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Detection of aerobic gram negative bacteria and bacterial endotoxins from the oral cavities of patients on cancer therapy

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2017
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DETECTION OF AEROBIC GRAM NEGATIVE BACTERIA AND BACTERIAL ENDOTOXINS FROM THE ORAL CAVITIES OF PATIENTS ON CANCER THERAPY

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A research report submitted to the faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in the partial fulfilment of the requirements for the Degree of Master of Science in Dentistry,

Johannesburg, 2017

DECLARATION

I, Juliana Mathews, declare that this research report is my own work. It is being submitted for the degree of Master of Science in Dentistry to the University of The Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.



(Signature of candidate)

……19…….day of…June……2017

DEDICATION

This research report is dedicated to God,

"I can do all things through Him who strengthens me." Philippians 4:13

my family, who did not stop believing in me

and who remained the "footprints in the sand".

My parents- who taught me to get up after a fall and start again

My husband, - where there is hope there is faith, where there is faith, miracles happen

and my two children- both young and young at heart who love to dream.

ABSTRACT

Chemotherapy and radiation therapy are the most widely used interventions for the treatment of cancer. Several adverse effects including mucositis are associated with these therapies, which affect a patient's quality of life, resulting in morbidity and mortality. Cancer therapy also causes an imbalance in the oral flora which allows colonization of the oral cavity with uncommon bacteria, including aerobic gram negative bacteria and less commonly, *Candida*. These organisms can become established in the lesions of oral mucositis. These gram negative bacteria produce endotoxins. Although the involvement of endotoxins in the development of systemic infections is well described, the role of endotoxins in oral mucositis is not known. Therefore, this study investigated the presence of aerobic gram negative bacteria and endotoxins in the oral cavities of patients receiving cancer treatment and their role in the development of oral mucositis.

Oral cavity rinse samples were collected from 100 cancer patients on cancer treatment and 50 healthy individuals. Ethical clearance was obtained from The Committee for Research on Human Subjects (Medical). The demographic and clinical data were recorded. Samples were serially diluted and cultured onto Mitis Salivarius Agar for Streptococci, Baird Parker Agar for *Staphylococcus aureus*, MacConkey Agar for aerobic gram negative bacteria (AGNB) and Chromagar for *Candida species*. Colony counts were obtained and the cultures of AGNB and *Candida species* were further identified at the species level using the API technique. An antimicrobial susceptibility test was performed on AGNB. Oral rinse samples were further tested for the presence of endotoxin using the Hycult Biotech Limulus Amebocyte Lysate (LAL) assay. Results were analysed using the Mann-Whitney and chi-square test. P-values of $\leq 0.05\%$ were considered significant.

Eighty percent of cancer patients were females with breast (58%) or cervical cancer (9%). The majority of these patients were on chemotherapy (61%), were receiving a combination of chemotherapeutic drugs and had completed many cycles of chemotherapy treatment. Twelve percent of patients had oral mucositis of various grades. Healthy individuals also comprised of mainly females (76%). All cancer patients and 98% of healthy individuals carried Streptococci. No significant difference in the carriage of *S. aureus* was found between the two groups. However, the *Candida* carrier rate was significantly high in cancer patients (55%) compared to the healthy (20%) individuals ($p<0.01$). Although the *Candida* counts were not different between the groups ($p>0.05$), cancer patients carried a variety of *Candida*

species and some patients carried more than one type of *Candida species* in their oral cavity. In addition, *C. glabrata* was only found in cancer patients. Between the groups of cancer patients and healthy individuals, no significant difference in the carrier rate of AGNB was found (cancer 24% vs 14% healthy). However, cancer patients carried a variety of AGNB. *Enterobacter cloacae* and *Klebsiella pneumonia* was isolated from the oral cavities of both the study groups. These bacteria were resistant to many antibiotics. When the prevalence of these oral bacteria was compared between patients with and without oral mucositis, it was found that the percentage prevalence of *Candida species* was significantly high in patients with oral mucositis.

A mean endotoxin concentration of 3.65ng/ml and 3.37ng/ml was detected in the oral rinse samples of cancer patients and healthy individuals respectively. The difference in the endotoxin between the two groups was not significant ($p=0.5$). Mean values of 4.1ng/ml and 3.53ng/ml were found in cancer patients with and without oral mucositis respectively. The difference in the quantities of endotoxins between the two groups was not significant ($p=0.6$). In addition, endotoxin present in cancer patients with AGNB (3.39ng/ml) and without AGNB (3.8ng/ml) was also not significantly different.

These results suggest that although cancer patients carry aerobic gram negative bacteria and endotoxins in their oral cavities, they may not contribute in the exacerbation of oral mucositis. However, *Candida species* may contribute in the exacerbation of oral mucositis and therefore, during cancer treatment, it is important to take preventative measures to reduce the colonization of *Candida* to prevent infection.

ACKNOWLEDGEMENTS

I wish to express my gratitude to the following people who contributed to my research project

- My supervisor, Prof M. Patel, senior scientist/lecturer at Oral Biological Sciences Department at the University of the Witwatersrand for mentoring me with patience guidance and incredible insight.

- The National Research Foundation for their financial support.

- The staff of the Oral Microbiology Laboratory, especially Malefaso Whitney, for their invaluable assistance in the laboratory experiments.

- My husband and children for their endless patience, encouragement and moral support.

- My parents for their ongoing encouragement and financial support to fulfil my research endeavours.

