well as for only one year contained various filamentous fungi capable of inducing occupational lung conditions during processing. The presence of both typical outdoor and indoor fungal genera on cotton fibres also highlights the varied nature of the contamination in this environment. Consequently, fungal contamination of cotton requires further research, in order to fully assess potential health risks.

Fungal profile of cotton trash and seed cotton

Comparison of fungi isolated from cotton lint and equivalent trash samples revealed variation between these two materials, and in several cases the predominant fungi also differed (Table 6.09). The trash material contained a higher average number of isolates, perhaps due to the availability of nutrients on this medium. However, despite these differences, the range of fungal genera found on trash material was the same as that found on the matching lint (Table 6.10). This further emphasises the limited number of different cotton contaminating fungal taxa. In addition, although the fungi identified on the two seed cotton samples varied, the fungi identified here were also the same varieties of fungi previously identified on cotton lint (Table 6.11). The predominant genus on both samples was *Cladosporium*, again reflecting the pattern described earlier, where the most prevalent fungus varies with production region. It will be interesting to investigate whether cotton trash also supports a higher number of fungal cells than cotton lint as well as a more varied population and this will be the research focus in the following chapter.

Summary

A limited variety of cotton fungal genera were isolated in this study. These consisted of eight morphologically different colonies, isolated from cotton lint, seed cotton and trash samples. Six of these colonies were successfully identified as common filamentous fungi, all of which had previously been isolated from cotton fibres and other occupational environments. *Aspergillus*

was the most commonly isolated genus, *Cladosporium* and *Fusarium* were also prevalent. A correlation between the most dominant fungal genus and the geographical origin of samples was indicated. *Alternaria* was the least widely distributed genus, compared to *Aspergillus*, *Cladosporium* and *Rhizopus*, which were present in samples from a range of different countries. The fungi from various Sudanese cotton samples mainly consisted of storage fungi, and variation between contamination of different cotton species and grades was revealed. Although the fungal taxa found on cotton trash and corresponding lint were generally identical, the mean number of isolates was higher on the trash material. Health implications of the contaminating fungi were discussed, with reference to allergies caused by inhalation of fungal spores and potential effects of mycotoxins. Finally, the need for further research into this area was highlighted.

CHAPTER 7

ENUMERATION OF COTTON FUNGI

7.1 INTRODUCTION

Plant material is an ideal substrate for the proliferation of fungal colonies. Many common plant fungi are soil-borne saprophytes, which survive on plant debris. If this decaying material is not cleared away at the end of a crop season, the organisms remain in the environment ready to colonise the following season's growing plants. There are a number of cotton plant diseases caused by fungal parasites, such as boll rot, wilts and leaf spots (Brock, 2000). The organisms responsible for these diseases are specifically adapted to colonise living plant material, and hence can be expected in high numbers on cotton plants, along with a range of symbiotic fungi. Therefore, these organisms are available to contaminate cotton fibres while they are exposed to field weathering prior to harvest. As with bacteria levels, it is likely to be the local environmental conditions such as humidity levels during weathering, which ultimately determine the numbers of fungal cells present on the cotton fibres (Ono, *et al.*, 1999; Peraica, *et al.*, 1999).

Previously, there has been little focus on the fungal contamination of cotton fibres, especially compared to the number of studies investigating bacteria and endotoxin levels. Indeed, examination of fungal contamination in raw organic materials generally, has been very limited, and data are only readily available on those shown in Table 7.01.

Material	Fungal count CFU/g	Location	Reference
Corn	$5.5 \times 10^3 - 5.2 \times 10^6$	Brazil	Ono, *et al.*, 1999
Cotton	3.3×10^5	Slovakia	Piecková & Jesenská, 1996
Flax	12.2×10^6	Slovakia	Piecková & Jesenská, 1996
Grain	$5 - 520 \times 10^3$	Poland	Krysińska-Traczyk, 2003

Table 7.01: Fungal cell counts on different industrial organic materials

However, several studies enumerating fungal cells in dusts created in a number of industrial environments have been carried out, and these include saw mills (Oppliger, *et al.*, 2005), rice mills (Desai and Ghosh, 2003a), and grain farms (Krysińska-Traczyk, 2003). A detailed study has also been carried out in a textile plant processing cotton, measuring airborne fungal cells at different processing stages (Table 7.02).

Processing stage	CFU/m^3
Blowing	7950
Carding	3709
Combing	3901
Drawing	3386
Roving	2581
Spinning	1562
Winding	799

Table 7.02: Fungal cell counts in airborne dust at progressive stages of processing, in textile mills handling cotton. Adapted from Su, *et al.*, 2002.

These data show that fungal cells are present on cotton fibres and in the mill environment in high numbers, especially during the earlier stages of

processing when the raw cotton lint is being handled. The enumeration of fungal spores on cotton lint is therefore of interest as it has not been addressed in detail previously. Furthermore, levels may be correlated with the concentrations of fungal toxins and other potentially hazardous fungal-derived compounds such as β-glucans, which may also be released from cotton during processing (Rylander and Lin, 2000). It would also be desirable to assess whether there is a correlation between fungal and bacterial cell counts, since the combined presence of these toxicants has further health implications.

Aims

- To perform viable counts on total fungal cells extracted from a range of cotton samples.
- To compare viable counts from cotton originating in a number of different countries and within one country, in order to consider potential risk to respiratory health.
- To examine patterns in cell numbers and consider factors which may affect the levels present.
- To investigate any relationship that exists between cotton fungal and Gram-negative bacterial cell counts.

7.2 MATERIALS AND METHODS

7.2.1 Materials

Equipment

All equipment was obtained from reputable sources, and was sterile wherever possible. Full details are available in Appendix I.

Cotton/trash samples

See Chapter 2 for details.

7.2.2 Methods

Fungal cell extraction

Fungal cells were extracted from 0.6000g (±0.0005g) cotton lint into 10 ml phosphate buffered saline, by shaking on a vortex multi-mixer at 1000 rpm for 60 minutes in a centrifuge tube (techniques adapted from Krysińska-Traczyk, *et al.*, 2001 following preliminary tests). The cotton fibres were then physically submerged using a sterile pipette and the supernatant removed to a fresh tube. Due to the limited availability of trash, 0.3000g (±0.0005g) material was shaken, as before in 5ml phosphate buffered saline in the extraction procedure, all other aspects of extraction and culture remained the same as those applied to the lint.

Dilutions

Required dilutions were prepared using phosphate buffered saline by 1 in 10 stepwise dilutions in sterile glass test tubes to 10^{-1} or 10^{-2} as required. The dilution tubes were vortexed for 30 seconds between each transfer step.

Agar plates

Malt extract agar (MEA) was made up following manufacturers instructions (33.6g/litre) and autoclaved for 1 hour at 120°C. It was then allowed to cool to approximately 40°C, at which point the antibiotic chloramphenicol (30μg/ml) was added in order to prevent the growth of both Gram-positive and Gram-negative bacteria (techniques adapted from Chun and Perkins, 1991; Fisher and Sasser, 1987) (see Appendix II for antibiotic action). The agar was then mixed gently by inverting the bottle several times, before 25ml molten agar was poured into each petri dish and allowed to solidify. Plates to be used for spread plating were 'over dried' in a laminar flow unit for 30 minutes prior to inoculation to remove excess water in order to ensure discrete colonies.

Spread plating

The required supernatant dilution was vortexed for 30 seconds prior to plate inoculation. A 0.1ml aliquot of supernatant was inoculated onto the centre of an over dried agar plate and evenly spread across the surface with a sterile (flamed) glass spreader. Plates were prepared in triplicate and all plates were left to stand at room temperature for 30 minutes prior to incubation.

Incubation

All plates were inverted and incubated at 25°C (±2°C) for three days.

Viable counts

Plates with 20-200 colony-forming units (CFU) had every colony counted by eye, using a pen to mark each colony in turn. Plates with 200+ colonies were divided into quarters; every colony in two opposite quarters was counted, the average calculated and multiplied by four.

7.3 RESULTS: GRAPHICAL/TABULAR REPRESENTATION

The range of inter-country fungal cell counts

Cotton lint samples from diverse countries exhibited a range of fungal cell counts, the highest count was in the sample from Benin (9250 ± 820 CFU/g), followed by samples from Zambia (4528 ± 394 CFU/g), Syria (3014 ± 208 CFU/g), Turkey (2333 ± 436 CFU/g), Paraguay (2181 ± 387 CFU/g), Zimbabwe (2014 ± 301 CFU/g), Ivory Coast (1139 ± 198 CFU/g), China Xinjiang (542 ± 64 CFU/g), Iran (500 ± 89 CFU/g), USA (472 ± 88 CFU/g), Tajikistan (375 ± 80 CFU/g), China 146 (306 ± 93 CFU/g), and the lowest count was on the sample from CIS (281 ± 29 CFU/g) (Figure 7.01).

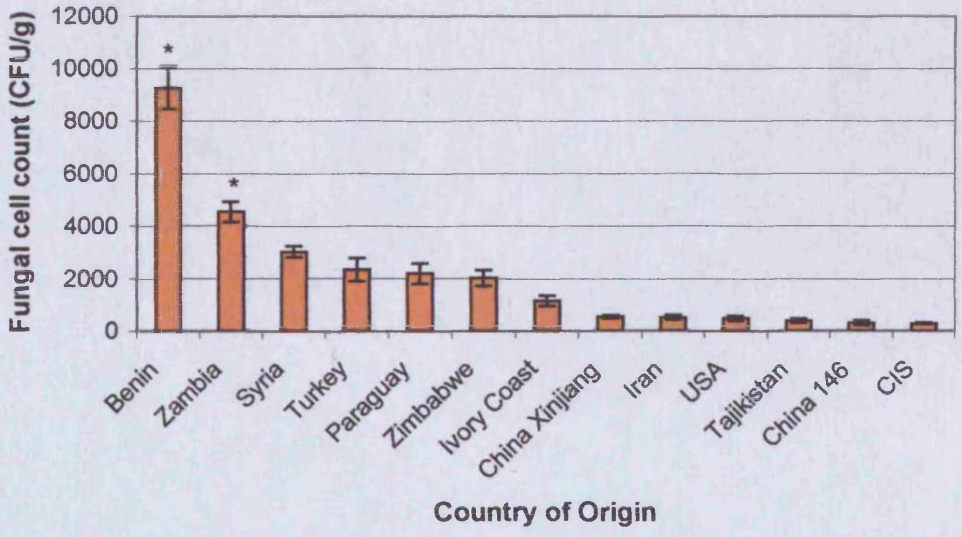


Figure 7.01: Total range of fungal cell counts (CFU/g), in aqueous washes from thirteen cotton lint samples from diverse countries of origin (2001/2002 production season). Results presented as mean viable count ± SEM (n=6). *Denotes statistically significant difference in fungal count compared to all other samples (P<0.05).

The influence of production region on fungal cell counts

Comparison of mean fungal cell counts on cotton samples from countries within Africa and Asia, revealed counts on African samples (4233 ± 668 CFU/g) were significantly higher than on those samples originating within Asia (1033 ± 67 CFU/g) ($P<0.05$) (Figure 7.02).

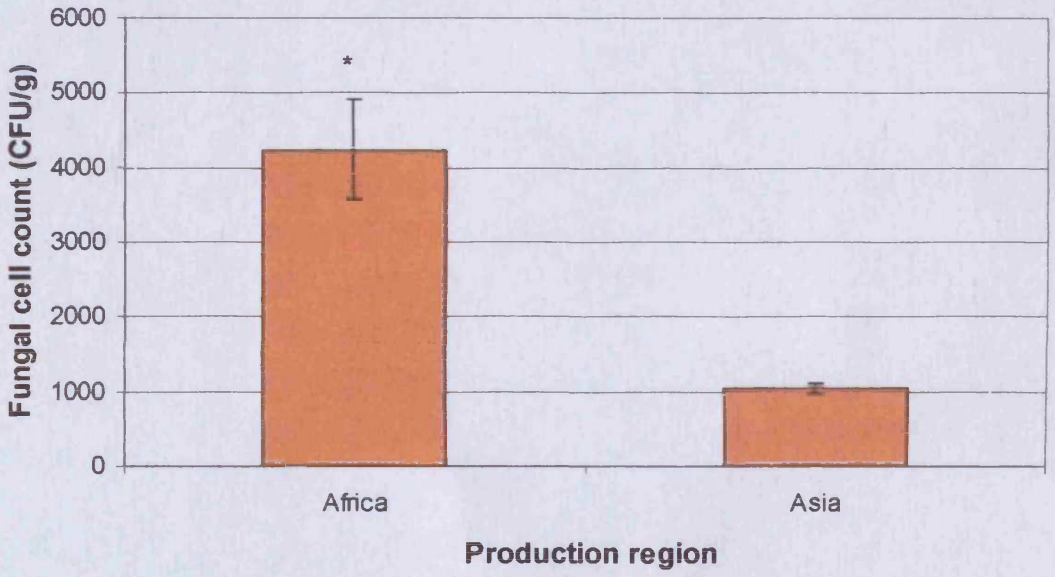


Figure 7.02: Fungal cell counts (CFU/g), in aqueous washes from cotton lint samples (2001/2002 production season), originating from two different cotton producing areas; Africa and Asia. African countries are Benin, Ivory Coast, Zambia and Zimbabwe (n=24). Asian countries are China, CIS, Iran, Syria, Tajikistan and Turkey (n=42). Results presented as mean viable count ± SEM. *Denotes statistically significant difference in mean fungal cell count compared to cotton samples from Asian countries ($P<0.05$).

Intra-country fungal cell enumeration; Turkey

The cotton lint samples from different production regions within Turkey possessed a range of fungal counts. The Efes 1 cotton sample had the highest count (6348 ± 1147 CFU/g), followed by the Efes 2 sample (1611 ± 360 CFU/g), the sample from Selçuk (1527 ± 293 CFU/g) and the sample from Bergama (833 ± 276 CFU/g), which all had similar counts (Figure 7.03).

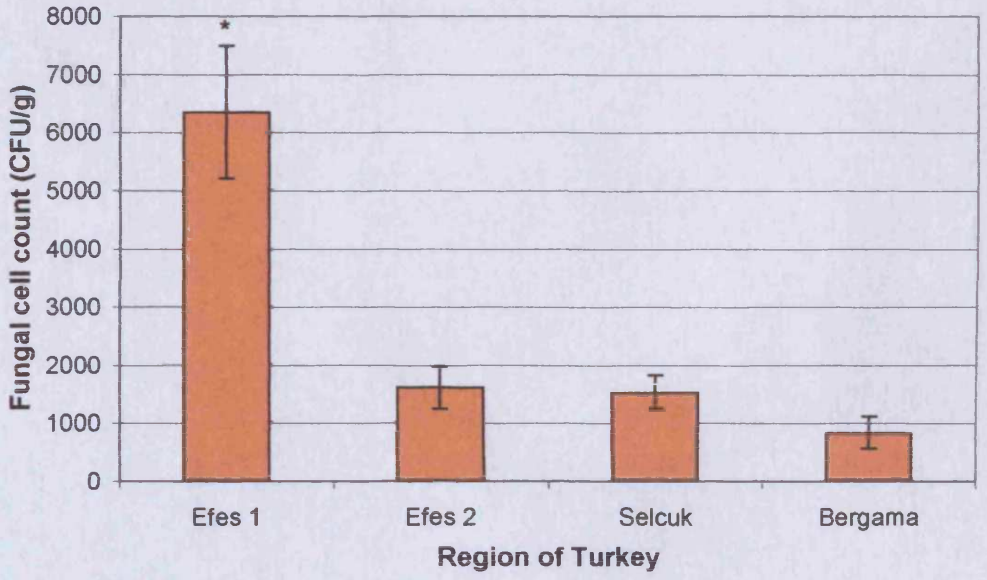


Figure 7.03: Fungal cell counts (CFU/g), in aqueous washes from cotton lint samples originating in different regions of Turkey (2002/2003 production season). Results presented as viable count ± SEM (n=6). *Denotes statistically significant difference in fungal cell count compared to other samples (P<0.05).

Intra-country fungal cell enumeration; Sudan

Cotton lint samples from within Sudan exhibited a range of fungal cell counts. Mean counts were highest on GAC/3SG/02 cotton (37361 ± 6574 CFU/g), decreasing through samples RAC/3SG/02 (28042 ± 4931 CFU/g), to GBA/4B/03 (8792 ± 882 CFU/g), GAC/4G/02 (3865 ± 566 CFU/g), GBA/6B/00 (3222 ± 934 CFU/g), GAC/3G/98 (1431 ± 236 CFU/g), GBA/4B/00 (931 ± 122 CFU/g), GBA/4B/98 (403 ± 148 CFU/g), GBA/6B/98 (333 ± 129 CFU/g), and GBA/6B/03 (292 ± 113 CFU/g) (Figure 7.04).

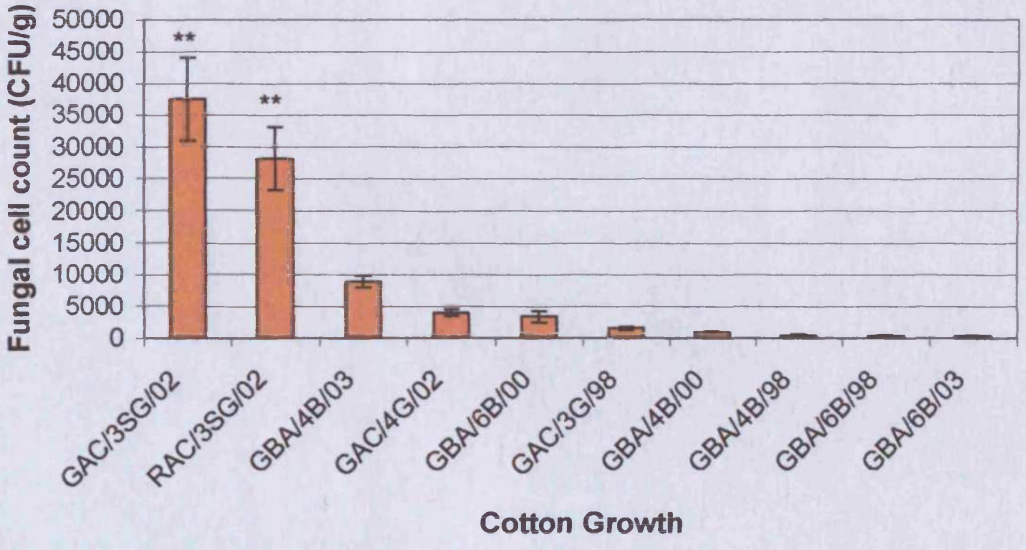


Figure 7.04: Total range of fungal cell counts (CFU/g), in aqueous washes from ten cotton lint samples from Sudan. Results presented as mean viable count ± SEM (n=6). Samples varied in production region, species and grade of cotton and harvest year. Production regions were Gezira (G) and Rahad (R), cotton species were Barakat (BA) and Acala (AC), grades were 4B, 6B, 3G, 3SG and 4G. Years of harvest were 1998, 2000, 2002, and 2003 (98/00/02/03 respectively). **Denotes statistically significant difference to other samples (but similarity to other **).

The influence of cotton species on fungal cell counts

Comparison of the mean fungal cell counts on different cotton species revealed a significant difference. Gezira Acala cotton had a significantly higher mean count (14219 ± 4481 CFU/g) than Gezira Barakat cotton (2329 ± 556 CFU/g) ($P<0.05$)(Figure 7.05).

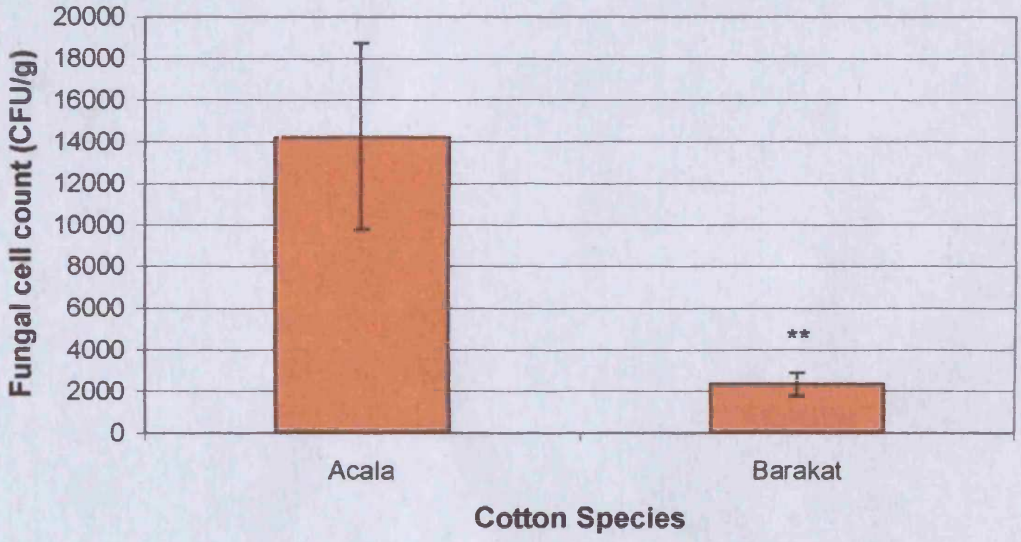


Figure 7.05: Mean fungal cell counts (CFU/g) in aqueous washes from various Sudanese cotton samples. Results presented as mean viable count ± SEM (n=18 (Acala) and n=36 (Barakat)). Samples are Gezira Barakat or Gezira Acala cotton species of several grades, from various harvest years. **Denotes statistically significant difference compared to Acala cotton ($P<0.01$).

The influence of cotton grade on fungal cell counts

The mean fungal count recorded on samples of Sudan Barakat 4B grade cotton (3375 ± 972 CFU/g), was not significantly different to Sudan Barakat grade 6B cotton (1282 ± 446 CFU/g) ($P>0.05$) (Figure 7.06).

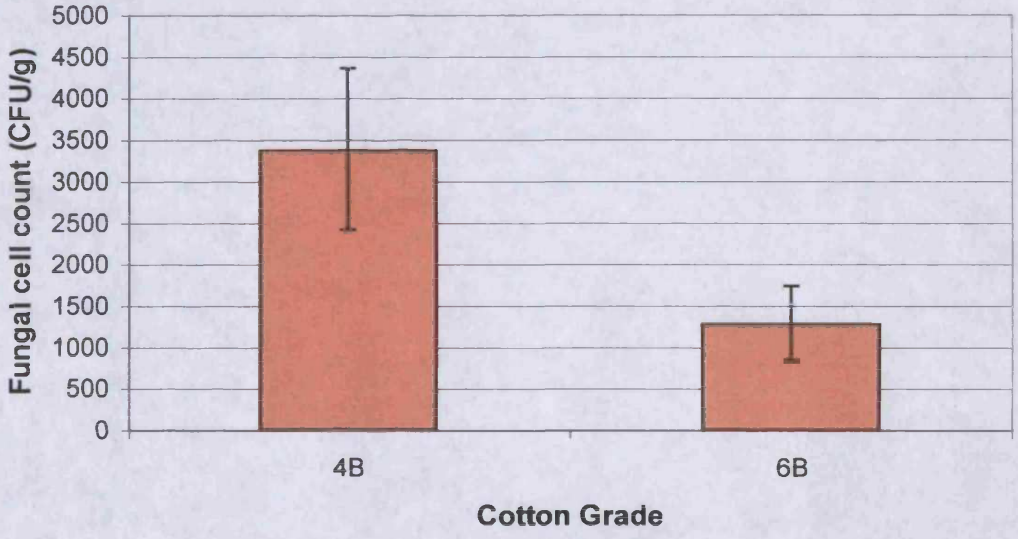


Figure 7.06: Mean fungal cell counts (CFU/g) in aqueous washes of cotton lint samples from within Sudan. Results presented as mean viable count ± SEM (n=18). Samples are Gezira Barakat cotton of two different grades (4B and 6B), each harvested from three different years: 1998, 2000 and 2003.

The possible effect of storage on fungal cell counts

Analysis of cotton samples harvested in three different years, revealed a trend between year of harvest and fungal count on the 4B cotton sample. Counts were lowest on 4B cotton from 1998 (403 ± 148 CFU/g); the sample from 2000 had higher counts than this (931 ± 122 CFU/g) and counts on 4B cotton harvested in 2003 increased further (8792 ± 882 CFU/g). However, although counts increased between 6B cotton harvested in 1998 (333 ± 129 CFU/g) and 2000 (3222 ± 934 CFU/g), 6B cotton from 2003 had lower counts (292 ± 113 CFU/g), and all counts were statistically similar (Figure 7.07).

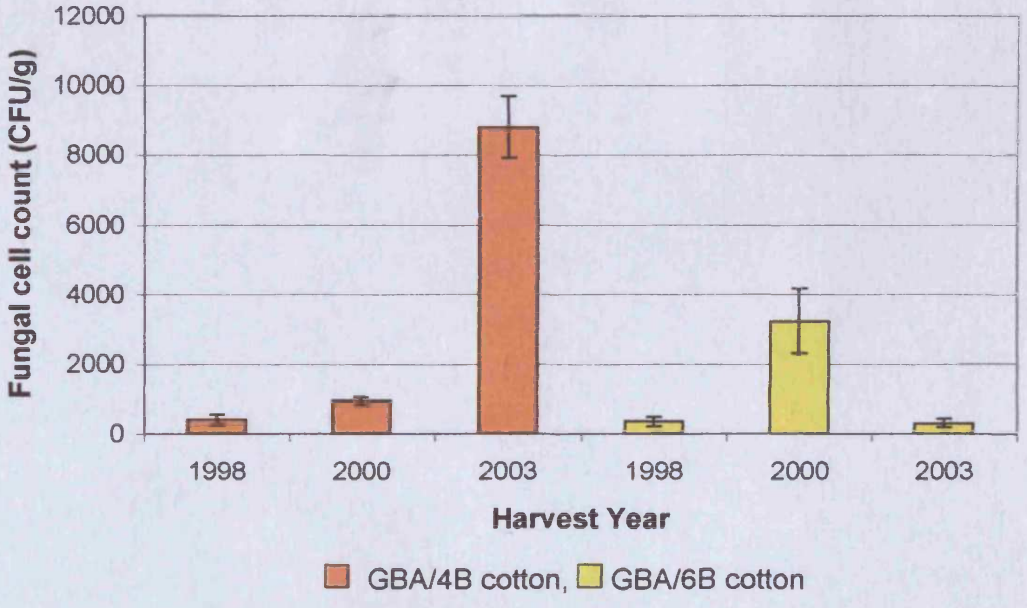


Figure 7.07: Fungal cell counts (CFU/g) in aqueous washes from Gezira Barakat (GBA) cotton samples of two different cotton grades (4B and 6B), produced in Sudan. Results presented as mean vible count ± SEM (n=6).

The fungal cell counts on cotton lint and equivalent trash samples

Fungal cell counts on four trash samples from Turkey (2002/2003 production season), revealed that this waste material harboured counts significantly higher than those on the corresponding cotton lint. Fungal counts on trash of various origins from the 2001/2002 production season was also consistently higher than on the associated lint, although these differences were not significant (Table 7. 03).

	Fungal cell counts (CFU/g)	
Sample Region	**Cotton lint**	**Cotton Trash**
Turkey 2002/03		
Bergama	833 ± 276	23217 ± 5019**
Efes 1	6348 ± 1147	29733 ± 10099*
Efes 2	1611 ± 360	60333 ± 20963*
Selçuk	1527 ± 293	7967 ± 3225*
Various 2001/02		
China 146	306 ± 93	608 ± 92
Iran	500 ± 89	917 ± 212
Turkey	2333 ± 436	3300 ± 1895

Table 7.03: Fungal cell counts (CFU/g) in aqueous washes from cotton lint samples and equivalent trash samples from different regions of Turkey (2002/2003 production season) and several samples from 2001/2002 production season. Results presented as mean viable count ± SEM (n=6). *Denotes statistically significant difference of trash fungal counts compared to those on lint (P<0.05), **denotes statistically significant difference of trash fungal counts compared to those on lint (P<0.01).

Fungal cell counts on seed cotton

Fungal cell counts on two samples of seed cotton from Eritrea were statistically similar, cotton varieties were Gedera 5A (127 ± 86 CFU/g) and SJ2G (183 ±127 CFU/g). Standard errors were high on this type of cotton (Figure 7.08).

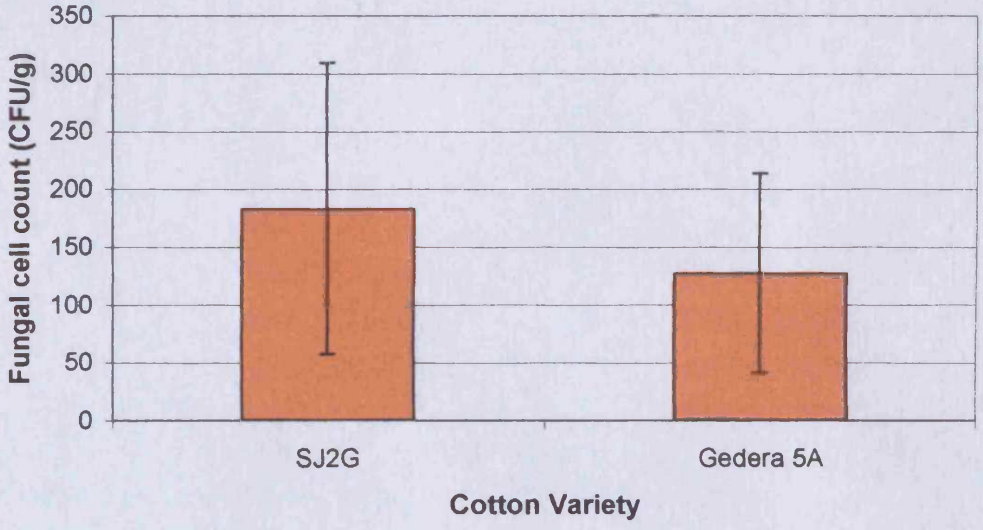


Figure 7.08: Fungal cell counts (CFU/g) in aqueous washes from two varieties of seed cotton from Eritrea (2001/2002 production season). Results displayed as mean count ± SEM (n=6).

Correlations between fungal and Gram-negative bacterial cells

There was a positive correlation between fungal cell counts and numbers of Gram-negative bacteria on the thirteen cotton samples from diverse countries. There was a statistically significant positive correlation between these two organisms on the ten samples from Sudan ($P<0.01$) and on all samples involved in the study ($P<0.05$). The result of the four samples from Turkey was not reliable due to the low number of samples involved, when these were analysed separately (Table 7.04).

Cottons	N	Correlation coefficient	Significance
Diverse samples (2001/2002)	13	0.451	0.122
Turkish samples (2002/2003)	4[a]	-0.600	0.400
Sudan samples (various)	10	0.879**	0.001
All samples in study	27	0.431*	0.025

Table 7.04: Spearman's rank correlation coefficients of fungal cell counts and Gram-negative bacterial counts measured in aqueous washes from various cotton lint samples. [a]Denotes too few samples for reliable statistical result. *Denotes statistically significant correlation ($P<0.05$), **denotes statistically significant correlation ($P<0.01$).

Linear regression analysis revealed a strong positive relationship between the fungal cell counts and Gram-negative cell counts recorded in the study on cotton samples from Sudan, despite variation in cotton species, grade and year of harvest. However, although the correlation coefficient was 0.93, this was not statistically significant (P>0.05) (Figure 7.09).

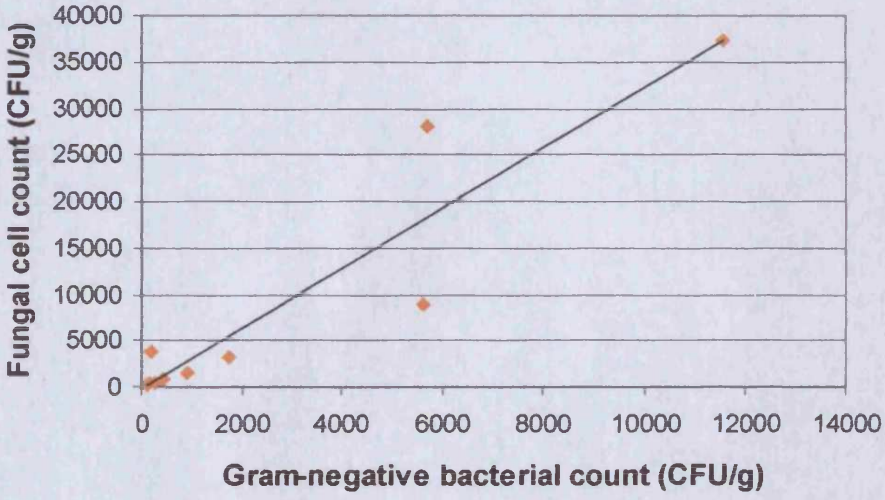


Figure 7.09: The linear relationship (y=a+bx, where a=393.04 and b=3.25) between viable Gram-negative bacterial and fungal cells in aqueous washes from various cotton samples from Sudan (n=10).