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LIST OF ABBREVIATIONS AND ACRONYMNS

Ab: Antibiotic

AK: Amikacin

AGNB: Aerobic gram negative bacteria

AP: Ampicillin

API: Analytical profile index

AUG: Amoxicillin-clavulanate potassium

BHI: Brain Heart Infusion

BP: Baird Parker

C: Chloramphenicol

C. albicans: *Candida albicans*

C. dubliniensis: Candida dubliniensis

C. famata: *Candida famata*

C. glabrata: *Candida glabrata*

C. krusei: *Candida krusei*

C. tropicalis: *Candida tropicalis*

CAZ: Ceftazidime

cfu/ml: Colony forming units

CIP: Ciprofloxacin

CLSI: Clinical and Laboratory Standards Institute

$CO_{2.}$ Carbon dioxide

COX-2: Cyclooxygenase-2

CPM: Cefepime

CTX: Cefotaxime

CXM: Cefuroxime

DNA: Deoxyribonucleic acid

E. cloacae: *Enterobacter cloacae*

E. coli:	*Escherichia coli*
E. sakazaki:	*Enterobacter sakazaki*
EFW:	Endotoxin free water
ETP:	Ertapenem
EU:	Endotoxin Units
EU/Kg/Hour:	Endotoxin Units/Kilogram/Hour
FDA:	Food and Drug Administration
FOX:	Cefoxitin
GM:	Gentamycin
Gy:	Gray
HIV:	Human Immunodeficiency Virus
HPV:	*Human papillomavirus*
HSCT:	Hematopoietic stem cell transplant
I:	Intermediate
IARC:	The International Agency for Research on Cancer
IL-1β:	Interleukin 1β
IL-6:	Interleukin 6
IMI:	Imipenem
K. oxytoca:	*Klebsiella oxytoca*
KDO:	2-keto-3-deoxyoctonoic acid
LAL:	Limulus Amebocyte Lysate
LPS:	Lipopolysaccharide
MEM:	Meropenem
ml:	Millilitre
MMPs:	Matrix metalloproteinases
MRSA:	Methicillin-resistant *S. aureus*

MSA: Mitis Salivarius Agar

NA: Nalidixic acid

N/D: Not done

NF-κB: Nuclear factor-κB

ng/ml: Nanograms per millilitre

nm: Nanometre

OD: Optical Density

P. aeruginosa: Pseudomonas aeruginosa

P. vulgaris: Proteus vulgaris

pg/ml: Picogram per millilitre

PTA: Polymyxin, tobramycin, and amphotericin B

PTZ: Piperacillin-tazobactam

R: Resistant

ROS: Reactive oxygen species

S Susceptible

S. aureus: Staphylococcus aureus

S. epidermidis: Staphylococcus epidermidis

S. pneumoniae: Streptococcus pneumoniae

S. sanguis: Streptococcus sanguis

SD: Standard deviation

TN: Tobramycin

TLR4: Toll-like receptor-4

TNF-α: Tumour necrosis factor- α

TS: Trimethoprim-sulfamethoxazole

5-FU: 5-fluorouracil

µl: Microlitre

CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Introduction

The development of cancer arises from the mutagenesis of normal cells from a particular organ as a result of the interaction of complex aetiologies. Various treatment modalities are used but intensive chemotherapy and radiotherapy are conventionally required. Several adverse effects including mucositis are associated with these therapies, which affect a patient's quality of life, resulting in morbidity and mortality. Oral mucositis is associated with immense oral discomfort, excruciating pain, an altered taste sensation, painful swallowing and subsequent dehydration and malnutrition. Due to the loss of balance between commensal bacteria and opportunistic bacteria, changes in the oral environment and microbiome occurs. Some of these opportunistic bacteria such as gram negative bacteria and *Candida species* become established in the lesions of oral mucositis. These gram negative bacteria produce endotoxins and *Candida species* produce hydrolytic enzymes as virulence factors. The endeavour to find an efficient solution to manage and prevent this pathology has been challenging. The efficacy of antimicrobials and other treatment modalities have been controversial. Although the involvement of endotoxins in the development of other bacterial infections is well described, the role of endotoxins in oral mucositis is not known. Therefore, this study investigated the presence of aerobic gram negative bacteria and endotoxins in the oral cavities of patients receiving cancer treatment and their role in the development of oral mucositis.

1 Literature Review

1.1 Cancer epidemiology

Cancer-related deaths have reached epic proportions, both in South Africa and internationally. This pandemic has amounted to approximately 8.2 million cancer-related deaths in 2012 and an incidence of 14.1 million cancer cases reported every year (GLOBOCAN 2012). By 2030, this is expected to rise to 21.7 million new cancer cases and 13 million cancer deaths (GLOBOCAN 2012). In recent articles (Stefan et al, 2013 and Sartorius et al, 2016), by 2030, South African cancer rates have been predicted to escalate by 78%. Cancer can arise from a complex aetiology affecting various cells and organs (Baskar et al, 2012). Although the incidence and mortality rates in western developed countries have decreased, almost half the number of reported incidences worldwide has been related to lung, female breast, colorectal and stomach malignancies (Jemal et al, 2010). Lifestyle factors such as smoking, alcohol, a sedentary lifestyle and obesity are risk factors seen in western countries, but increased incidences in cervical, liver and stomach cancers, have been related to cancers developing from infectious agents in underdeveloped countries and improved socioeconomic countries (Jemal et al, 2010).

The habitual use of tobacco and alcohol are predominant risk factors in the development of head and neck carcinomas. In less developed countries, carcinogenic infection is an important cause of cancer. Of concern, oncogenic viruses such as the *Epstein- Barr virus, Human papillomavirus virus* (HPV) and *Helicobacter pylori* have been linked to endemic nasopharyngeal cancer, oropharyngeal cancers and gastric cancer respectively (Jemal et al, 2010; Schoenfeld, 2015 and Plummer et al, 2016). The development of HIV-related cancers has been associated with immunosuppression and the increased expression of carcinogenic infectious agents (IARC, 2012 and Plummer et al, 2016). Many cases of fatalities could be prevented in countries where HIV and HPV are prevalent, by implementing screening procedures, proper diagnosing and treatment of precancerous lesions (Crosbie et al, 2013 and Plummer et al, 2016).

Various other risk factors are commonly associated with cancer, including prolonged sun exposure, demographic factors, occupational inhalants, marijuana use, poor nutrition and immunosuppression (Khan and Khan, 2015). Family history and pre-existing medical conditions play a significant role (Pai and Westra, 2009; and Khan and Khan, 2015). Hormone replacement therapy and oral contraceptives may increase the risk of some cancers but decrease it for others. Ironically, specific chemotherapy agents such as Chlorambucil have been found to allow growth of cancer at a different anatomical site (American Cancer Society, 2014).