7.4 DISCUSSION

Method appraisal

Fungal cell counts on cotton samples were carried out using standard spread plating methods. Similar extraction methods to those utilised successfully for the culturing of fungi for identification procedures (Chapter 6) and for the enumeration of Gram-negative bacterial cells (Chapter 4), were employed in this instance for fungal cell counts, hence only water-extractable cells were enumerated. Malt extract agar was used to culture the fungal cells, as this is a general-purpose growth medium, upon which a large variety of fungal colonies can develop, especially at 25°C, the optimum growth temperature for the majority of fungi. Other studies enumerating environmental fungal cells, including a previous paper isolating these cells from cotton fibres, have utilized similar methods. In this chapter, colonies were counted after three days incubation, however, the incubation time used by different groups to culture environmental fungal cells has varied, for example from 3 days (Lugauskas, *et al.*, 2004), 7 days (Desai and Ghosh, 2003a; Piecková and Jesenská, 1996), 10 days (Gehring, *et al.*, 2001) and 14 days (Andersson, *et al.*, 1997). Colony numbers in the present study were initially counted upon appearance and then on consecutive days until 14 days after growth appeared. Three days was chosen as the optimum incubation period, because after this time, no additional fungal colonies appeared and those already present simply grew larger and began to overlap, confusing the count.

Each set of sub-sample culture plates was prepared in triplicate and the mean count calculated, this was repeated six times on different sub-samples. Although the density of contamination is likely to have fluctuated throughout a sample, this technique was used to give an accurate representation of individual cotton samples. However, despite success on cotton lint, large errors were displayed in the results on the two seed cotton samples from Eritrea (see Figure 7.08), this was also seen with the results of Gram-negative bacterial counts, and may again be ascribed to the affect of the added step of tearing the fibres away from the seeds prior to analysis.

The range of fungal counts

The fungal cell counts measured in the study varied over a wide range. Counts on cotton samples from diverse countries (2001/2002 production season), and those from Turkey (2002/2003 production season) ranged similarly from 10^2 – 10^3 CFU/g (Figures 7.01 and 7.03), whereas the largest range (10^2 – 10^4 CFU/g) was seen in the samples from Sudan (Figure 7.04). This was surprising, as the variables involved in cotton production, many of which could influence fungal contamination levels within this one country, would have been similar for all samples involved, as in the case of those from within Turkey. Whereas procedures and conditions would have varied to a greater extent between the diverse countries of origin, potentially leading to highly variable fungal counts. However, the cotton samples from Sudan varied in harvest year and hence storage period, therefore it may have been this factor, which contributed to this diversity.

It is notable that the results recorded in this study were generally lower than that recorded in the only previous study available which has measured fungal cells on cotton lint (Piecková and Jesenská, 1996). This study utilised similar extraction and culture methods to those applied in the current study to record an average of 3.3 $x10^5$ CFU/g on 45 cotton samples, 39 of which were from Uzbekistan, 3 from the USA, 1 from China, 1 from Egypt and 1 from Russia, all of which were processed in a cotton mill in Slovakia. The mean fungal count in the current study from a total of 27 cotton samples from various countries was a hundred times lower (4.5 $x10^3$ CFU/g). However, Piecková and Jesenská (1996) did not give details regarding cotton sample age or variety. Clearly more studies, which analyse this cotton contamination parameter, are required as currently there are no further studies with which to compare results on this particular material.

The influence of production conditions on fungal cell counts

Cotton samples from countries within Africa demonstrated a higher mean level of fungal contamination, than cotton samples from within Asia (Figure 7.02), hence these environments have an increased probability of harbouring mycotoxin producing fungal strains. This is the same pattern seen in previous chapters with Gram-negative bacterial cell counts (Chapter 4) and endotoxin concentrations (Chapter 5). Therefore, it is likely that fungal growth on cotton fibres is influenced by the same variables as bacterial contamination. Both fungi and bacteria require warmth, moisture and a source of food in order to proliferate (Brock, 2000), and clearly these conditions are present at favourable levels for both on cotton plants, leading to the numbers of

microbes reported in this project. Due to interest in both Gram-negative bacteria and fungi with regard to lung inflammation and respiratory disease, a number of studies have measured numbers of both these cells in various indoor enviroments (e.g. Dutkiewicz, 1997; Su, *et al.*, 2002). Despite this, there appear to be no studies of this kind, which have analysed the relationship or calculated any correlation between the two parameters.

There is also a lack of studies analysing the effects of field weathering on cotton fungal growth, especially compared to the number carried out in order to study cotton bacterial growth, for example after rainfall (Fischer and Sasser, 1987; Heintz, *et al.*, 1990), periods of dry weather (Simpson and Marsh, 1985) and differing relative humidity (DeLucca, *et al.*, 1990), as discussed in Chapter 4. However, changes in temperature and water activity (a_w) were found to affect fungal growth on green coffee beans in Spain (Pardo, *et al.*, 2005), temperature and relative humidity influenced fungal populations on banana chips in the Philippines (Sales, *et al.*, 2005), and climatic conditons such as high rainfall stimulated fungal growth during the field weathering of Brazilian corn (Ono, *et al.*, 1999). These studies infer that differences in climate during cotton field weathering may also influence the fungal growth on cotton fibres as has been demonstrated with levels of Gram-negative bacteria.

However, analysis of four cotton samples from Turkey (Figure 7.03) revealed a significant difference between the fungal counts on two samples taken from the Efes region. These samples are likely to have been produced under

similar climatic conditions and subjected to the same production techniques, they were also harvested in the same year and are probably of the same cotton type, hence reasons for this difference in fungal contamination level are difficult to identify. This highlights the complexities of studying cotton fibres, and again demonstrates the multifactorial influences that affect the biological contamination of cotton.

Correlation between fungal cells and Gram-negative bacterial counts

A postive rank correlation was observed between Gram-negative bacteria and fungal cell counts on cotton from diverse origins (Table 7.04). Although this was not statistically significant, the fungal counts reflected the same pattern as bacteria cells, in that samples from within Africa had significantly higher mean levels of contamination than samples from Asia (Figure 7.02), as discussed above. The rank correlation coefficient between bacteria and fungi for all 27 samples in the project was similar to that shown with cotton from diverse sources, but this was of statistical significance ($P<0.05$), probably due to the higher number of samples involved in the analysis. Further to this observation, there was a strong and highly significant rank correlation between GNB and fungal cells on the ten cotton samples from within Sudan (Table 7.04). This strong correlation was also reflected by linear regression analysis (Figure 7.09), although this relationship was not significant, perhaps again due to the limited number of samples involved.

As a result of this relationship, the same patterns were seen in the results of fungal cell counts on Sudanese cotton samples, as those discussed

previously in Chapter 4, (with reference to Gram-negative bacterial counts). In this regard, Gezira Acala cotton contained significantly higher mean fungal cell counts than Gezira Barakat cotton (Figure 7.05), however, no significant difference was observed between the fungal counts on Gezira Barakat 4B grade cotton and 6B grade cotton (Figure 7.06). Although, as with the Gram-negative bacterial counts, the 4B grade cotton samples exhibited a slightly higher contamination level than those of 6B samples. Therefore, these results also imply that fungal cells are influenced by the same variables as Gram-negative bacteria, for instance the bushier fibres of the Acala cotton (Khalifa, 2001), again harboured higher levels of contamination compared to Barakat cotton. Moreover, the mean fungal cell counts on cotton samples harvested in different years also mirrored that of Gram-negative bacterial counts (Figure 7.07), where it was indicated that storage time may have resulted in a decrease in contaminating cells. Although this may be a more complex issue with regard to fungi as storage fungi are known to thrive in the period after storage of organic material (Dutkiewicz, 1997; Kaese, *et al.*, [WWW] 2003). Again, the lack of data from the time of harvest for samples stored for a number of years makes the effects of storage difficult to isolate.

In nature, bacteria and fungi often occur together, and when this occurs they can associate in different ways. Co-operative synergistic relationships have been observed between the two, as well as competitive antagonistic relationships, hence these interactions are often complex (Kanegsberg, *et al.*, [WWW] 2005). No previous studies have analysed the relationship between cotton bacteria and fungi levels. However, correlations have been recorded

between bacterial endotoxin and the fungal cell wall component (1-3)-β-glucan, in cotton dust (Rylander, *et al.*, 1989a), and other indoor environments (Gehring, *et al.*, 2001; Rylander, *et al.*, 1989b; Rylander, *et al.*, 1999). This may reflect a relationship between their source organisms i.e. Gram-negative bacteria and fungi, although further research is needed in order to explore this possibility.

Contamination of trash material

Also following the trend shown by Gram-negative bacterial counts and endotoxin levels, trash samples available from Turkish cotton (2002/2003 production season), exhibited statistically higher numbers of fungal cells than equivalent lint samples (Table 7.03). Although the differences were not statistically significant, trash from three samples from 2001/2002, originating in China, Iran and Turkey, also showed higher counts in each case compared to those recorded on lint samples. No previous studies have been carried out to analyse the fungal content of cotton trash material. However, the consistently higher levels of biological contamination on trash samples, highlights the increased health risk posed to cotton workers at the very early stages of cotton procesing. The increased levels of fungal cells in this particular production area, implies a greater risk of mycotoxin producing strains being present. Hence, workers at this point may require higher levels of respiratory protection and health monitoring, than those in subsequent stages.

Summary

Similar patterns were observed in this chapter to those demonstrated in previous chapters. Fungal cell counts varied significantly between samples, from 10^2-10^4 CFU/g and cotton samples from within African countries exhibited statistically higher fungal counts compared to those from within Asia. A signifcant correlation was also revealed between fungal cell counts and the Gram-negative bacterial counts performed in Chapter 4, implying that fungal growth is influenced by the same factors as bacteria, such as humidity and rainfall. The trash samples analysed also contained higher numbers of fungi compared to cotton lint, as seen with Gram-negative counts and endotoxin levels, suggesting that cotton workers at the early stages of cotton processing are most at risk from health effects. The lack of previous studies focussing on cotton fungal contamination was also highlighted.

CHAPTER 8

ASSESSMENT OF ENZYME ASSAYS USED TO MEASURE (1-3)-β-GLUCAN LEVELS ON COTTON LINT

8.1 INTRODUCTION

(1-3)-β-D-glucans are polyglucose compounds with various molecular weights and degrees of branching. They have been isolated from a wide variety of sources including most fungi and yeasts, where they have a structural role in cell walls, constituting 48-52% of cell wall polysaccharides (Pérez and Ribas, 2004). They are also present in small amounts in fungal cytosol, in the majority of higher and many lower plants as well as being excreted as extracellular polysaccharides from certain bacteria (Douwes, *et al.*, 1996). More recently, they have been isolated from bird droppings and tartar on teeth (Rylander, *et al.*, 1994; Young, *et al.*, 1998). Figure 8.01 shows the position of these compounds in the fungal cell wall.

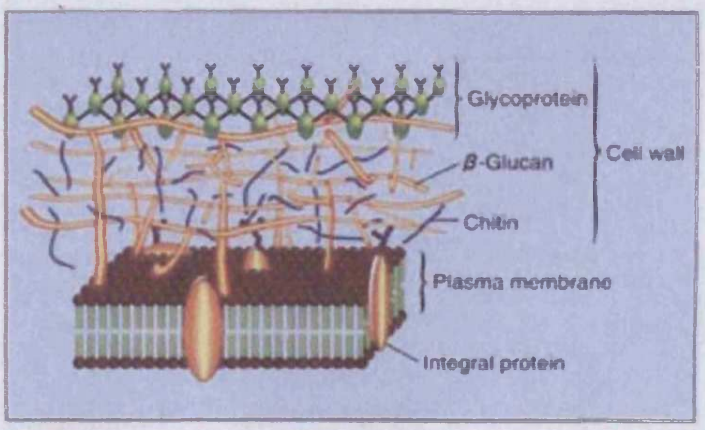


Figure 8.01: The structure of a fungal cell wall showing the layer consisting of β-glucan. Adapted with permission from Sambo Medical Company, [WWW] 2005.

Interest in β-glucans was heightened when it was discovered that they are specific modulators of the immune system and possess anti-tumour, anti-microbial and radioprotective activity. They have been classified pharmacologically as Biological Response Modifiers (BRMs), and they appear to function by indirect activation of the host's immune system (Kulicke, *et al.*, 1997). As a result, a variety of (1-3)-β-D-glucans have been isolated, and research identifying and characterising new polymers of this type is ongoing. A recent study has demonstrated the biological activity of these compounds in the common cotton fungus *Aspergillus* (Ishibashi, *et al.*, 2004). Table 8.01 lists some of the most well characterised (1-3)-β-D-glucans.

(1,3)-β-glucan	Links/Branches	M_w	Solubility	Source
Curdlan	(1-3)-β-D	>136000	NaOH	Bacteria
Carboxymethylcurdlan	(1-3)-β-D	>95000	H_2O	Bacteria
Grifolan	(1-3)(1-6)-β-D	5×10^5	H_2O, 120°C	Fungus
Laminarin	(1-3)(1-6)-β-D	NA	H_2O, 120°C	Algae
Lentinan	(1-3)(1-6)-β-D	94700	H_2O	Fungus
Pachyman	(1-3)(1-6)-β-D	80000	NaOH	Fungus
Pustulan	(1-3)(1-6)-β-D	NA	H_2O, 120°C	Lichen
Schizophyllan	(1-3)(1-6)-β-D	76800	H_2O	Fungus
Scleroglucan	(1-3)(1-6)-β-D	16800	H_2O	Fungus

Table 8.01: Some well-characterised (1-3)-β-D-glucans, their branching, molecular weights, solubility and source. NA = data not available. Derived from Aketagawa, *et al.*, 1994; Brown and Gordon, 2003; Douwes, *et al.*, 1996; Kim, *et al.*, 2000.

Despite initial interest in (1-3)-β-D-glucans as possible therapeutic agents, and some success in this area; schizophyllan and lentinan have been used clinically in Japan for the treatment of cancer for almost twenty years (Bohn and BeMiller, 1995), (1-3)-β-D-glucans (referred to simply as β-glucans or glucans hereafter) have emerged relatively recently as toxicants capable of causing lung disease upon inhalation. However, experimental results examining these effects are often contradictory, due to variations in the source and structure of the glucans studied, as well as differing exposure protocols. Briefly, large molecular weight (particulate) glucans have been shown to activate leukocytes, stimulating their cytotoxic and phagocytic activities. These particular glucans are also capable of initiating an inflammatory response in the form of mediators such as cytokines. Lower molecular weight molecules are also biologically active, although they do not induce the release of cytokines, they have been shown to activate leukocytes, priming these cells for enhanced responses to a further challenge, for example of endotoxin (Gordon and Brown, 2003). This may be an important effect in heterogeneous environments,such as those containing organic dusts, where both glucans and endotoxin are likely to be present.

There are no standardised measurement procedures for glucans in occupational environments. However, general levels of this toxicant can be measured in a similar way to endotoxin; by adapting the LAL assay. (1-3)-β - glucans also trigger an enzymatic pathway upon contact with American Horseshoe Crab amoebocytes, which results in the formation of a gel clot. Glucans interact with this system via a different pathway than endotoxin (the

G-factor pathway) (Roslansky and Novitsky, 1991). This non-specificity was initially considered a large drawback of the endotoxin measurement assay. In light of this apparent limitation, commercial LAL reagents have now been developed, which are rendered endotoxin-specific by adding a glucan blocker (Figure 8.02).

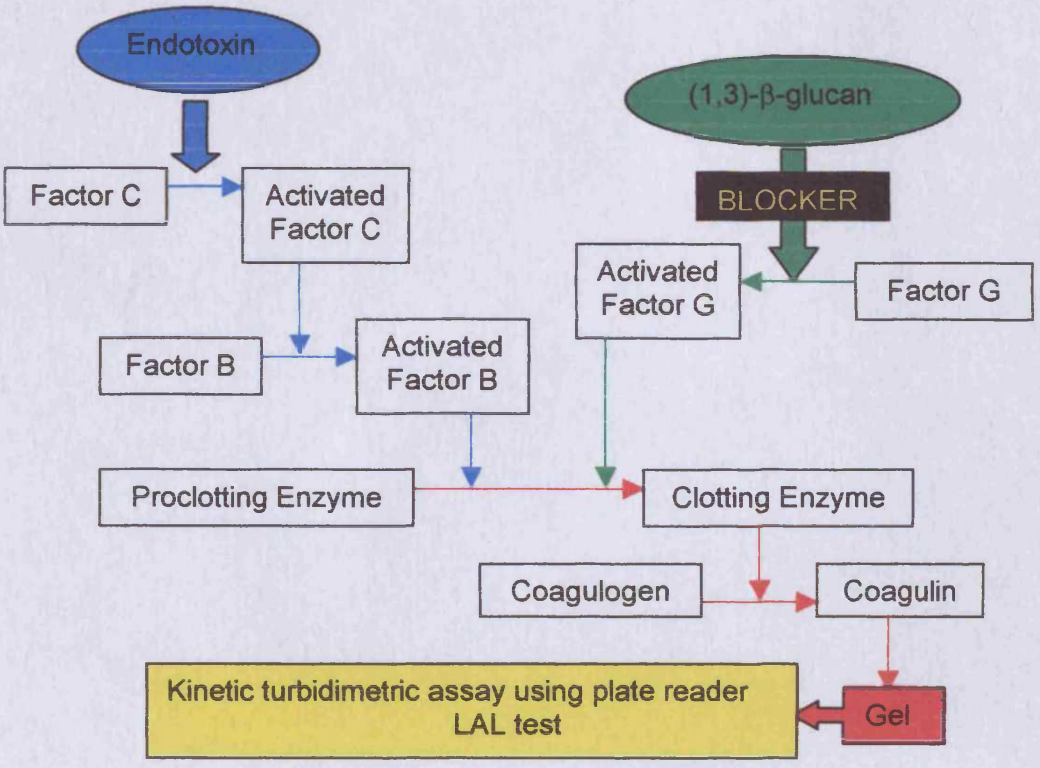


Figure 8.02: The separate reaction pathways of endotoxin and glucan with *Limulus* amoebocyte lysate used in quantification.

The blocker is a carboxymethylated curdlan (a type of glucan); glucans only activate LAL in the range 1-1000 ng/ml, and are inactive in concentrations above this (Cooper, *et al.*, 1997). Hence, addition of curdlan to the reaction vessel with a test sample has the effect of 'swamping' the system and rendering those glucans present in the sample, undetectable. By exploiting

this biochemistry, it is possible to measure the glucan contamination of a sample by carrying out the assay simultaneously in the presence and absence of the blocker. The results yield an endotoxin-specific measurement that can be subtracted from the total LAL reactive material value, to give the remainder, which is assumed attributable to glucan.

This 'subtraction method' is endorsed by producers of LAL reagents (Foster Jordan, Charles River Endosafe - personal communication), and the principles have been applied in clinical research into fungal infections (Kohno, *et al.*, 1993; Mitsutake, *et al.*, 1996; Miyazaki, *et al.*, 1992), yielding what is termed the 'fungal index', or the difference between endotoxin-specific and non-specific assays. Simultaneous LAL analyses with and without added glucan blocker have also been applied to measure (1-3)-β-glucan in dust from a sewage treatment plant (Krajewski, *et al.*, 2004). However, this technique has not previously been applied to samples extracted from cotton fibres, hence it required validation for use on this material. In addition to the required internal validation controls, the subtraction method could be assessed by attempting to correlate results from the same cotton samples, with those from a more widely used glucan-specific assay. Glucan-specific reagents are commercially available for the diagnosis of deep-seated mycosis in patients with fungal infections. These specific tests are also derived from the LAL assay, but the lysate has been processed to remove Factor C (in the endotoxin-reaction pathway). An example of this type of reagent is Glucatell™ (Associates of Cape Cod Incorporated, Falmouth, Massachusetts), which is formulated as a chromogenic lysate by

adding Boc-Leu-Gly-Arg-pNA to the substrate. (1-3)-β-D-glucan in the test sample activates Factor G, resulting in the pro-clotting enzyme, then the clotting enzyme which cleaves p-nitroaniline (pNA) from the chromogenic peptide substrate and the free pNA is measured spectrophotometerically (ACCI, Glucatell™ product insert, 2002).

(1-3)-β-glucan is present in the secondary walls of cotton fibres in the form of callose (Andrawis, *et al.*, 1993). However, as this is a complex molecule, which also has (1-4) and (1-6) links, the more easily extractable glucans are likely to be present on the surface of cotton fibres through the contamination of fungi, which have been shown to be present on cotton in high numbers (Chapter 7). Measuring glucan on cotton will be useful not only to assess potential risk in the cotton production environment, but also to analyse the relationships between this and other cotton contaminants. There may be a correlation between glucan and the fungal cells from where it originates. Ideally, if this were the case, fungal cell counts could be used to indicate glucan levels on cotton and hence toxicity (Rylander and Lin, 2000). This would be of benefit as fungal cell counts are more cost effective to carry out on a large number of samples.

Further to this, a possible relationship has been shown between numbers of bacterial and fungal cells on cotton (Chapter 7), this implies that samples contaminated with glucan, may also have high levels of endotoxin. A positive correlation between these toxicants is of interest, since glucans are capable of modifying host responses to endotoxin. Although data regarding the nature of modification is conflicting, there is evidence that glucan acts as an

endotoxin adjuvant and increased inflammatory responses have been demonstrated in animal experiments after simultaneous glucan/endotoxin exposure (Cook, *et al.*, 1980; Fogelmark, *et al.*, 1994). Therefore, high levels of both endotoxin and glucan in cotton samples may exacerbate the risks to respiratory health.

Aims

- To investigate the applicability of a subtraction method using endotoxin-specific and non-specific assays for measuring glucan on cotton samples.
- To test the validity of this method using a glucan-specific assay.
- To investigate any correlation between glucan levels and fungal cell counts measured in Chapter 7 on equivalent cotton samples.
- To examine any relationship between glucan contamination and endotoxin concentrations measured in Chapter 5.

8.2 MATERIALS AND METHODS

8.2.1 Materials

Equipment

All equipment was obtained from reputable sources, and was guaranteed pyrogen free to at least <0.005 EU/ml, full details are available in Appendix I.

Cotton samples

See Chapter 2 for details.

Control standard endotoxin

Endosafe® control standard endotoxin was used, this was prepared from E.coli strain 055:B5. Each vial contained 10ng of purified LPS, freeze dried in a stabilised matrix. Potency of this standard was determined as 19 EU/ng. Lot EX13172 was used throughout the study.

LAL reagent

Lyophilised Endosafe® KTA^2 Limulus Amebocyte Lysate (LAL) reagent, Lot S2632L was used throughout the study.

Endotoxin-specific buffer

Endosafe® Endotoxin-specific buffer solution consisted of carboxymethylated curdlan in a pH-buffering solution containing Tris(hydroxymethyl)amino-methane at pH 7.4. The buffer was terminally sterilised and endotoxin-free.

8.2.2 Methods

Template protocol

Templates were programmed using Dynex Revelation version 4.22 software on an MRX Revelation microplate reader.

Glucan extraction

Glucan was extracted from 0.2000g (± 0.0005g) cotton lint into 4 ml pyrogen free water by shaking on a vortex multi-mixer at 1500 rpm for 60 minutes in a centrifuge tube. The cotton fibres were then physically submerged using a sterile pipette and the supernatant removed to a fresh tube.

Standard curve preparation

Control standard endotoxin was reconstituted according to lot specific Certificate of Analysis (3.8 ml LAL reagent water added to 10ng vial to obtain 50EU/ml). The vial was then vortexed vigorously for 5 minutes. Four concentrations were used for the curve; 50EU/ml, 5.0EU/ml, 0.5EU/ml and 0.05EU/ml. These were prepared by performing stepwise dilutions using pyrogen free water (PFW) in depyrogenated borosilicate glass tubes. Tubes were vortexed for 30 seconds between each transfer step.

Lysate reagent

Each vial of KTA^2 was rehydrated immediately before use with either 5.2ml of Endotoxin specific buffer or 5.2ml PFW and gently swirled until dissolved into a colourless liquid.

Negative controls

PFW was used as the negative control in each assay.

Positive controls

Positive controls were test samples spiked with 10µl of the 5.0EU/ml standard endotoxin to test for interference (inhibition/enhancement).

Preparation for analysis

100µl of negative and positive controls, standards, and samples were added to wells of a 96 well microtiter plate guided by the template, which assigns each component. Each well was run in duplicate.

Kinetic-turbidimetric test procedure

100µl of reconstituted endotoxin specific lysate or standard (PFW) lysate was added quickly to each well using a repeating pipette and syringe. PFW lysate was used with endotoxin standards. The plate was then placed without lid into the reader pre-heated to 37°C, and mechanically shaken for 10 seconds to mix reagents. The assay was run according to manufacturers instructions at 37°C and test wavelength 340nm for 60 minutes with automated readings every 30 seconds. The time taken for each well to reach 0.03 OD was recorded and this value subsequently read off the standard curve to give the equivalent endotoxin concentration (EU/ml). Standard curves were only accepted as valid when the absolute value of the correlation coefficient, r, was greater than or equal to 0.98.

Calculation of glucan contamination

Data from wells containing endotoxin specific lysate, measured only the endotoxin present in the samples in EU/ml, this value was subtracted from that of the equivalent sample, assayed with standard (PFW) lysate, as this measured the total reactive material in the sample (endotoxin + glucan), results were reported in reactive units per gram of fibre (RU/g).

8.3 Validation of subtraction method

A commercially available (1-3)-β-D-glucan detection reagent kit was used to assess the validity of the subtraction method (detailed above). This was the Glucatell™ kit (Associates of Cape Cod Incorporated), although it is also based around the LAL assay, the lysate has been processed to remove the enzyme pathway through which endotoxin reacts, rendering it glucan-specific.

8.3.1 Materials

Cotton samples

Ten cotton samples from diverse origins (all from 2001/2002 production season) were selected as these had shown a range of glucan levels as measured by the subtraction method, these were samples from Benin, China Xinjiang, CIS, Ivory Coast, Paraguay, Syria, Tajikistan, USA, Zambia, and Zimbabwe. For full details, see Chapter 2.

Glucatell reagent

Freeze-dried chromogenic glucan detection reagent, lot number 855010 was used throughout the study.

Pyrosol reconstitution buffer

The buffer used to reconstitute the Glucatell reagent was tris(hydroxymethyl)aminomethane hydrochloride, 0.2M, pH 7.4. Lot number 226-231 was used throughout the study.

(1-3)-β-D-glucan standard

Lyophilized (1-3)-β-D-glucan standard, lot number 962002 was used throughout the study.

8.3.2 Methods

Template protocol

As for subtraction method

Glucan extraction

As for subtraction method, followed by dilution of supernatant to 10^{-3} in pyrogen free water.

Standard curve preparation

Glucan standard was reconstituted with 1.7 ml pyrogen free water to provide a solution of 100pg/ml solution and vortexed for five minutes. Four concentrations were used for the curve; 100pg/ml, 50pg/ml, 25pg/ml, 12.5pg/ml. These were prepared by performing stepwise dilutions using pyrogen free water in depyrogenated borosilicate glass tubes. Tubes were vortexed for 30 seconds between each transfer step.

Glucatell reagent

Each vial of reagent was reconstituted with 2.8ml pyrogen free water and 2.8ml pyrosol buffer. The vial was swirled gently until the powder was completely dissolved, reagent was used within ten minutes as per manufacturers guidelines.

Negative controls

Pyrogen free water was used as the negative control in each assay.

Positive controls

Positive controls were test samples spiked with 10µl of the 50pg/ml standard glucan.

Preparation for analysis

25µl of negative and positive controls, standards and test samples were added to the wells of a 96 well microtiter plate guided by the software template. Each well was run in triplicate.

Kinetic assay procedure

100µl of Glucatell reagent was added to each well using a repeater pipette and syringe. The plate was then placed without lid into the reader (pre-heated to 37°C) and shaken for five seconds before being read at 405nm, every 20 seconds for 60 minutes. The time taken to reach the onset density of 0.03 OD was recorded and this value subsequently read from the standard curve to give the equivalent glucan concentration in pg/ml. Standard curves were only accepted as valid when the absolute value of the correlation coefficient, r, was greater than or equal to 0.980.

8.4 RESULTS: GRAPHICAL/TABULAR REPRESENTATION

The range of glucan levels measured by the subtraction method

Cotton lint samples from diverse countries displayed a wide range of glucan levels, which statistically fell into seven homogeneous subsets. Mean levels in decreasing order were from Paraguay (2964.42 ± 313.90 RU/g), Ivory Coast (2493.25 ± 248.87 RU/g), Benin (2231.08 ± 135.09 RU/g), Zimbabwe (2015.52 ± 160.52 RU/g), Zambia (1430.72 ± 174.73 RU/g), Iran (969.26 ± 113.65 RU/g), USA (920.85 ± 208.40 RU/g), China Xinjiang (598.77 ± 262.55 RU/g), Syria (349.29 ± 199.18 RU/g), China 146 (151.77 ± 23.80 RU/g), Tajikistan (74.18 ± 23.75 RU/g), CIS (36.20 ± 19.62 RU/g), and Turkey (15.96 ± 5.18 RU/g) (Figure 8.03).

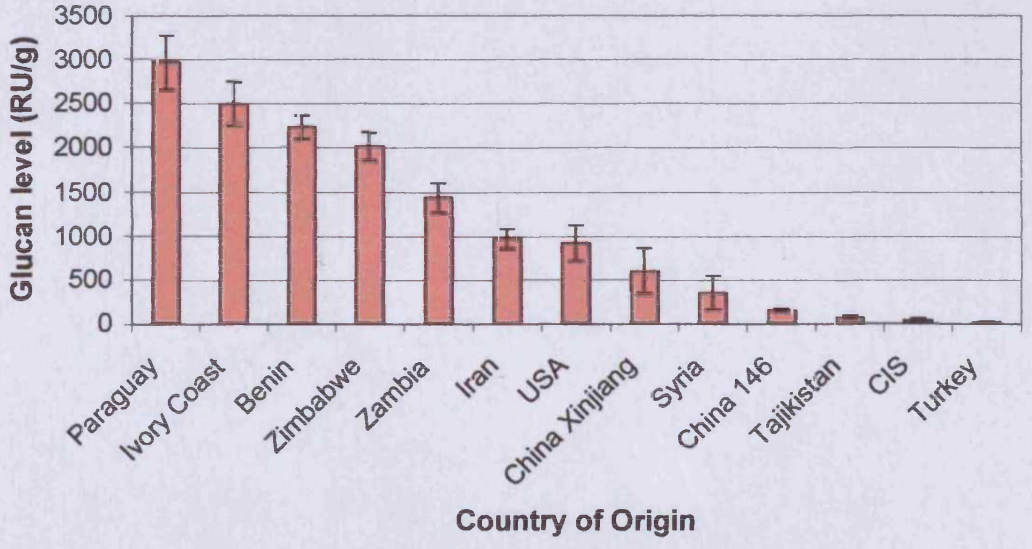


Figure 8.03: Total range of glucan levels (RU/g) measured by the subtraction method in aqueous washes from thirteen cotton lint samples from diverse countries of origin (from 2001/2002 production season). Results presented as mean level ± SEM (n=6).