1.2 Cancer treatment

Most cancers are treated with surgery, radiotherapy, chemotherapy and immunotherapy (Payne and Miles, 2008). The choice of treatment depends on the site, grade and stage of tumor, as well as the patient's age and general medical condition (Khan and Khan, 2015). Surgery with complete tumour resection, radiotherapy and /or chemotherapy are used to treat most early stages of cancer, but stage III and IV malignancies may require adjuvant radiotherapy and chemotherapy for effective tumour regression and organ preservation for better survival (Caballero et al, 2009 and Alvarez et al, 2015). Surgery can be aggressive, consequently affecting aesthetics and function and therefore can affect the patient both socially and psychologically. Regardless of treatment, recurrence of the tumour can occur at the same region and if distant metastasis occurs, death can be inevitable (Alvarez et al, 2015).

Radiotherapy requires high doses of ionizing radiation to directly damage the cellular DNA or indirectly damage DNA by free radicles. This deprives cancer cells and interferes with its multiplication potential to cause eventual death of cancer cells (Baskar et al, 2012). The rationale of using radiotherapy prior to surgery is to shrink the size of the tumour, but post-surgical radiation will target undetectable tumour cells that may have persisted (Baskar et al, 2012). This can be applied internally either in close proximity of the tumour or into the tumor (brachytherapy) or more commonly externally (teletherapy) (Baskar et al, 2012 and Alvarez et al, 2015). Administration of external radiotherapy varies from a short duration or up to several weeks. A cumulative dose between 50-70 Gy for solid epithelial tumours is received, which is sub-divided over a period of approximately two months, of which a daily dose of 2Gy is received from Monday to Friday with a rest period of two days (Jham and Freire,

2006). Moreover, this allows the tumour to receive the maximum required dosage of radiation, thereby excluding the adjacent healthy tissues from toxicity of the treatment. The period of rest during the weekend allows for re-oxygenation of tumour cells, increases its sensitivity towards the treatment and allows for healthy cells to repopulate tissues (Joiner and van der Kogel, 2009; and Alvarez et al, 2015).

The principle of chemotherapy is to interfere with replicating cancer cells within the various phases of the cell cycle, thereby causing selective cell death and inhibiting tumour growth (Sak, 2012 and Alvarez et al, 2015). This cocktail of drugs, work in synergy to reduce the development of resistance and to allow for better efficacy of treatment, without injuring the host (Sak, 2012 and Alvarez et al, 2015). Cytotoxic drugs such as methotrexate and vincristine are active at specific periods in the cell cycle to kill proliferating cells and are affected by the duration of exposure. Chemotherapeutic agents such as chlorambucil and cisplatin can efficiently target tumour cells throughout the whole cell cycle and their degree of cytotoxicity is dose dependant (Payne and Miles, 2008 and Sak, 2012). Adjuvant chemotherapy is required if there is a known risk of relapse, due to the aggressive nature of the cancer. As there is a lack of specificity towards cancer cells and normal rapidly dividing cells, with certain cytotoxic drugs such as 5- fluorouracil and cisplatin, these result in adverse effects of bone marrow suppression, loss of hair, mucositis, nausea and vomiting, which have been associated with the morbidity of this treatment (Payne and Miles, 2008).

1.2.1 Oral effects of radiotherapy

Intraorally, acute complications can arise as a result of salivary hypofunction from direct injury of the salivary glands by radiation. This can be reversible, although irreversible and permanent xerostomia can affect the volume and quality of saliva produced. Xerostomia can temporarily affect the sense of taste which affects a patient's appetite and nutritional status, but permanent side effects of a reduction or loss in taste can persist (Alvarez et al, 2015).

The sparse volume of saliva becomes more viscous and the buffering capacity is reduced, with a change in the salivary electrolyte concentration and the compromise of the host's oral defence system (Kielbassa et al, 2006, Naidu et al, 2004 and Gupta et al, 2015). Oral

tenderness, tooth decay, oral disease and difficulty in swallowing are common complaints associated with this treatment. It accounts for a reduction in oral clearance, allowing for an acidic environment to persist and the emergence of cariogenic bacteria such as *Streptococcus mutans* and *Lactobacillus,* as seen in a study by Epstein *et al*, (1998).

These cancer patients are more likely to develop periodontitis, bacterial and viral infections and oral candidosis (from *Candida species*) associated with immunosuppression (Gupta et al, 2015 and Jham and Freire et al, 2006). The emergence of opportunistic infections, largely due to *Candida* infections, is associated with radiation-related hyposalivation, the presence of oral prostheses, poor oral care and vices such as tobacco smoking and the consumption of alcoholic beverages. Although topical antifungal drugs are the treatment of choice for this infection, systemic medication is frequently recommended for patients undergoing radiotherapy (Meurman and Grönroos, 2010 and Alvarez et al, 2015). One of the most common complications associated with patients undergoing head and neck radiotherapy is oral mucositis. Late complications of radiotherapy can result in trismus and osteoradionecrosis (Alvarez et al, 2015).

1.2.2 Oral complications of chemotherapy

The direct effects of stomatotoxicity, depends on the type of chemotherapeutic agent used, the dosage and frequency. Agents such as cisplatin, 5- fluorouracil and methotrexate are used in the treatment head and neck cancers, are known to cause mucosal injury as seen in a study by Kasettya *et al*, (2012) and consequently, conditions such as oral mucositis and microbial infections develop (Kasettya et al, 2012). The outcome of this mucosal atrophy is attributed to the cytotoxic effects of these drugs on the genetic replication and proliferation of basal cells of the mucosa (Lopez et al, 2011 and Kasettya et al, 2012). Lopez *et al*, (2012) concluded that indirect stomatotoxicity was related to signs of bone marrow suppression, immunosuppression and a loss of the host's salivary defence mechanisms against infection (Lopez et al, 2011 and Kasettya et al, 2012).