8.5 DISCUSSION

Method appraisal

In order to assess the subtraction method for cotton glucan analysis, glucan was firstly extracted by a simple procedure (vigorous shaking in pyrogen-free water). Glucan extraction from cotton fibres has not previously been carried out, so no standard procedures exist. However, it is noteworthy that different methods have been applied to extract glucan from various dust filters, such as those containing cotton dust, house dust and saw dust. These have involved a range of procedures, including agitating the filter in distilled water with 0.02% saponin, followed by autoclaving at 122°C for 90 minutes (Rylander, *et al.*, 1989), shaking on ice for 10 minutes in pyrogen-free water with added 0.3 M NaOH (Thorn and Rylander, 1998; Wan and Li, 1999) and in distilled water with added 0.05% Tween-20 at 120°C (Douwes, *et al.*, 2000; Ronald, *et al.*, 2003). Other methods utilised to increase the solubility of glucans include derivatization procedures such as carboxymethylation and sulfation, and techniques such as acid and alkaline hydrolysis, enzymatic digestion and ultrasound irradiation have been applied in order to lower the molecular weight of the compounds thus increasing solubility (Šandula, *et al.*, 1999).

A concern with these methods is that treatments may alter the activity of glucan in the LAL assay. For example, in order to render glucan more water soluble with alkali treatment, the triple-helix structure of the molecule is unwound (Aketagawa, *et al.*, 1994), inevitably altering its biochemistry. As

the focus of this chapter was to assess the assay method, a straightforward, simple extraction procedure that has previously been used successfully with endotoxin LAL analysis was utilised. Although this method would only remove the more water-soluble glucans from the fibres, it ensured there was nothing present in the extraction media that could interfere with either the glucan activity, or the enzymes involved in the reaction pathways. However, it should be noted it is likely that this method greatly underestimated the true glucan content of the cotton samples.

Validation of the subtraction method

The Glucatell™ assay kit was chosen to assess the subtraction method, as it is specific for (1-3)-β-D-glucan, and is carried out in the same way as the traditional LAL assay method, thus it required similar expertise and equipment. Although recently approved by the FDA for clinical use, the Glucatell™ assay has not been applied to environmental glucan measurement, as it is a new product, which has superseded the discontinued Gluspecy or G-test (Seikagaku Co., Tokyo, Japan), which has been employed to measure glucan in various dusts in several studies (e.g. Rylander, 1999; Thorn and Rylander, 1998; Wan and Li, 1999). An enzyme immunoassay, which quantifies glucan using affinity-purified rabbit anti-(1-3)-β-glucan has also been developed to measure glucan in dust samples (Douwes, *et al.*, 1996). In this assay, immunospecific rabbit antibodies are produced by immunization with bovine serum albumin-conjugated laminarin and affinity chromatography (Rylander, 1999). Although this method has been applied with success to measure glucan in occupational dusts

(Douwes, *et al.*, 2000; Ronald, *et al.*, 2003), the simpler assay procedure of the Glucatell™ kit was deemed preferential in this instance.

The subtraction method is the more cost effective of the two assays, particularly because it allows endotoxin levels to be measured concurrently. This is useful, since endotoxin is also a respiratory hazard found in cotton fibres in high concentrations. The cost of reagents required for the Glucatell™ assay render it almost prohibitively expensive for use on a large number of samples. However, a drawback of the subtraction method is the necessity to read glucan levels from an endotoxin standard calibration curve. Thus, no conversion to concentration is possible and the RU (reactive units) adopted in this chapter to designate levels of LAL reactive material (i.e. glucan), were essentially relative values. These units can however be utilised for comparison between different samples tested similarly, a tool which could be useful in assessing human risk from exposure to different cottons.

Both assays fulfilled the internal quality control criteria required to ensure a LAL-derived assay is valid; in that the correlation coefficient of the standard curves were greater than 0.98, and the negative controls were significantly lower than the lowest point of the standard curve. Also, the positive control spike recoveries were in the range of 50-200% of the expected value for the Glucatell™ assay, though this calculation was not possible for the subtraction method due to the endotoxin, rather than glucan positive control. Despite fulfilment of these internal quality controls, a statistically insignificant negative correlation was revealed between the two sets of results (Table 8.02). The

Glucatell™ results revealed similar glucan concentrations in all samples analysed (see Figure 8.05); this was unexpected, given the diverse geographical sources of the cotton samples tested. In contrast, the results from the subtraction method, displayed varying levels of glucan in the different samples (see Figure 8.03) as had been seen with the Gram-negative bacteria, endotoxin and fungal cell counts, measured in previous chapters of this thesis. This similarity was further extended to the mean results of cotton samples from within Africa and Asia; the results of the subtraction method displayed a significantly higher glucan level on the cotton from African countries compared to those from Asian samples (Figure 8.04), and this pattern has been found in previous chapters with bacteria, endotoxin and fungi. However, in contrast, the Glucatell™ assay results did not demonstrate any difference between mean glucan concentrations from African and Asian cotton samples (see Figure 8.06).

Not only did the subtraction method yield graded, quantitative results, as might be expected, but also, the values correlated positively and significantly with the fungal cell counts performed in Chapter 7 (see Table 8.02). Since the majority of glucans present on cotton fibres would have been derived from contaminating fungi, this relationship is easily explained. A correlation between glucan and fungal cells has also been demonstrated in other situations, such as in wood dust (Alwis, *et al.*, 1999) and house dust (Chew, *et al.*, 2001; Gehring, *et al.*, 2001). Glucan levels, as measured by the subtraction method also showed a highly significant positive correlation with endotoxin (Table 8.02), the health implications of which will be discussed

below. A relationship between glucan and endotoxin levels has also been reported previously in cotton dust (Rylander, *et al.*, 1989a), house dust (Gehring, *et al.*, 2001) and indoor air (Rylander, *et al.*, 1989b), as well as in the air of other occupational settings such as the paper industry (Rylander, *et al.*, 1999) and a sewage treatment works, where the rank correlation coefficient between the two toxicants was 0.86, following application of a subtraction method (Krajewski, *et al.*, 2004). The results from the Glucatell™ analysis did not correlate with any other parameter measured in the project (Table 8.02).

It seems from the current findings that the Glucatell™ assay reacted in an 'all-or nothing' fashion to the glucan present in the samples, and despite dilution of the supernatant to levels within the range of the standard curve prior to analysis, it was unable to differentiate between concentrations, yielding only qualitative results. Whereas, the subtraction method yielded results that reflected expected patterns, in relation to the findings of previous chapters in this project, and other studies. In light of this, the correlations between the glucan levels as measured by the subtraction method and the other toxicants investigated in the project will be analysed below.

Correlation with other toxicants

The correlation between glucan levels and fungal cell counts implies that the glucan in this study originated from fungi that naturally contaminated the cotton plants, during field weathering and subsequent storage. The rank correlation coefficient of this relationship was significant ($P<0.05$) (Table

8.02), however, linear regression analysis did not show a significant relationship between glucan levels and fungal cell counts (Figure 8.06). This suggests that although a high level of fungal cells may be a preliminary indicator of glucan contamination, fungal cell counts are not highly predictive of the glucan concentration. This may be because glucan levels are more stable than living fungal cells, remaining active in the environment after the death of their source cells (Fogelmark and Rylander, 1997; Rylander and Lin, 2000), as with endotoxin and Gram-negative bacteria discussed in Chapter 5. The viable counts carried out in Chapter 7, did not reflect dead cells and hence, the glucan level is more likely to reflect the maximum living fungal contamination, which was at one time present on the samples, probably at the time of harvest.

The above explanation is given in the literature for weaker than expected correlations between glucan and viable fungal counts in several instances (e.g. Gehring, *et al.*, 2001; Wan and Li, 1999). Consequently, current, research tends to focus specifically on chemical biomarkers for total mould biomass (alive and dead cells), which itself can only be assessed by a trained microbiologist, via time-consuming microscopy (Reeslev, *et al.*, 2003). (1-3)-β-glucan has been highlighted as such a chemical marker (Gehring, *et al.*, 2001; Rylander, 1999a; Rylander and Lin, 2000), along with ergosterol, a fungal sterol (Miller and Young, 1997; Reeslev, *et al.*, 2003) and fungal extracellular polysaccharides (Chew, *et al.*, 2001; Schram, *et al.*, 2005). Of these, glucan has been shown to correlate well with a variety of respiratory and other symptoms in non-industrial environments (Douwes, *et*

al., 2000; Rylander, *et al.*, 1998; Thorn and Rylander, 1998; Wan and Li, 1999). Therefore, studies that facilitate glucan measurement in occupational settings may prove valuable.

The positive rank correlation between levels of glucan and endotoxin concentrations was highly significant (Table 8.02), and linear regression analysis of these toxicants was also of statistical importance (Figure 8.07). Although such a correlation has been reported before in various organic dusts and indoor environments (Gehring, *et al.*, 2001; Krajewski, *et al.*, 2004; Rylander, *et al.*, 1989a; Rylander, *et al.*, 1989b; Rylander, *et al.*, 1999), the reasons underlying this relationship have not been well explained. In light of the findings of this project, a possible explanation for this trend, is that there is primarily, a strong positive correlation between total Gram-negative bacterial and fungal biomass on cotton, however due to the fluctuating levels of living cells and variation in cotton production procedures, this correlation is disrupted down to the level reported in Chapter 7, between only *viable* Gram-negative and fungal cells ($r=0.43$, $P>0.05$ for samples from diverse countries). However, due to the fact that both endotoxin and glucan retain their biological activity after the death of their source organisms, the initial strong positive correlation is still reflected in the levels of these stable compounds, long after the number of living cells has peaked and begun to decline. This last statement also explains why only weak positive correlations were seen between endotoxin and Gram-negative bacterial cell counts in Chapter 5 ($r=0.41$, $P>0.05$ for diverse samples) and between glucan and fungal cell counts in this chapter ($r=0.65$, $P<0.05$). Although there is a lack of

direct evidence in support of this hypothesis, neither is there any available information that contradicts it.

The strong correlation between glucan and endotoxin levels on cotton is important with respect to occupational health. Conflicting effects of simultaneous glucan/endotoxin exposure have been reported, and some studies have revealed that glucans can "protect" against endotoxin exposure by suppressing the release of inflammatory mediators (Hoffman, *et al.*, 1993; Rylander, 1994). However, it is now emerging that this effect is likely to be caused by cytotoxicity of glucan, which reduces macrophage function, as opposed to a truly protective effect (Milanowski, 1997; Young, *et al.*, 2002). Simultaneous inhalation of glucan and endotoxin has been shown to elevate airway neutrophils, macrophages and lymphocytes, compared to endotoxin alone (Fogelmark, *et al.*, 1994; Rylander, 1994). It has now largely been accepted that airway eosinophilia occurs in response to inhalation of glucan, and this differs to the neutrophil influx, which is characteristic of a response to endotoxin (Fogelmark, *et al.*, 2001).

Clearly, these toxicants interact in a complex manner and the nature of their relationship is not fully understood. However, the heterogeneous environment of the cotton mill is undoubtedly influenced by the raw cotton, which enters the production chain at this point, along with the contamination entrained within it. Hence, it is possible that high levels of endotoxin, glucan and possibly mycotoxins from fungal cells will all be present simultaneously in some cotton production environments. This has serious implications for the

health of cotton workers, especially as none of these toxicants can be controlled by monitoring levels of inert dust. As this is currently the only component with an imposed safety limit in the industry, further research is desperately needed.

Summary

In order to assess the validity of a LAL subtraction method to measure glucan levels on cotton fibres, data were compared to those measured using a purified glucan-specific assay kit. Although the results from these two methods did not correlate, those from the subtraction assay were quantitative and reflected expected patterns, whereas the glucan-specific assay yielded only qualitative results. Glucan levels measured by the subtraction method correlated with fungal cell counts and more strongly with endotoxin concentrations. This was explained by the fact that glucan and endotoxin are more stable compounds than their source organisms, and as such are probably a better reflection of total microbial biomass than viable cells. The presence of simultaneous high levels of glucan and endotoxin has significant health implications, as glucan may exacerbate responses to inhaled endotoxin.

CHAPTER 9

IDENTIFICATION OF BIOMARKERS FOR COTTON QUALITY

9.1 INTRODUCTION

Currently, in a single year, raw cotton worth over 20 billion US dollars is grown worldwide (Answers, [WWW] 2005), and it is estimated that the 2004/2005 harvest season, will result in the production of 21,857,000 tons of cotton (Bremer Baumwollbörse, 2004). Cotton is an important high value commodity in many countries. For example, in Uzbekistan, one of the top five cotton producers in the world, cotton constitutes over 40% of the country's total exports. As a result, the economy of Uzbekistan is dependent on the value of this crop, and hence rises and falls along with fluctuations in world cotton price (Encarta, [WWW] 2005). To further highlight the economic significance of cotton, in countries of Central and Western Africa, such as Benin, Chad, Mali and Senegal, cotton can constitute up to 75% of export earnings and an estimated 10 million people are dependent on this 'white gold' as their only source of cash income (Cotton Incorporated, [WWW] 2005). A poor quality cotton harvest in these regions is devastating.

Cotton quality testing is therefore important in order to allow standardised cotton classing, as the class of cotton ultimately determines the all-important price. The term 'cotton classification' refers to "the application of standardized procedures for measuring the physical attributes of raw cotton that affect the quality of the finished product and/or manufacturing efficiency" (Cotton

Incorporated, [WWW] 2005). Cotton quality is traditionally assessed by experienced cotton classers, who examine the fibres manually to decide characteristics such as colour, trash content and length. However, the use of automated, objective quality classing methods is increasing. The International Cotton Association (ICA), based in Liverpool, UK, provides a worldwide service for the international sale, purchase and movement of raw cotton between consumers, traders and producers, and over 60% of the world's cotton trade is bought and sold under the International Cotton Association Bylaws and Rules (ICA, [WWW] 2005). Many contracts for international trade are agreed subject to ICA Rules, and ICA resolution procedures are brought into practice in the event of a dispute concerning quality. In order to carry out independent cotton arbitration, the ICA has a subsidiary company, the Liverpool Cotton Research Corporation (LCRC), which carries out the routine technical testing of cotton quality parameters.

9.1.1 HVI analysis and quality testing

The LCRC carry out High Volume Instrument (HVI) analysis for the routine assessment of cotton quality. The HVI spectrum is a fully automated means of measuring a number of different cotton fibre properties (described below).

Micronaire

This is a measurement of fibre fineness, represented by the air permeability of a mass of cotton fibres compressed to a fixed volume. Measurement is performed on an instrument called a micronaire and the measurement is commonly known as 'micronaire' or mike (values are typically 2.5-6.5). The

result determines the relative size or fineness of fibres. The reading can also provide a relative indication of fibre maturity or cell wall thickness. The maturity of the fibre is related to the degree of cell thickening, which describes the amount of cellulose that has been deposited into the fibre lumen during the cotton's development. This is specific for a cotton variety, as is fibre length. Mature fibres (with high micronaire values) give a better eveness of yarn with less end-breakages. Dyes are deposited on the cellulose when fibres are coloured, and immature fibres cause differences in the end product as they hinder absorbance.

Fibre length

This is a measurement of the average length of the longer one-half of the fibres (upper half mean length), reported in inches or mm (typically 0.90-1.40 inches or 23-36 mm). This is one of the most important aspects of the fibres as it defines their spinnability, if the fibres are longer in length they can be spun into finer counts of yarn that fetch higher prices. During the development of cotton, the fibres first grow in length, which is ultimately determined genetically by cotton variety. Thus, initially, all fibres have similar length while still on the cotton plant. However, length is reduced by any mechanical treatment such machine harvesting, and ginning.

Fibre strength

Fibre strength is reported in grams per tex, to the nearest tenth. A tex unit is equal to the weight in grams of 1,000 metres of fibre. Therefore, strength is the force needed in grams to break a bundle of fibres one tex unit in size (typically 18-35 grams/tex). Stronger fibres give stronger yarns, so

processing speeds can also be higher giving increased productivity with less end-breakages.

Elongation

Elongation is the percentage increase in fibre length at point of breakage during strength testing (typically 5-8%). A higher value of elongation reduces end-breakages during spinning giving higher productivity with low wastage of raw material. Yarn elongation is also beneficial for weaving efficiently.

Colour

The colour of cotton is measured by the degree of reflectance (Rd) and yellowness (+b). Reflectance (also called greyness) indicates how bright or dull a sample is i.e. the reflectiveness of the surface of a sample (typically 70-80%), a higher value means the sample can reflect light better and the yarn will have a better appearance. Yellowness indicates the degree of yellow colour pigment, measured on Hunter's scale (typically 8-13), a higher value gives a lower grade, resulting in inferior yarns.