1.2.3 Oral complications of chemoradiation

The combination of radiotherapy and chemotherapy improves therapeutic results, allows for better tumor control in the region and controls distant metastasis (Joiner and van der Kogel, 2009; and Alvarez et al, 2015). Consequently, high daily doses and total cumulative doses of radiotherapy to an exposed area, in combination with cytotoxic treatment, can affect the incidence, severity and duration of the complication (Jham and Freire et al, 2006). The complications of chemoradiation are similar to the oral complications associated solely from either treatment, but are significantly accentuated (Kasettya et al, 2012).

1.3 Oral Mucositis

The complications associated with cytotoxic treatment can have a significant impact on various mucosal tissues in the gastrointestinal tract, including the oral cavity, oesophagus, stomach and intestine. Symptoms of pain, ulceration, nausea and vomiting, diarrhoea and rectal bleeding have been reported, depending on the area affected. Individuals experience different degrees of mucosal injury across different sites within the gastrointestinal tract (Al-Dasooqi et al, 2013).

Oral mucositis is a debilitating, acute, multifactorial complication associated with cancer patients receiving radiotherapy, chemotherapy or hematopoietic stem cell transplantation (HSCT) (Vozza et al, 2015). Clinical symptoms of severe oral pain, bleeding and ulceration can affect the person's quality of life and delay the management of cancer. Hospitalization and additional use of antibiotics and parenteral opioid analgesics delays cancer treatment (Naidu et al, 2004 and Vozza et al, 2015). As the severity of oral mucositis and pain heightens, their oral intake is restricted and patients may require the insertion of a feeding tube for their nutritional requirements and the intake of multiple drugs. Severe oral mucositis is commonly found in patients that receive radiation of the oral cavity and surrounding structures and those treated with HSCT, as these patients have prolonged and intense myelosuppression. It is less frequently seen as a complication of chemotherapy in treating solid tumours, but it is most severe when the patient has underlying neutropenia (Redding, 2005).

With the initiation of treatment, when doses reach up to 20Gy at the field of radiation or within two weeks of initiating chemotherapy, either transient hyperkeratinisation or erythema

develops. Multiple, daily, fractioned radiotherapy may result in repeated tissue damage (Redding, 2005 and Vozza et al, 2015) and eventually lead to ulceration covered by a pseudomembrane and healing can take up to six weeks following the completion of treatment (Vozza et al, 2015). The severity of radiotherapy-related oral mucositis is affected by factors surrounding the treatment such as the cumulative dose, the volume of area undergoing radiation and predisposing patient factors such as habitual use of cigarette smoking, alcohol consumption (Köstler et al, 2001 and Naidu et al, 2004) and radiation-induced xerostomia as seen in a study by Franzén et al, (1992).

Oral mucositis associated with chemotherapy is restricted to non-keratinized oral mucosal tissue with healing and resolution occurring three weeks after the end of treatment (Redding, 2005). This severity is also related to the neutropenic state of the patient. The combined effects of the type of chemotherapeutic agent used, the therapeutic regimen, the dosage and the concomitant medication, can affect the severity of ulceration. Lower doses of cytotoxic agents with prolonged or repetitive administration and prior episodes of chemotherapy-induced oral mucositis have been implicated in affecting the severity (Köstler et al, 2001).

1.3.1 Aetiology of oral mucositis

Some of these risk factors associated with oral mucositis include age, particularly younger patients, poor oral health care and hyposalivation before and during treatment. Compromised nutrition, the type of malignancy, the neutrophil count before treatment and the underlying genetic predisposition are factors contributing to this pathology (Naidu et al, 2004). There is a higher chance of stomatotoxicity with the use of 5-fluorouracil (5-FU) in combination with anthracycline-based, taxane-based or platinum-based chemotherapeutic drugs (Redding, 2005). Methotrexate found in saliva contributes to the development of oral mucositis. When used in combination with drugs such as antidepressants, which have an anticholinergic effect, can promote xerostomia and enhance the symptoms of oral mucositis (Naidu et al, 2004). Chemoradiation is a choice of treatment to allow for better cancer response, but consequently, it is proportionally responsible for the high oral mucositis rates (Redding, 2005). Any form of iatrogenic trauma such as ill-fitting dentures and defective restorations can initiate ulceration. Cancer patients are prone to oral mucosal infections of bacterial, viral and fungal aetiology, and these can exacerbate oral mucositis with the resultant ulceration acting as a portal of entry for these organisms into the systemic circulation (Redding, 2005).

Other factors include the substitution of gram-positive bacteria for gram-negative bacteria as a result of the tumour, the cancer treatment and supportive therapies (Donelly et al, 2003 and De Sanctisa et al, 2016). Alcohol consumption predisposes the patient to malnutrition and immunosuppression; and the accumulation of alcoholic toxic metabolites allows for mucosal damage, thereby enhancing the progression of oral mucositis. Cigarette smoking elicits mucosal inflammation and causes the host defence mechanism to be vulnerable to infectious agents and therefore compromising the healing of oral mucositis (De Sanctisa et al, 2016).

1.3.2 Pathophysiology of oral mucositis

The pathology of oral mucositis can be divided into various phases. The first phase involves the initiation of oral mucositis. Direct injury to the basal cellular epithelium occurs due to DNA strand breaks and the generation of oxidative stress and reactive oxygen species (ROS) by radiation and/or chemotherapeutic agents. This ROS directly damages the mucosal cells, tissues and blood vessels and it also stimulates the release of transcription factors which initiates this toxicity in the mouth (Sonis et al, 2004).

During the second phase, injury to the DNA strands and the ROS, activate transcription factors such as Nuclear factor-κB (NF-κB). Proinflammatory cytokines such as tumour necrosis factor- α (TNF-α), Interleukin-1β (IL-1β), and Interleukin-6 (IL-6) are produced contributing towards tissue injury and apoptosis. Apoptosis can also be activated by the ceramide pathway with the production of sphingomyelinase or ceramide synthase. With the destruction of fibronectin, the activated macrophages activate matrix metalloproteinases (MMPs) that cause direct tissue injury (Sonis et al, 2004 and Redding, 2005). NF-κB also upregulates genes to activate cyclooxygenase-2 (COX-2), which causes inflammation, oedema and tissue damage with the production of prostaglandin from the submucosal fibroblasts and endothelial cells after two weeks of radiation (Al-Dasooqi et al, 2013).

In the signalling and amplification stage, proinflammatory cytokines indirectly exaggerate mucosal injury. A further production of cytokines allow further tissue injury via other pathways like the ceramide and caspase pathway and NF-κB activates the transcription pathway (Sonis et al, 2004).

The ulcerative phase is the most clinically significant phase associated with pain, oral discomfort and loss of function, as the mucosa is breached and the neuronal endings are exposed (Redding, 2005). Pathogenic bacterial colonization of gram positive, gram negative and anaerobic bacteria, occur. The presence of endotoxins released from the cell walls of gram negative bacteria, further stimulate the production of proinflammatory cytokines such as TNF-α , IL-1β , IL-6, which amplify and accelerate local tissue damage to form ulcers and allows the ingress of endotoxins through the mucosal tissues and into the systemic circulation and possibly causing sepsis (Sonis et al, 2004). Secondary infection is common in this phase and patients with neutropenia are more likely to develop complications such as bacteraemia, viral, fungal infections and sepsis (Al-Dasooqi et al, 2013).

In the healing phase, there is renewed cell production and angiogenesis. The commensal bacteria are re-established and the leukopenia is resolved. Although the mucosa eventually has a healthy appearance, the epithelium remains significantly altered increasing the risk for future episodes of oral mucositis (Redding, 2005).

1.3.3 Clinical presentation of oral mucositis

Oral mucositis may begin within two weeks of commencing chemotherapy and it resolves within three weeks of completing the cancer treatment. This may depend on the dose, duration, course of treatment and the patient's personal experience. The initial presentation begins with generalized burning or sensitivity of the delicate oral mucosa or in some cases with leukoedema (Köstler et al, 2001 and Redding, 2005). Areas of erythema and atrophy of the mucosa lead to the emergence of ulcers that may be covered by a pseudomembrane. These ulcers present with a tendency to bleed as many of these patients have thrombocytopaenia (Naidu et al, 2004).

With the emergence of ulcers in the oral cavity and oropharyngeal region, mild or intense pain may be experienced, which result in poor nutrition and hydration and this affects their oral care. Decreased salivary flow as a complication of cancer treatment allows poor lubrication with the accumulation of debris in the mouth and signs of a hairy tongue may be observed.

The World Health Organization Oral Toxicity Scale (WHO, 1979) is routinely used to evaluate oral mucositis clinically. Grade 0 has no signs or symptoms of oral mucositis. Grade I oral mucositis presents with painless ulcers, erythema and oedema but patients are able to eat. Grade II is categorized by its painful erythema and ulcers and the presence of oedema but they are able to eat. With progression, the ulcers become more painful, erythematous and oedematous and the patient is unable to eat in Grade III. If the previous symptoms persist, but without the ability to eat or drink, Grade IV oral mucositis is present, where patients may require parenteral or enteral support (Sonis et al, 2004 and Naidu et al, 2004).



Adapted from [http://www.prothelial.com/oral_mucositis/index.html]

Figure 1.1 Various grades of oral mucositis

1.3.4 Management of oral mucositis

The first approach taken in the management of oral mucositis has been to practice better oral care, to ultimately reduce the frequency and extremity of this pathology. Studies have shown that enforcing strict oral care protocols and managing pre-existing periodontal and dental disease, showed better control of this oral injury (Eilers and Million, 2011 and Lalla et al, 2008). By delivering a known volume of radiation to a precisely demarcated irregular-shaped diseased region, alleviates high doses of radiation to surrounding healthy tissue, as used when defining the Planned Target Volume of the radiation beam (Kouloulias et al, 2013 and De Sanctisa et al, 2016).

Treatment modalities such as cryotherapy, the use of Amifostine and glutamine, have been suggested, but these have proven ineffective (De Sanctisa et al, 2016). Controversy also exists with the use of topical antibacterial mouthwashes such as 0.12% chlorhexidine digluconate to reduce oral mucositis and candidosis (Lopez et al, 2011 and De Sanctisa et al, 2016).

Several combinations of topical antimicrobials have been incorporated to develop a solution to prevent and treat oral mucositis, but results have been controversial. By integrating polymyxin, tobramycin, and amphotericin B (PTA) in the form of an antimicrobial lozenge, this eliminated selective oral flora, but it did not accomplish the role of reducing the severity of oral mucositis (Saunders et al, 2013 and Stokman et al, 2003). Similarly, many other antibiotics and treatment modalities have been suggested (Donnelly et al., 2003; Laheij et al, 2012 and Spijkervet et al, 1990). Studies have shown that systemic antibiotics, antivirals or antifungals are recommended for preventative use, but only in the presence of neutropenia (Saunders et al, 2013). Therapeutic treatment may be required if a prominent infection has been diagnosed and the causative agent is identified (Saunders et al, 2013 and Bergmann et al, 1995). As most antibiotic interventions have not been consistently successful, this may suggest that the microbial flora might not be the primary culprit in initiating oral mucositis (Napeñas et al, 2010 and Al-Dasooqi et al, 2013).

1.4 Microbiology of the oral cavity during cancer treatment

Chemoradiation may induce an alteration of the oral environment resulting in a loss of balance between oral commensal bacterial flora and the emergence of opportunistic bacteria. The systemic and oral mucosal immunity is compromised by the residing tumour, antineoplastic treatment and by the recommended adjunctive treatment, thus leading to an imbalance and changes to the oral environment (De Sanctisa et al, 2016, Napeñas et al, 2010) and Donnelly et al, 2003).

In addition to the cytotoxic effects of cancer treatment, factors such as neutropenia, xerostomia and the use of multiple drugs including antibiotics can affect this harmony. Patient-related factors such as the compromised oral hygiene, pre-existing periodontal disease and the presence of nosocomial pathogens can disrupt this delicate balance (Meurman et al., 1997).