9.1.2 Biological contamination and cotton structure/quality

An individual cotton fibre is a single cell seed hair, which forms in two stages, the first is the development of the cuticle and primary wall from the outer layer of the cotton seed, followed by secondary wall thickening as layers of cellulose are deposited inside the primary wall. During the first stage, the fibre reaches its full length and diameter taking about 25 days from plant flowering. The second stage begins 18 days after flowering and takes 30-50 days (Wakelyn, 1986). Figure 9.01 illustrates the essential elements of the cotton fibre structure.

wall. In addition, several cotton fungi are cellulolytic, i.e. they produce the enzyme cellulase, which breaks down cellulose and could potentially damage the primary and secondary cell walls of the fibres (Kaese, *et al.*, [WWW] 2003).

The majority of previous studies investigating correlations between cotton biological contamination and specific fibre quality parameters have focussed on GNB or endotoxin on US cottons. Therefore, it would be of interest to examine whether these relationships extend to cottons from diverse sources and whether fungal cell counts, or glucan levels as a marker of total fungal contamination, demonstrate the same patterns. Methods employed to reduce the presence of organisms that cause the deterioration of cotton fibres, could also help decrease the risk of exposure to byssinosis-causing factors once the cotton reaches the mill, hence the benefits may be two-fold.

Aims

- To measure a range of cotton quality parameters using cotton industry approved methods.
- To investigate relationships between the structural quality of cotton samples and biological contamination measured during the project.
- To examine whether biological contamination can be used as an initial indicator of overall cotton quality.

9.2 MATERIALS AND METHODS

9.2.1 Materials

Equipment

All testing facilities and equipment were provided by the International Cotton Association (ICA), through the Liverpool Cotton Research Corporation, at the Cotton Exchange Building, Liverpool, UK. Testing was carried out under the supervision of Dr. Robert Jiang (Quality Arbitration and Laboratory Manager), with assistance by Albert Purcell (Arbitration and Laboratory Technician). The HVI machine was calibrated daily using United States Department of Agriculture (USDA) standard cottons (for testing length, strength, elongation and micronaire) and a series of ceramic tiles (for testing colour components).

Cotton samples

See Chapter 2 for origin details. Cotton samples of 80-100g were stored in the ICA arbitration room at 20°C prior to testing. Samples were also tested for moisture content to ensure they were all within the range 6.75-8.25% prior to analysis.

9.2.2 Methods

HVI Spectrum

An HVI (High volume instrumentation) machine was utilised to measure micronaire, length, strength, elongation, reflectance (greyness), and yellowness of cotton fibres (see Figure 9.02 below). All measurements were performed on the fully automated HVI machine, with the results recorded simultaneously on a linked computer, using HVI software.

attached computer. The measurements were repeated three times on separate sub-samples and the mean calculated.

Length

20-30g cotton was placed over a plate with a series of holes ([3] on Figure 9.02 above), under which a rotating comb collected a sample of fibres, which were pulled into the measuring zone and aligned into a tapered fibre beard. This was then optically scanned to produce a fibrogram and the length was derived from this image. The process was repeated three times on different areas of the same sub-sample.

Strength/Elongation

These were again measured automatically, simultaneously with length. Following length measurement two pairs of mechanical clamps positioned at a known distance, grasped the fibre bundle and one pair of clamps moved away from the other at a constant speed, stretching the fibre to breaking point, giving strength data. Elongation values were measured by the distance travelled by the clamps before breakage occurred. The mean of triplicate readings was also calculated for these measurements.

Colour (reflectance and yellowness)

Fibre reflectance and yellowness were measured simultaneously, 20-30g of cotton was placed over a photographic plate ([4] on Figure 9.02 above) so that the plate was entirely covered. Light was shone from inside the machine at the cotton, enabling measurement of reflected light (reflectance) and the degree of colouration (yellowness). This was repeated three times on different areas of each sample.

9.4 DISCUSSION

Method appraisal

In order to assess the quality of a range of cotton samples, high volume instrumentation (HVI) methods were employed. This fully automated system is approved by the United States Department of Agriculture (USDA) for the measurement of a range of cotton fibre properties. HVI data is routinely utilised for assessing cotton fibre properties across the world, with more than 1400 systems in place in over 70 countries (Hunter and Spies, 2002). Although a number of other tests have been applied in the past for the assessment of cavitoma, such as the use of microscopy to study fibre swelling. This examination is carried out following addition of 18% sodium hydroxide, as damaged fibre cuticles demonstrate increased swelling. Decreased levels of reducing sugars are also associated with microbial damage and these can be measured by a variety of simple chemical reactions. Methods focussing on the accessibility of the secondary cellulose in the interior of cotton fibres, due to cuticle damage, have also been applied to highlight microbial damage (Allen, *et al.*, 1995). However, HVI analysis is the only available standardised method, allowing generation of quantitative data on a range of fibre parameters, and was used here for these reasons.

It should be noted that cotton fibre parameter values are often very similar, with only slight variations in any particular characteristic across many different fibres, hence factors influencing these changes may be difficult to isolate in some cases.

Influence of production parameters on cotton quality

Throughout the project, cotton samples from Africa exhibited significantly higher levels of biological contamination i.e. had greater Gram-negative bacterial counts, endotoxin concentrations, fungal cell counts and glucan levels, than samples from Asia. If the presence of high levels of these micro-organisms leads to decreased cotton quality, it would be expected that the samples from Africa would be of a generally poorer quality than those from Asia. However, there was no significant difference between mean fibre length, micronaire, reflectance, strength, or yellowness values on cotton from these two regions (see Figure 9.03). Although a marginal decrease in strength and reflectance values, and slightly increased yellowness was seen with the more contaminated samples (i.e. these were somewhat poorer quality fibres). Whereas, Asian cotton samples had a significantly higher mean elongation measurement, a desirable quality in cotton fibres as it gives higher productivity during spinning processes.

Continuing this theme, throughout the project Sudanese Barakat cotton demonstrated significantly lower mean levels of Gram-negative bacteria, endotoxin, and fungi when compared to Acala samples, and as such would be expected to have fibre properties that indicate higher quality. In this regard Barakat cotton did demonstrate significantly improved length, strength and elongation data than Acala samples, although Acala samples displayed better reflectance than Barakat cotton, and there was no difference in yellowness data between the two groups (Figure 9.04). Acala cotton also

exhibited higher micronaire values than Barakat cotton. Micronaire is a complex issue that reflects fibre fineness and maturity, it is largely determined by genetic factors relating to the specific variety of cotton plant grown. Hence it would be unlikely to be greatly influenced by contamination levels. However, a number of indirect factors related to micronaire, may have an influence on contamination levels and these will be addressed in detail below. These findings indicate that increased levels of biological contamination may decrease certain aspects of cotton fibre quality, particularly elongation and possibly fibre strength. In light of this, correlations between quality parameters and specific contamination levels are discussed below.

Gram-negative bacterial counts and HVI data

There were no significant correlations found between cotton fibre quality parameters and Gram-negative bacterial cell counts performed in Chapter 4 of this thesis (see Table 9.01). This was surprising, as several earlier studies have reported a relationship between Gram-negative bacterial counts and fibre quality parameters. For example, a relationship has been shown previously between decreasing cotton colour quality, as assigned by United States Department of Agriculture (USDA) classers and levels of Gram-negative bacteria on US cottons (Morey, *et al.*, 1984) (Table 9.05 below).

desirable. However, the significant correlations between both reflectance and yellowness values and endotoxin concentrations with the Sudanese samples were unexpected. These samples displayed a positive correlation between endotoxin and reflectance, and a negative correlation between endotoxin and yellowness. On face value, these results imply that increased contamination actually improves fibre colour quality. This would be difficult to explain, and the relationships may be related to other factors, which could not be considered here. For example, cotton with high levels of yellowness, may possess a characteristic, which is unfavourable for the growth of Gram-negative bacteria, resulting in lower counts, and an apparently inverse relationship. Gram-positive bacteria are also capable of causing fibre damage, and some types produce cellulase enzymes, including the cotton bacteria *Clostridium*, which may damage the cellulose cotton fibres. The relationship between cotton Gram-positive and Gram-negative bacteria warrants further examination in order to elucidate the effect of bacterial competition on these relationships.

Similarly, the positive relationship demonstrated between fibre reflectance and endotoxin concentrations, may be the result of fibres with increasing reflectance possessing a factor such as nutrient availability, which encourages the growth of Gram-negative bacteria. Moreover, numerous other factors such as climate, presence of trash particles, and genetic influences may also have a modifying influence, disrupting any direct 'cause and effect' relationships.

Micronaire is also a complex issue; immature fibres are fine in diameter, hence these fibres give a low micronaire reading, but as a cotton fibre matures, increasing amounts of cellulose are added to the interior of the fibre, leading to more mature, coarser fibres and increased micronaire value. A desirable micronaire reading, giving a premium cotton price, derives from a balance between these two types of fibres. In this project, correlations between endotoxin concentrations and micronaire values were subject to conflicting results between inter and intra country sample groups. The diverse samples showed a negative correlation with micronaire, and the Sudanese samples demonstrated a positive relationship with this measurement. Again, cause and effect is hard to define from these results.

It is known that immature cotton fibres (with low micronaire values) have higher levels of free plant sugars than mature fibres (University of Arizona, [WWW] 2005). These are simple sugars, including glucose, fructose, and sucrose, which are all excellent nutrition for bacteria, and higher levels could support larger numbers, giving a negative correlation between endotoxin and micronaire data. Additionally, coarse fibres (having increasing micronaire readings) may carry less microbial contamination than finer fibres because they have a lower surface area per gram of cotton (Sasser, 1997). Both of these factors provide some explanation for an inverse relationship between micronaire and endotoxin. However, fibres with increasing maturity (and increasing micronaire) are likely to have spent longer periods of time maturing in the field, than finer, less mature fibres. This prolonged exposure to field weathering would be conducive to increased bacterial contamination,

(i.e. a positive correlation between endotoxin and micronaire data). This factor appears to have been more important in the case of the Sudanese samples. The conflicting results regarding micronaire and biological contamination, demonstrate that further investigation is required into this complex issue.

The significant negative correlation between fibre length and endotoxin concentration on the Sudanese cotton samples, may also initially appear unexpected. Due to the fact, that fibre length is also determined by genetic factors, and the finding that shorter fibres have higher levels of endotoxin contamination again contradicts what was expected (i.e. longer fibres have increased surface area and hence will support higher levels of contamination). However, cotton fibre length is also affected by mechanical treatment, where processes such as machine harvesting and ginning break fibre ends, decreasing their length. High endotoxin concentrations (reflecting high GNB levels) may cause fibres to weaken, and hence to break more readily under these physical stresses. The negative correlation with elongation data discussed above supports this hypothesis, as does strength correlation data, which shows a negative, though not significant relationship with endotoxin concentrations.

Previous studies have analysed the relationship between endotoxin content and fibre properties measured by HVI analysis; some of these results are summarised below in Table 9.07. These data demonstrate significant negative correlations between endotoxin and all fibre parameters except

with endotoxin concentrations. Again, the reasons for this relatively strong correlation are hard to isolate, but may also be ascribed to other factors not considered in this study as described above. Negative correlations were demonstrated with fibre elongation and fungal counts. As with endotoxin levels, fibre length was inversely related to fungal contamination, perhaps also for the reasons described above, where increased fibre breakage occurs with more contaminated fibres. The negative correlations between fungal counts and both elongation and strength data (though not all significant) are further evidence for this conclusion.

A previous study focussing on the effect of fungal contamination on cotton fibre strength, in which sterilised cotton fibre samples were incubated with a range of aqueous fungal cultures at 25°C for 14 days, reported that different fungal species decreased fibre strength to varying degrees (Kaese, *et al.*, [WWW] 2003). It was also found that nutrient availability also had a species-specific effect on cotton fibre degradation, and increased nutrient availability provided via the culture medium, was detrimental to fibre strength (Table 9.08 below). Hence, nutrient availability on cotton fibres in the field situation may influence the degree of fibre damage. It is notable from these data, that *Aspergillus niger* is one of the most potent species to affect fibre quality, particularly since this was the most commonly isolated fungal species in this project (see Chapter 6). However, fibre strength did not seem to be significantly affected by fungal cell counts in this project, although weak negative coefficients were noted. The data outlined below, were gathered

Glucan levels and HVI data

Glucan levels revealed similar correlations to those demonstrated by fungal cell counts, but the statistical significance of these relationships differed. Glucan data was only available for the samples from diverse sources, and these possessed a significant negative correlation with both reflectance and micronaire data (see Table 9.04). The reasons behind these correlations have been discussed previously in the context of endotoxin concentrations, and are likely to also apply to the fungal contamination as reflected here by glucan levels. Viable fungal cell counts from diverse samples also showed negative relationships with these two properties, but these coefficients were weaker than with glucan, and not significant (Table 9.03). As with fungal counts, glucan levels showed no discernable effect on fibre strength data, perhaps also due to the reasons given above, in relation to fungal counts, regarding the inducible production of cellulase enzymes.

As discussed elsewhere in this thesis (Chapter 8), glucan levels may be more stable than fungal cell counts, giving a better indication of total fungal contamination levels. However, there was a significant correlation between fungal cell counts and glucan levels reported in Chapter 8, although this was not very strong (0.653). This may explain why similar correlations were observed between fibre properties and both fungal cells and glucan levels.

Preserving cotton quality

Treatments that render cotton fibres safer to production workers, such as cotton washing (Wakelyn, 1986), may conflict with the need to retain fibre quality because harsh treatments will inevitably effect fibre properties. This highlights the need to explore methods, which will remove biological contamination in a manner that will benefit both cotton quality and safety. Such methods should attract higher investment, as they will be of mutual benefit to two sectors of the industry. Methods that decrease the biological contamination of cotton fibres will be discussed in detail in the next chapter.

Summary

HVI methods were utilised in order to assess a range of cotton fibre properties. Correlation coefficients were then calculated between these data and biological contamination parameters reported elsewhere in the thesis. Samples produced in different geographical regions did not demonstrate significantly differing qualities, but cotton samples from different plant species were shown to vary in several ways. Gram-negative bacteria showed no significant correlations with fibre properties, but endotoxin concentrations were correlated with fibre elongation, length, micronaire and colour values. There was some conflict whether certain parameters had a negative or positive relationship with endotoxin, and reasons for this were discussed. Similar patterns were also demonstrated with fungi and glucan data, but it was concluded that further focussed studies are required, to fully understand these associations.

CHAPTER 10

GENERAL DISCUSSION AND FUTURE FOCUS

10.1 CONCLUSIONS

This study analysed four biological cotton contamination parameters on samples from fourteen different countries with a worldwide distribution. In this respect it is unique, principally because previous studies have focussed upon only one contaminant or on cotton from one region (in many cases the United States). This focus has had two effects; due to this previous attention on single parameters, investigation into the relationships that exist between different contaminants have been neglected and thus our understanding remains poor. Furthermore, the priority given to analysis of US cotton in the past has been misplaced, even though this is one of the largest cotton producing nations, the health problems associated with cotton processing are now almost of negligible proportions in North America. Whereas, many other countries would benefit from research into the causes of cotton contamination, and its health effects. Future research should focus on other major cotton producers such as China, India, Pakistan, Brazil and the former Soviet Union, whose large cotton industry workforce does not necessarily correspond to high standards of occupational safety. Smaller cotton producers who lack the means of monitoring their workforce due to financial and political constraints, for example the African countries of Benin, Ivory Coast, Sudan and Zimbabwe, should also be included. Espcially since the findings of this study indicated high levels of cotton contamination in some of these regions, and cotton production in these countries is expanding.

The findings from individual chapters of this study were discussed at the appropriate place in each chapter. Therefore, these discussions will not be repeated. However, this study has highlighted some general issues, which have direct relevance to the health of cotton workers, and these are summarised below:

- Cotton samples from every country analysed, including samples stored for up to six years and of different species, contained significant levels of Gram-negative bacteria (GNB), endotoxin, fungi and glucan.
- Cotton samples from African countries consistently carried higher mean levels of all four contamination parameters than those from Asia.
- *Gossypium hirsutum* contained higher mean levels of Gram-negative bacteria, endotoxin and fungi than *G. barbadense*.
- Similar GNB species were identified on cotton samples from around the world; the most common was *Enterobacter,* which is associated with one of the most potent LPS molecules.
- Identified fungal genera were also similar on all cotton samples analysed, the most common being *Aspergillus*; a fungus capable of producing a range of mycotoxins, including the carcinogen aflatoxin.
- Trash material often contained 10-100 times higher levels of contamination than equivalent lint samples.
- Significant positive correlations existed between the contamination parameters, particularly endotoxin and glucan.