When the bacterial flora is investigated in the presence of oral infection, the total number of commensal oral Streptococci is reduced with the colonization of opportunistic pathogens (Napeñas et al, 2010). This can be also observed in immunocompromised cancer patients with radiation-induced xerostomia, as higher counts of *Streptococcus mutans*, *Lactobacillus*

spp., *Candida* (mainly *C. albicans*) and *Staphylococcus spp.* have been found, whereas the number of *S. sanguis*, *Neisseria spp*. and *Fusobacterium spp*. has been shown to decline (De Ryck et al, 2015 and Brown et al, 1975). These changes allow for a higher prevalence of radiation-induced caries in these patients due to the poor saliva present, the altered pH and buffering capacity in saliva (Brown et al, 1975 and Almståhl et al 2001). The oropharyngeal mucosae is covered by a glycoprotein fibronectin that possesses attachment sites for gram positive microorganisms including *S. pneumoniae, S. aureus* and *Actinomyces* spp., whereas receptor sites for aerobic gram negative bacteria are thought to emerge after denudement of oral mucosae from fibronectin by the underlying disease (Yoneda et al, 2007).

In a study done by Panghal *et al*, (2012), a predominance of gram positive bacteria (*S. aureus* and *S. epidermidis*) was found in the oropharynx and in the bloodstream of patients with varying grades of oral mucositis undergoing intensive chemotherapy and radiation. During the ulcerative phase of oral mucositis, bacterial translocation through the bloodstream may induce fever, infection and possible sepsis (Al-Dasooqi et al, 2013). These organisms could also enter into the bloodstream with the use of a central venous line (Panghal et al, 2012). Alteration in the microbiome has also been noted where proper sanitation is compromised in overcrowded hospitals or where oral and personal hygiene has been neglected (Panghal et al, 2012). When oral pain is heightened to a degree that no alimentation is possible as seen in Grade IV oral mucositis, cancer patients have to be tube-fed and hence the emergence of other opportunistic gram positive pathogens such as *Corynebacterium striatum* and *Streptococcus agalacticae* (Renom et al, 2014).

Moreover, immunocompromised patients undergoing antineoplastic chemotherapy, radiation and HSCT frequently develop infections arising from candida and have been associated with factors such as the immunosuppression of the host, the alteration of the oral microbiome and xerostomia (Naidu et al, 2004). The colonization of *Candida spp*. as seen in candidiasis and invasive candidemias, has been frequently seen in patients with neutropenia (Laheij et al, 2012). As candidiasis can present clinically in its pseudomembranous and erythematous forms, patients with the erythematous forms complain of pain with or without a burning sensation and this can be confused with symptoms of oral mucositis (Jham and Freire, 2006). Although *Candida albicans* has been the most prevalent *Candida species*, non-albicans Candida species such as *C. glabrata, C. krusei* and *C. dubliniensis* have been identified in

radiation patients and patients undergoing HSCT (Laheij et al, 2012), and studies has shown variations of certain species according to geographical locations (Jham and Freire, 2006, Ramla et al, 2016).

Theories of an association between periodontitis and the severity of oral mucositis have been considered by Laheij *et al*, (2012) as they both have similar features of local and systemic inflammation and periodontal gram negative anaerobic bacteria have also been found in patients with oral mucositis. Evidence of this theory was disproved in a pilot study by Khaw *et al*, (2014) where even though a greater portion of patients with oral mucositis had periodontitis, this risk factor did not demonstrate any significance. Reports have shown that colonisation of bacteria in the ulcerative phase can enhance the severity of oral mucositis but evidence of antimicrobials failing to treat oral mucositis may prove that the microbial flora may not be the primary causative factor for this condition (Barasch et al, 2006 and Al-Dasooqi et al, 2013).

1.4.1 Cancer treatment and aerobic gram negative bacteria

The presence of aerobic gram negative bacteria in the oral cavity is less frequently found in healthy individuals. The cells lining the oral cavity to the gastrointestinal tract are influenced by similar microbiological and immunological characteristics. This is illustrated by the presence of indigenous *Escherichia coli* in the gastrointestinal tract and the absence of aerobic gram negative bacteria in the oropharynx of healthy individuals. Individuals, who are carriers of *Pseudomonas aeruginosa* orally, are generally faecal carriers of the same strain (Leenstra et al, 1996).

1.4.2 Factors associated with aerobic gram negative bacterial colonization and oral mucositis

This colonization of gram negative organisms and the release of endotoxins in the ulcerative phase of oral mucositis occur on damaged mucosal tissue, which may be further complicated by concomitant neutropenia (Naidu et al, 2004). Inflammatory mediators are released which influence the progression of oral mucositis (Köstler et al 2001).

Studies have demonstrated an overall increase in the prevalence of aerobic gram negative bacilli such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella* and *Enterobacter* spp. in cancer patients (Soares et al, 2011, Gaetti-Jardim et al, 2011 and Panghal et al, 2012). Panghal *et al*, (2012) describes that in developing countries like Lebanon, Malaysia and India, gram negative bacteria were the most predominant pathogen observed in febrile neutropenic patients. In a study done by Anirudhan *et al*, (2008) similar bacterial pathogens were found, but their blood culture showed bacterial sepsis due to the presence of *Escherichia coli*, *Klebsiella* and *Enterococcus* spp. In a systemic review by Napeñas *et al*, (2007) although paediatric cancer patients undergoing chemotherapy were more susceptible to gram positive oral bacteria such as *Streptococci* and *Staphylococci*, adult oncology patients were associated with changes involving gram negative bacteria such as *Enterobacteriaceae* and *Pseudomonas spp.*

Similarly, bacterial translocation of aerobic gram negative bacteria, as described by Leenstra *et al*, (1996) can take place at the oropharynx and gastrointestinal tract regardless of presence of indigenous gram negative flora. Factors such as bacterial overgrowth, immunosuppression, and physical disruption of the gastrointestinal tract by cancer treatment, trauma and endotoxins are contributing factors of bacterial translocation (Jubelirer, 2011).

1.4.2 Treatment associated with gram negative bacteria and oral mucositis

Several suggestions have been made to prevent and treat oral mucositis by the reduction of the total bacterial count. The effect of PTA lozenges (Saunders et al, 2013 and Stokman et al, 2003) and chlorhexidine gluconate may have eliminated selective oral flora but they were not efficacious in reducing the acute symptoms of oral mucositis (Wijers et al, 2001). In a study by Sano *et al*, (2015), various combinations of antibiotics such as ceftazidime plus piperacillin/tazobactum and ampicillin plus aztreonam, were some of the antibiotics that could be used. In a study by Baskaran *et al*, (2007) these antibiotics were also combined with an aminoglycoside and /or vancomycin for better results. The fact that most antibiotic interventions have failed, may suggest that other factors may be involved in the progression of oral mucositis (Napeñas et al, 2010 and Al-Dasooqi et al, 2013).

1.5 Endotoxins

Gram negative aerobic and anaerobic bacteria possess endotoxins which are composed of lipopolysaccharides (LPS) found in the bacterial cell membrane (Leenstra et al, 1996). As these endotoxins are released, it may intensify the inflammatory process and promote or exacerbate ulcer formations in oral mucositis (Napeñas et al, 2007). Pathogens such as *Escherichia coli*, *Pseudomonas* and *Vibrio cholerae* have been associated with endotoxins. Routinely, in conjunction with the intestinal barrier, Kupffer cells in the liver can remove endotoxins by a process of detoxification and phagocytosis, but high prevalence of these endotoxins are seen in the systemic circulation if these cells cannot cope with the overwhelming burden of the presence of endotoxin (O'Brien and Bruce, 2007).

In general, one endotoxin unit/ml (EU/ml) equals to 0.1ng/ml. It has been reported that the normal endotoxin levels circulating in healthy individuals is between 0.3-10.4pg/ml (O'Brien and Bruce, 2007). In endotoxemia, plasma endotoxin concentrations are greater than 2.5EU/ml or 0.25ng/ml (O'Brien and Bruce, 2007). There are recommendations for the allowable endotoxin limit for biological products, drugs and devices. According to the Food and Drug Administration (FDA 2012), water added to drugs that require dilution prior to injecting, sterile water for parenteral use and sterile, non-pyrogenic water for irrigation of body wounds or medical devices have an allowable endotoxin limit of 0.25 EU/ml. The maximum allowable endotoxin exposure for humans is calculated as 5 EU/Kg/Hour (or 350 EU per adult for 70Kg person per hour) to avoid fever and hypotension from endotoxin contamination (FDA, 2012). For drugs gaining access to the cerebrospinal fluid (intrathecals), an endotoxin limit of 0.2 EU/Kg/Hour has been determined and 2.5 EU/Kg/Hour for radiopharmaceuticals (FDA, 2012).

Endotoxin testing is crucial in establishing endotoxin limits for pharmaceutical and medical devices, in establishing procedures for validating the use of the bacterial endotoxin testing in the laboratory and establishing procedures for conducting routine testing. The Limulus Amebocyte Lysate (LAL) Test is utilized to test many drug and device products (FDA, 2012). The most frequently used test is the LAL Chromogenic endpoint test which is a sensitive and specific test based on the chromogenic detection and measurement of endotoxin.

The lipopolysaccharide complex consists of three regions. The first region consists of O-specific chains (O- antigens), which is known as the antibiotic-combining site affecting the immunogenicity of the pathogen cell wall. Diverse antigenic variations of the O side chains can occur between species and strains of pathogens as seen in *Salmonella* and *E. coli* and other strains of gram negative species. Absence of this region or parts of this region in *E. coli* and *Salmonella* allows for partial loss in virulence, making them susceptible to phagocytosis, serum bactericidal reactions, antibiotics and hydrophobic compounds as the permeability of the outer membrane is affected (Todar, 2002).

The second component consists of the Core (R) antigen, which supports the LPS structure. It consists of various sugars including heptose which binds to 2-keto-3-deoxyoctonoic acid (KDO) (Koga et al, 1985**).** KDO is one of the sugars present in the Lipid A portion of LPS which is used as a positive index in investigating the presence of LPS. LPS from the *Bacteroides species (*anaerobic gram negative bacteria) lack composition of KDO and heptose (Leenstra et al, 1996). This region is less variable but they may not have identical cores (Todar, 2002).

The Lipid A structure forms part of the third component of the LPS and affects the toxicity among *Enterobacteriaceae*. It is the least variable region (Peterson, 1996). They are associated with the toxicity of gram negative bacteria and when released in the circulation (Beveridge, 1999; Fry, 2013), are responsible for causing fever, diarrhoea, leukopenia, disseminated intravascular coagulation, hypotension and possible fatal endotoxic shock (Dey and Dey, 1978). Both Lipid A and the polysaccharide side chains determine the virulence of gram negative bacteria. They are liberated in large quantities only after the death, disintegration or autolysis of the organism (Dey and Dey, 1978). The endotoxicity of LPS for anaerobic gram negative bacteria such as *Bacteroides species* is much lower (1000 times less) than that of endotoxin from AGNB such as *E.coli.* This explanation can be justified by the course and outcome of septicaemia caused by *Bacteroides species* being more favourable than those with septicaemia caused *E. coli* (Leenstra et al, 1996).

LPS binds to lipid binding protein, which is associated with Toll-like receptor-4 (TLR4) for the recognition of the endotoxin in the host (Lundin and Checkoway, 2009). Endotoxic shock is the outcome as a result of the secretion of pro-inflammatory cytokines and nitric oxide by macrophages and endothelial cells. Several cytokines (IL-1, IL-6, IL-8 and TNF-α) and platelet- activating factor stimulate the production of prostaglandins and leukotriene, which are responsible for inflammation and septic shock. T-lymphocytes affect cell-mediated and humoral immunity and the complement cascade promotes histamine release leading to vasodilation. These patients are susceptible to blood clotting, thrombus formation and acute disseminated intravascular coagulation with the depletion of platelets and various clotting factors. Hypotension arises with the release of bradykinins and other vasoactive peptides. These sequences of events lead to inflammation, intravascular coagulation, haemorrhage and shock which is characteristic of endotoxemia (Lundin and Checkoway, 2009).