Several of these findings, suggest that the cotton production environment is more hazardous than previously thought. Certain cotton bales may introduce simultaneously high levels of bacterial and fungal cells, potent endotoxin, glucan and mycotoxins into the cotton mill. Currently, it is only inert dust levels, which are monitored to safe levels within production facilities. Controlling levels of dust has little bearing on the concentrations of any of these agents. Hence, further research is required to monitor and reduce their levels.

This project also highlighted the number of different factors involved in cotton production, which may have an influence on the contamination levels of the fibres. In turn, these variables indicate the complexities involved in studying natural cotton fibres. The factors implicated in the biological contamination of cotton are detailed below (Figure 10.01). It is likely that some of these variables have a more significant influence than others do. Although controlled studies would be difficult, they are needed to fully elucidate the impact of each of these factors. This is important, as small changes in cotton production techniques may be a straightforward and economic way of decreasing incidences of byssinosis.

Also highlighted in this thesis, was the lack of information regarding prevalence rates of byssinosis. Reports are often out of date, and do not come from official sources, because of this the extent of the problem may be worse than currently estimated. There have been trials to assess methods,

stages during the production of cotton fibre, and should be aimed at trying to decrease the effects of factors displayed in Figure 10.01 above. Whereas, secondary methods include epidemiological surveillance, identification of susceptible individuals, and early diagnosis of the disease (Bates, *et al.*, 1997).

There are numerous steps that can be adopted to modify production practices in order to reduce the biological contamination of cotton fibres, and decrease the risk of production operatives developing byssinosis. The results of this project showed that the micro-organisms, which contaminate cotton originate in the soil and plant material. Thorough preparation of crop land, such as removal of decomposing plant material from the previous season and treatment of weeds may decrease the reservoir of microbes present in the environment and slow the contamination of new plants. The species of cotton planted also appeared to influence the levels of contamination, as Sudan Acala cotton *(G. hirsutum)* had higher levels than Barakat cotton *(G. barbadense)*. Perhaps use of the species that harbour less microbes should be promoted. This is also linked to insect infestation, since *G. hirsutum* is more susceptible to whitefly (Khalifa, 2001), which aids the dispersal of bacteria and produces large amounts of honeydew sugars; the use of insecticides may decrease contamination by removing this food source.

Harvesting practices have also been examined with a focus on the microbial colonisation of cotton. It seems the longer cotton remains exposed in the field prior to harvest, the greater the levels of biological contamination when it is

harvested, due to field weathering (Fischer and Sasser, 1987; Millner, *et al.*, 1984). Hence, the use of chemical defoliants to allow machine harvesting to proceed as early as possible after boll opening may help to decrease the risks, especially as climatic conditions have been shown to be important factors. Cotton exposed to rainfall and frost has increased contamination levels (Morey, *et al.*, 1983), and the early harvesting of cotton may minimise these effects. Trash particles have been found to be highly contaminated with microbes and their toxins in this study, therefore harvest methods which involve the collection of as little trash material as possible may also be important. In this regard, hand picked cotton is preferred over machine harvested fibres. However, due to the fact that handpicking is labour intensive and time consuming, machines are used increasingly for practical reasons. Further research into these small adjustments to production procedures is warranted, as alterations at this level are practical and likely to be cost effective. However, their use may be of limited value, given the prolific growth of micro-organisms on cotton, and the high densities reported in this thesis.

Thirty years ago, prior to the identification of any specific causative factors for byssinosis, prevention methods were focussed on reducing the production of general cotton dust. Investigations included a number of procedures to wash the cotton prior to processing. Cotton washing studies involved a number of different test methods including batch and continuous protocols (Wakelyn, 1986). For example, using a rayon rinse system, which consisted of passing cotton on a wire mesh conveyor through a series of soaking chambers,

where water at 60°C was applied by gravity to the cotton. This was followed by several rollers, which squeezed the cotton to expel water, before it was dried by hot blown air. Variations included passing cotton through a series of tanks filled with steam heated water (the wool scouring method) and a batch system where a sequence of three treatments; wash-scour-bleach were applied to the cotton before it was squeezed by rollers and dried in 140°C compressed air (Sasser, *et al.*, 1986). Methods to expose cotton to high temperatures in steam chambers were also developed (Imbus and Suh, 1974; Merchant, *et al.*, 1974).

These processes were found to significantly decrease the amount of dust released during processing (Perkins, *et al.*, 1986) and a reduced decline in lung function was observed when human subjects were exposed to washed cotton in an experimental card room (Castellan, 1986; Petsonk, *et al.*, 1986). However, there are drawbacks with the commercial use of these systems, in that they are expensive, time-consuming, use large amounts of water and energy, and are detrimental to the structural quality of the cotton. Further to this, if the cotton is not fully dried after the washing process, large numbers of micro-organisms may quickly recontaminate the fibres (Jacobs, 1987). Therefore, these methods are not feasible in the high-throughput industrial situation. The need to retain the quality and processibility of treated cotton is an underlying problem when developing byssinosis prevention methods. Over-spraying cotton with oil has been utilised in a pilot study, which reduced dust levels by 50% in the early processing stages but there was an increase

in levels after carding and unfortunately oil covered cotton is difficult to process (Jacobs, 1987).

Since the identification of endotoxin as a causative agent for byssinosis, methods to render this agent harmless upon inhalation have been examined. Studies attempting heat detoxification of endotoxin by applying heated metal plates to cotton fibre for several minutes, found temperatures of 200°C reduced endotoxin levels, but caused significant damage to the cotton fibres (Rouselle, 1990). However, by exposing cotton to 250°C for 60 seconds in a forced-draft oven, the endotoxin content of the fibre was reduced by 76% and less damage occurred to the fibres (Rouselle, 1991), although a 6-11% reduction in fibre tenacity, a 3-6% reduction in fibre length and slightly increased fibre yellowness were still recorded following this treatment. Again, the practicalities of these methods in the cotton industry are questionable.

More recently, a promising study has analysed the use of bactericidal treatment of raw cotton as a method of byssinosis prevention (Hend, *et al.*, 2003). Opened cotton capsules were sprayed with an aqueous solution of benzododecinium bromide, the cotton samples were then exposed to warm temperature (40°C) and humidity (95%) to simulate conditions during cotton transport and storage, which promote the growth of bacteria. Endotoxin concentrations measured by LAL assay on sprayed cottons remained low, while those on untreated samples increased significantly following several weeks in storage (Table 10.01).

One of the most successful ways of preventing byssinosis employed to date has been the introduction of permissible dust exposure levels. The US cotton dust standard was introduced in 1978 and revised in 1985, by OSHA. To ensure this standard is maintained, employers must follow a dust surveillance program, where dust levels are measured every 6 months. Enforcement is through site inspections and those not adhering to the standard can be fined. Modern cotton mills are engineered to ensure that safe exposure levels are maintained by efficient exhaust and ventilation systems. Health monitoring is also an integral part of these programs; employees undergo an annual medical review to identify any adverse effect on lung function. Education and training is also paramount, so workers are aware of safe working practices and what they can do to minimise risks; for example, the wearing of respirators and cessation of smoking is strongly recommended (Jacobs, 1987).

Similar cotton dust standard programs now operate in many developed countries, and have been met with success. Mortality due to byssinosis in the US had decreased to just 7 deaths in 1999, a rate of 0.03 deaths per million population (CDC, [WWW] 2005). However, byssinosis persists in less developed countries, where suggestions for dust control are often beyond the means of governments to efficiently enforce. Here, information development programs providing education, training and health promotions are an inexpensive way to begin combating the problem, by bringing the dangers to the attention of those at risk. The International Labour Organisation (ILO) and

World Health Organisation (WHO) have initiated occupational hygiene programs in Africa, Southeast Asia and Latin America (Ahasan, *et al.*, 2000).

10.3 INDUSTRIAL TOXICANT MONITORING

Several early papers addressing the issue of byssinosis prevention, cite the lack of knowledge regarding specific causative factors as a stumbling block to competently confronting the disease (Bates, *et al.*, 1992; Jacobs, 1987; Wakelyn, 1986). Now that several causative agents have been identified, including endotoxin and glucan, this stumbling block has been removed; only to be replaced by several others. The environmental measurement of endotoxin is plagued by a lack of standards, as detailed in Chapter 5 of this thesis. The influence of protocol variation on the reproducibility of LAL-measured endotoxin levels has been examined by Chun, (1999) whereby endotoxin content was assessed in dust samples taken from a common source using in-house extraction and assay protocols in several laboratories. These data were then compared to results obtained using a unified extraction protocol, followed by in-house assay. The outcome was that less variation occurred when the common extraction protocol was adopted. Reproducibility within each laboratory was high, indicating that further standardisation i.e. identical assay kits, protocol and detection equipment, would have led to a further reduction in variability (Chun, 1999). This study therefore, highlighted the sizable impact a uniform protocol would have on assessment of endotoxin in the environment.

The results of this project demonstrated the validity of the LAL assay on cotton samples and showed it to be reproducible when applied to cotton lint fibres as opposed to dust levels, on samples from across the world. However, there are shortcomings of the assay; qualities such as stability, sensitivity, and linearity depend on the individual manufacturers formulation. Even from one manufacturer, different LAL test batches may yield fluctuating results due to biovariation in the source population, hence the use of an internal reference standard i.e. control standard endotoxin (CSE), is imperative. Other drawbacks of the assay include the production of the lysate reagent, which still relies on the harvesting of Horseshoe Crab amoebocytes by capture and passive bleeding. Although these animals are subsequently released with a mortality rate of <10%, there have been calls to develop another means of enzyme production, which would eliminate inter-batch variation, spare the use of this ancient species, and reduce costs (Cambrex, 2003).

This concept has been realised by genetic engineering of proteins within the enzyme pathway. In this context, recombinant Factor C (rFC) has been engineered and validated (Ding and Ho, 2001) and there is now a recombinant factor C endotoxin detection system commercially available (Pyrogene™ from Cambrex). This system, which is produced entirely independently of Horseshoe Crabs, also demonstrates batch uniformity (Cambrex, 2003). A production technique of such sophistication for a LAL assay reagent from a perpetual source can only facilitate the use of this test in the environment. However, currently the new assay is untested in the

occupational setting and prohibitively expensive for use on even a moderate number of samples.

Fungal agents such as mycotoxins and glucans, only emerged as occupational hazards more recently, and have not yet attracted the levels of research focussed on endotoxin. Therefore, methods of decreasing these toxicants are as yet unknown. Modified LAL assays may be a means of monitoring environmental glucan levels, although as demonstrated in this thesis, these are not always adaptable to this setting. However, very little is known about airborne mycotoxins and other fungal-derived volatile organic compounds, despite the likelihood that they pose a significant threat to cotton and other industrial workers. Although it is unlikely that these agents are a cause of byssinosis, inhalation may lead to other serious illnesses such as damage to the central nervous system, reproductive problems and cancer (Bennett and Klich, 2003). Hence there is a need to assess the risks posed by these extremely toxic low molecular weight compounds, and to examine whether they can be filtered out by the use of respirators or if other means of protection is required.

Establishment of endotoxin exposure limits alone may appear simplistic. However, endotoxin is currently the most widely characterised of the multiple toxic factors present in these complex dusts, hence a focus upon it is justified. Continued profiling of occupational respiratory toxicants and their respiratory effects will be of future value. A more detailed appreciation of the reactions involved when endotoxin, glucan and as yet unidentified agents,

are inhaled, will assist with future disease prevention. Recent progress in this area is described below.

10.4 RECENT PROGRESS IN THE FIELD

There remains a lack of knowledge regarding the actions of both endotoxin and glucan when inhaled into the respiratory system. This awareness is required in order to identify possible therapeutic targets. However, progress has been swift and several recent findings have significantly advanced scientific comprehension.

10.4.1 LPS and the TLR4 signalling pathway

Recognition of LPS by its primary targets such as the alveolar macrophage is the subject of current investigation and a unifying theory has only recently become clear. CD14, a 55 kD glycoprotein expressed in monocytes, macrophages and neutrophils, binds with high affinity to LPS. CD14, which can exist in membrane and soluble forms, is central to the recognition process and crucial to the subsequent response to LPS (Erridge, *et al.*, 2002). Along with CD14, a second receptor was identified some time ago; lipopolysaccharide-binding protein (LBP) an acute phase protein expressed on several host cell types, enhances the capacity of LPS to bind and activate macrophages and neutrophils, and binds specifically to the lipid A section of LPS (Kirkiae, *et al.*, 1994). Along with LBP and CD14, the β_2 integrins, CD11b/CD18 (subunits of complement receptor CR3) are also involved in LPS recognition and subsequent signalling (Dobrovolskaia and Vogel, 2002).

The discovery of the toll-like receptor TLR4, a type I transmembrane protein, by Medzhitov, *et al.*, (1997), was a significant advance in understanding LPS signalling, as its involvement in LPS recognition quickly became evident (Dobrovolskaia and Vogel, 2002). There has been a surge of research into the TLR family since the discovery of TLR4, and ten members have now been characterised (Akira, 2003). TLRs are present on antigen presenting cells, and form part of the innate immune system. They are known as pattern recognition receptors (PRRs), which recognise conserved molecular features of certain microbial groups, called 'pathogen-associated molecular patterns' (PAMPs) (Horner and Raz, 2003). For example, LPS is a TLR4 ligand, whereas TLR2 has several ligands including peptidoglycan from Gram-positive bacteria and TLR9 is required for responses to bacterial DNA (Akira, 2003). TLRs are characterised by extracellular leucine and cysteine-rich regions, as well as a cytoplasmic Toll/IL-1 receptor (TIR) domain (Dobrovolskaia and Vogel, 2002). All TLRs activate a complex universal signalling pathway, which via either of the adaptor proteins MyD88 or TIRAP (TIR domain-containing adaptor protein, also known as Mal) ultimately leads to the activation of nuclear factor kappa B (NFκB) transcription factors and mitogen-activation protein (MAP) kinases. This results in the recruitment of leukocytes, and release of inflammatory cytokines and chemokines such as various interleukins, TNFα, and reactive oxygen species (Akira, 2003; Barton and Medzhitov, 2003; Cook, *et al.*, 2004; Horner and Raz, 2003).

Research focussing on TLR4 and LPS recognition, lead to the discovery of another molecule, the MD-2 protein, which is required to associate with TLR-4 to allow it to interact with LPS (Akira, 2003). MD-2 also has a role in the intracellular distribution of TLR4 (Miyake, 2003). In light of these recent findings, a model has been proposed for LPS recognition in mammalian phagocytes, where the combined actions of the LPS-binding protein (LBP), the membrane bound or soluble forms of CD14, along with the recently identified TLR4-MD-2 complex, cofacilitate rapid molecular recognition (Heumann and Thierry, 2002). Figure 10.02 below illustrates the current understanding of LPS recognition and signalling via the MyD88-dependent pathway.

TLR4 can also activate a MyD88-independent signalling pathway, which requires the different Toll/interleukin 1 receptor adaptor proteins, TRIF and TRAM (TIR domain-containing adaptor inducing IFNα, also known as TICAM-1 and Toll-like receptor adaptor molecule 2, or TICAM-2). These activate interferon regulatory factors (IRF3 and IRF7), resulting in production of interferon inducible genes (e.g. IFN-α/β) and the late phase activation of NFκB and MAP kinases (Zughaier, *et al.*, 2005). Specific interaction mechanisms between endotoxin and these different adaptor molecules remains poorly characterised. However, this aspect of endotoxin pharmacology is advancing rapidly and the attainment of a widely accepted, detailed hypothesis regarding LPS signalling is imminent.

confounded decisions on 'no effect levels'. This variability could stem from polymorphisms in the genes encoding either endotoxin-released cytokines or the LPS receptor CD14 (Michel, 2000). Genetic evidence has also been reported showing that common mutations in TLR4 are linked to variations in human responses to inhaled and systemic LPS (Schwartz, 2001; Michel, *et al.*, 2003 respectively). Consequently, further characterisation of additional genes involved in the deleterious effects of endotoxin inhalation is essential to any further conceptual understanding of this phenomenon. The genetic regulation of endotoxin-induced airway disease is now being examined by a combination of quantitative trait locus analysis, micro array-based gene expression studies and recombinant inbred mice studies (Cook, *et al.*, 2004).

10.4.2 Glucan recognition

Reports on the biological effects of β-glucans in the literature can be conflicting, this is partly due to variations in the source and structure of the glucans studied, as well as differing exposure protocols. These contradictions are exacerbated by a lack of understanding concerning the innate recognition of glucan. The first cellular target of glucan is the macrophage, which is subsequently activated. There are thought to be two phases in this interaction: an initial binding phase followed by internalisation (Williams, *et al.*, 1996). Pattern recognition receptors (PRRs) for glucan have also been identified on various other leukocytes such as neutrophils and eosinophils as well as non-immune cells like endothelial and alveolar epithelial cells (Brown and Gordon, 2003). A number of glucan receptors have been identified,

reaction, which becomes phosphorylated during the process (Brown and Gordon, 2003).

TLR2 only recognises specific components of zymosan and does not bind to other β-glucans, hence Dectin-1 remains the central receptor for these compounds, and mediation through the pathway suggested above is likely to be key to human responses (Willment, *et al.*, 2005). However, it is worth noting that some of the potent inflammatory responses demonstrated in various glucan studies, may be due to contamination by unknown TLR2 ligands. Perhaps explaining the contradictory nature of some of these results. Studies on Dectin-1 knockout mice may further elucidate its role in these responses (Brown and Gordon, 2003).

These rapidly advancing areas of research will lead to a greater understanding of some key areas of occupational toxicant exposure. For example, this thesis has revealed that the simultaneous presence of high levels of endotoxin and glucan in cotton production facilities is very likely. Although it is known that glucan may exacerbate the pharmacological effects of endotoxin, how and to what extent this occurs is currently unknown. Further elucidation of the action mechanisms of these agents may aid understanding of this phenomenon. Advances may also assist in the development of therapeutic targets for occupational lung disease.

10.4.3 Potential therapeutic routes

A fuller understanding of how endotoxin exerts its biological effects has enabled the development of more sophisticated methods of preventing airway inflammation. Although in the early stages, examples of areas of very recent research include attempts to block the actions of NFκB transcription factors resulting from the pathways described above. This has shown some success in studies with asthma (Desmet, *et al.*, 2004) and in experiments to prevent lung injury during endotoxic sepsis (Baetz, *et al.*, 2005; Matsuda, *et al.*, 2005). In these studies, oligonucleotides have been tested as NFκB decoys, which compete with NFκB binding elements to interfere with gene transcription, thus blocking the inflammatory response. Thalidomide may also be an agent with potential as a therapeutic agent against neutrophil-mediated inflammation such as that caused by endotoxin. Thalidomide is thought to selectively suppress NFκB activation, and administration of thalidomide has been shown to inhibit production of TNFα and promote recovery from symptoms associated with neutrophilia (Yasui, *et al.*, 2005).

Further methods of prevention may stem from pre-treatment with Toll-like receptor antagonists, for example E5564, a lipid A analog. Intratracheal treatment with 10 and 100 μg of this agent has been shown to inhibit airway responses to LPS for up to 48 hours after administration in mouse-models of environmental airway disease (Savov, *et al.*, 2005). High levels of vitamin E have also been shown to inhibit proinflammatory cytokine and endotoxin-induced release of nitric oxide in alveolar macrophages (Khanduja, *et al.*, 2005). Hence, agents of this type may have a therapeutic role in chronic

endotoxin-induced airway disease. These recent examples highlight the progress that can be made to prevent occupational lung diseases such as byssinosis, once a full understanding of the causes is obtained.

10.5 WIDER APPLICATIONS

This thesis has focussed specifically upon endotoxin and glucan as respiratory toxicants of the cotton industry. However, both these agents are being identified in an increasing number of environments, where derivatives of the LAL assay are proving useful in assessing the levels present and their potential effects on health. The LAL assay has been used to quantify airborne endotoxin in such environments as farming and agriculture (Lundholm *et al.*, 1986; Olenchock, *et al.*, 1986), metal working (Laitinen, *et al.*, 2001; Park, *et al.*, 2001), refuse handling (Neumann, *et al.*, 2002), and wood processing (Laitinen, *et al.*, 2001; Simpson, *et al.*, 1999). Table 10.03 below displays the wide variation in endotoxin concentrations measured in a range of occupational environments by the LAL assay. Despite this widespread research use of the LAL assay, as with the cotton industry there are no standard methods for the collection, extraction or measurement of endotoxin, and no industry has implemented a safety program involving the monitoring of endotoxin. Progress in this area in the textile industry may act as a precedent for other industries.

is an increased incidence of respiratory disease (Heinrich, 2001; Gehring, *et al*, 2001; Michel, 2000). In particular, high levels of bacterial and fungal contamination are implicated as a major factor linking damp housing with lung problems. Levels of these agents in the non-industrial setting are generally much lower than those recorded in industrial environments. Nevertheless, accumulating evidence from homes, offices, schools and day-care centres suggests that these levels are potentially detrimental to human respiratory health.

Household endotoxin testing has been more widely performed over recent years, where the LAL assay has been applied to aqueous extracts either from settled or airborne dust samples collected respectively by vacuum cleaner or gravimetric filter (Gehring, *et al.*, 2001; Heinrich, *et al.*, 2001; Park, *et al.*, 2000; Su, *et al.*, 2001; Wan and Li, 1999). Household pets such as cats and dogs or vermin such as cockroaches have also been associated with higher endotoxin levels in house dust (Heinrich, *et al.*, 2001). In such studies, coupling basic endotoxin data with respiratory symptom profiles collected by questionnaire survey, is important in identifying correlations between endotoxin level and health risk (Vojta, *et al.*, 2002).

In a large-scale indoor study, a correlation was also revealed between elevated concentrations of β-glucan and the proportion of individuals with nasal and throat irritation, dry cough, headache and excessive tiredness (Rylander, 1996). In a similar investigation comparing two schools, it was shown that the prevalence of respiratory symptoms was significantly higher in

the school with β-glucan elevation (Rylander, *et al.*, 1998). These findings explain why glucans are now strongly associated with "sick building" syndrome, where particular buildings are linked with higher levels of staff absence due to illness.

Domestic endotoxin has several implications, and evidence suggests that it could have a synergistic effect on IgE-mediated immune responses (Michel, *et al.*, 1996). Low-level domestic endotoxin exposure in children has been coupled with increased incidence of non-specific respiratory illness. It has also been reported that inhalation of endotoxin increases the severity of chronic conditions such as asthma (Su, *et al.*, 2001; Thorn, 2001b), especially in individuals sensitive to the house dust mite (Fernández-Caldas, 2002). It may also increase the susceptibility of asthmatics to rhinovirus-induced colds, chronic bronchitis and emphysema (Reed and Milton, 2001). In contrast, a certain degree of endotoxin exposure may actually be beneficial in young children, where it appears to protect against such conditions as atopic eczema, asthma and allergic rhinitis later in life. This may arise from promotion of T_H1 responses, conferring greater tolerance to common allergens (Liu, 2004; Reed and Milton, 2001), but the timing of the exposure is likely to play an important role in mediating this effect (Singh and Schwartz, 2005). Specific sources of the toxin in the home, as well as seasonal and climatic patterns of endotoxin fluctuation are poorly understood, and further investigation here is warranted (Park, *et al.*, 2000).

Endotoxin concentrations in tobacco have also been reported using the LAL assay (Hasday, *et al.*, 1999). This work was prompted by comparison between the effects of cigarettes on the lungs and agricultural dust inhalation, since both lead to chronic bronchitis and airway obstruction. Similar levels of endotoxin in cigarettes (6-9 μg/g tobacco) to those reported in other agricultural products were demonstrated. Although temperatures at the tip of a lit cigarette are sufficiently high to inactivate LPS, tobacco upstream from the tip is at a lower temperature, and it is thought that 1% of biologically active LPS in unsmoked cigarettes remains as an active component of the smoke (Hasday, *et al.*, 1999). This example stresses the diversity of materials that can be analysed for their endotoxin content using the LAL assay, and it is certain that additional important sources of this toxin will be revealed in the future by the employment of this method.

Therefore, research into the respiratory toxicants, endotoxin and glucan is now being carried out in a variety of scientific disciplines and a number of aspects are being examined, such as their source, structure, pharmacology, quantification, prevention and potential benefits. The more knowledge available regarding these compounds, the more equipped the world will be to combat their detrimental effects in both the occupational and non-industrial environments.

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PERSONAL COMMUNICATIONS

Chun, David; Microbiologist, USDA/ARS Cotton Quality Research Station, PO Box 792, Clemson SC 29633, received 30/09/2002.

Jiang, Robert; Arbitration supervisor, Liverpool Cotton Research Corporation, UK, received 19/11/2004.

Jordan, Foster; Head of *in vitro* products, Charles River Endosafe, U.S., received 12/02/2003.

Metcalfe, Kim; Technical application specialist – UK South, Charles River Endosafe, Europe, received 04/06/2004.

APPENDIX I: EQUIPMENT LIST

Reagents/chemicals and suppliers

Acetone, Sigma, UK

API reagents, Biomérieux, France

Chloramphenicol, Sigma, UK

Control Standard Endotoxin (19 EU/ng), Charles River, France

Crystal Violet, Sigma, UK

Cycloheximide, Sigma, UK

Endotoxin-specific buffer, pyrogen-free <0.005EU/ml, Charles River, France

Ethanol, Fisher, UK

Glucatell reagent kit, Associates of Cape Cod Incorporated, USA

Iodine, Sigma, UK

Lactophenol blue solution, Sigma, UK

LAL reagent, Charles River, France

Malt extract agar, Sigma, UK

Mineral oil, Biomérieux, France

Oxidase reagent, Biomérieux, France

Phosphate buffered saline, Sigma, UK

Pyrogen-free water, <0.001EU/ml, Charles River, France

Safranin O, Sigma, UK

Sodium Chloride Solution, Biomérieux, France

Tryptic Soy Agar, Sigma, UK

Tween-20, (polyoxyethylene-sorbitan monolaurate), Sigma, UK

Vancomycin, Sigma, UK

Equipment and suppliers

API dehydrated identification strips, Biomérieux, France

API reference book, Biomérieux, France

Centrifuge tubes, PET, 15ml, pyrogen-free, Fischer, UK

Dilution tubes, borosilicate glass, (<0.001EU/ml), Charles River, France

Filter paper circles, Whatman 55mm, Fisher, UK

Microscope slide cover slips, circular, glass, Fisher, UK

Microscope slides, Menzel superfrost 76 X 26mm, Fisher, UK

Microtiter plates, 96 well, individually wrapped, pyrogen free, Falcon, UK

Petri dishes, 18cm, non-vented, Fisher, UK

Pipettes, 10, 5, 2 and 1 ml, glass (<0.001EU/ml), Charles River, France

Pipette tips, 100µl, Eppendorf (<0.001EU/ml), Fisher, UK

Swabs, sterile individually wrapped, Biomérieux, France

Syringes, 5ml, Eppendorf, individually wrapped (<0.001EU/ml), Fisher, UK

Apparatus

Balance, Precisa 125A

HVI Spectrum machine, Uster

HVI software, Uster

Incubator, Gallenkamp, economy size 7

Light microscope, Leica with QWIN windows analysis software

Light microscope, Olympus SP

Microplate reader, MRX Revelation

Moisture meter, Drycom

Open balance (HVI), Monobloc

Pipette, 1-200µl, Gilson

Pipette, 0.5-10µl, Jencon

Repeating pipette, 1-200µl, Eppendorf, multipette plus

Shirley Analyser, SDL International Ltd

Vortex Genie 2, Scientific Industries

Vortex multi-mixer, Vibrax VXR Basic

APPENDIX II: ISOLATION OF MICROBIAL CELLS

The isolation media was engineered to be selective; Tryptic soy agar (TSA) is a general-purpose bacterial growth medium, as is maltase extract agar (MEA) for fungal cells, specific antibiotics were added to make these selective.

Antibiotics

Chloramphenicol: Inhibits translation of the bacterial 50S ribosomal subunit at the peptidyltransferase step (elongation inhibition) it is therefore bacteriostatic and was added to MEA to select for fungal cells.

Cycloheximide: A protein synthesis inhibitor (translocation step) of eukaryotic cells, it is a fungicide and was added to TSA to inhibit the growth of fungi.

Vancomycin: Inhibits the biosynthetic pathway of the cell wall peptidoglycan layer in Gram-positive bacteria, causing the cell to lyse. It is unable to pass through the outer layer of Gram-negative bacteria hence is specific for Gram-positive bacteria. It was added to TSA to select for Gram-negative cells.

APPENDIX III: BASIS OF THE GRAM STAIN

The Gram differentiation is the most fundamental division of bacterial species. It is based upon the colour reactions of bacteria in a fixed smear when treated with the primary dye crystal violet, followed by a mordant such as iodine solution. Certain organisms subsequently lose the violet colour rapidly when a decolourising agent such as ethyl alcohol is applied. Others lose their colour more slowly. After decolourisation, a counterstain (usually the red dye safranin) is used. Bacterial cells resistant to decolourisation will retain the primary dye and exhibit a blue/purple colour. These are referred to as Gram-positive bacteria. The decolourised cells take up the counterstain and exhibit a pink/red colour; these are described as Gram-negative cells (Brock, 2000). See Table A1 below for procedure details.

Reagents in order of use	Time applied	Gram-positives	Gram-negative
Crystal violet (CV)	1 minute	1. CV taken up 2. Cells appear violet	1. Same 2. Same
Iodine solution (I)	1 minute	1. Iodine fixes CV 2. CV-I complex formed 3. Cells remain violet	1. Same 2. Same 3. Same
Ethanol/acetone Decolouriser	Dropwise	1. CV-I complex dissociated 2. Diffusion of dye proceeds slowly 3. Cells remain violet	1. Same 2. Diffusion of dye proceeds fast 3. Cells become clear
Safranin Counterstain	½ -1 minute	1. Some displacement of CV may occur but in general cells not affected 2. Cells appear purple/blue	1. Displacement of any CV left occurs as cells take up counterstain 2. Cells appear pink/red

Table A1: Specific steps involved in the Gram Stain

APPENDIX V: ABBREVIATIONS/ACRONYMS

ABPA	Allergic bronchopulmonary aspergillosis
API	Analytical Profile Index
BCGA	British Cotton Growing Association
CFU	Colony forming units
CIS	Commonwealth of Independent States
CR	Compliment receptor
CRD	Carbohydrate recognition domain
CSE	Control standard endotoxin
DECOS	Dutch Expert Committee on Occupational Standards
FEV_1	Forced expiratory volume in the first second
GAC	Gezira Acala cotton
GBA	Gezira Barakat cotton
GNB	Gram-negative bacteria
GPB	Gram-positive bacteria
HP	Hypersensitivity pneumonitis
HSE	Health and Safety Executive
ICA	International Cotton Association
IL	Interleukin
ILO	International Labour Organisation
IRAK	Interleukin 1 receptor-associated kinase
IRF	Interferon regulation factor
ITAM	Immunoreceptor tyrosine-based activation motif
KDO	2-keto-3-deoxy-D-manno-octonic acid
LAL	*Limulus* amoebocyte lysate
LBP	Lipopolysaccharide binding protein
LCRC	Liverpool Cotton Research Corporation
LPS	Lipopolysaccharide
MAP	Mitogen activation protein
MEA	Maltose extract agar
MIP	Macrophage inflammatory protein
MMF	Maximum mid-expiratory flow
NFκB	Nuclear factor kappa B

NIOSH	National Institute for Occupational Safety and Health
OA	Occupational asthma
OD	Optical density
ODTS	Organic dust toxic syndrome
OSHA	Occupational Safety and Health Administration
PAF	Platelet activating factor
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PFW	Pyrogen-free water
PRR	Pattern recognition receptor
RAC	Rahad Acala cotton
RADS	Reactive airways dysfunction syndrome
SEM	Standard error of the mean
TIR	Toll/IL-1 receptor
TIRAP	TIR domain containing adaptor protein
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TSA	Tryptic soy agar
TRAF	TNF receptor associated factor
TRIF	TIR domain-containing adaptor inducing IFNα (TICAM-1)
TRAM	Toll-like receptor adaptor molecule 2 (TICAM-2)
WHO	World Health Organisation