The virulence and the rate of release of endotoxins are affected by variance in its biological activity, its potency between the different species and its pathogenic property among similar species. This may also be affected by host factors such as leukopenia (Hurley, 1995). On the contrary, if after the initial exposure to endotoxins, the body tolerates subsequent exposure of continuous small doses of LPS without adverse symptoms, this phenomenon is known as endotoxin tolerance (Lundin and Checkoway, 2009).

The control of endotoxin absorption is crucial at the oropharynx and gastrointestinal tract. Neutralization of endotoxin occurs with the macrophages and lymphoid tissue in these tissues. Endotoxins that enter into the systemic circulation are neutralized by platelets, proteins and leucocytes in blood. Intraorally, the action of swallowing, reflex movements, tongue movement, salivary flow and salivary mucins affect the clearance of aerobic gram negative bacteria and endotoxin. Gastric motility and bile are important for the removal of endotoxins in the gastrointestinal tract (Leenstra et al, 1996). Bacterial translocation of aerobic gram negative bacteria can occur regardless of the presence of indigenous gram negative flora which elevates the LPS concentration systemically and attributes towards the high endotoxicity (Leenstra et al, 1996).

1.5.1 Endotoxins and oral mucositis

Although the involvement of endotoxins in the development of bacterial infections is well described, the role of endotoxins in oral mucositis is not known. Cancer patients develop oral mucositis due to the cytotoxicity of chemotherapy and the changes in the oral flora with the alteration in the oral environment. The abnormal presence of aerobic gram negative bacteria which produce endotoxins, has also been reported, which interact with the host's cells to induce the production and secretion of proteases and pro-inflammatory cytokines (Gaetti-Jardim et al, 2011 and Ramachandran, 2014). Nevertheless, the role of these endotoxins in the exacerbation of oral mucositis has not studied.

1.6 Aim

The aim of this study was to isolate and identify all aerobic gram negative bacteria and endotoxins in the oral cavities of patients receiving cancer treatment; and to determine whether the concentration levels of endotoxins affect the severity of oral mucositis within its various grades.

1.6.1 Study Objectives

- To isolate Streptococci, *Staphylococcus aureus, Candida species* and aerobic gram negative bacteria (AGNB) from the oral cavities of patients on cancer treatment and healthy individuals.
- To identify all the aerobic gram negative bacteria and perform antimicrobial susceptibility tests.
- To detect endotoxin from the oral cavities of cancer patients with and without oral mucositis.

CHAPTER 2

METHODS AND MATERIALS

2 Study population

A cross-sectional study was done on patients diagnosed with any type of malignant solid tumor, who were scheduled for either radiation and chemotherapy or only chemotherapy that were attending clinics at the Department of Radiation Oncology and the Department of Oncology, at the Charlotte Maxeke Johannesburg Academic Hospital, were approached and asked to volunteer for the study.

A sample size of one hundred cancer patients was determined, based on previous studies on cancer patients undergoing treatment (Panghal et al, 2012 and Anirudhan et al, 2008), and studies done on the presence of gram negative bacteria in healthy individuals who smoked or wore dentures (Conti et al, 2009). In both studies a large sample size was required, as the percentage of gram negative bacteria isolated were lower in proportion as compared to other bacterial isolates.

2.1 Exclusion and inclusion criteria

These patients were chosen based on the criteria as per the data collection sheet (Appendix B). The exclusion criteria included HIV positive patients, diabetic patients, smokers and patients who wore oral prostheses. Patients who were presently on antimicrobials and patients who had less than 2 weeks of cancer treatment were also not eligible to participate in the study. At the time of sampling, the procedure was explained to the selected patients and written consent was obtained (Appendix A and B). The demographic data and clinical parameters such as the type of cancer, type treatment and duration of treatment were recorded. The presence or absence and the grade of oral mucositis (WHO, 1979), was observed by inspecting their oral cavity. Oral observation, periodontal and caries screening procedures were used to detect halitosis, periodontitis, dry mouth and dental caries. Eligible patients were asked to rinse their oral cavity with 10 ml of sterile distilled water and this was collected back into a sputum jar. All samples collected were processed at the Oral Microbiology Laboratory.

A sample size of fifty healthy individuals over the age 18 years old and of either gender from the Oral Biological Sciences Department and Wits Oral Health Department, was used as a control group and chosen as per the criteria from the data collection sheet (Appendix A and B). The exclusion criteria included individuals who did have any systemic diseases (such as diabetes), smokers, those who wore oral prostheses and individuals who were presently on antimicrobials. Similarly, an oral rinse was collected from the control group. All samples that were collected were processed at the Oral Microbiology Laboratory.

2.2 Ethics

Ethical clearance (Certificate number: M160562) for the study was obtained from The Committee for Research on Human Subjects (Medical), University of the Witwatersrand, Johannesburg (Appendix C).

2.3 Microbiological analysis

2.3.1 Microbiological plating procedure

Media preparation procedures are described in Appendix D.

The oral cavity rinse samples were vortexed and one hundred microlitres of this sample was serially diluted in 0.9ml of phosphate buffered saline and mixed using a vortex mixer to create dilutions of 1/10 to 1/1000. One hundred microlitres of the oral rinse and the respective serially diluted saliva were plated using an air displacement micropipette with disposable pipette tips on Mitis Salivarius Agar (MSA), Baird Parker Agar (BP), *Candida* Chromagar and MacConkey Agar (Figure 2.1). In addition, 0.5 ml of oral rinse was added into Brain Heart Infusion broth (BHI) and this was incubated at 37°C for 24 hours as an enrichment step to allow low numbers of aerobic gram negative bacteria to be detected (Stokman et al, 2003). The Mitis Salivarius Agar plates were incubated at 37°C for 48 hours under CO_2 to isolate Streptococci. The Baird Parker Agar plates (to isolate Staphylococci), the *Candida* Chromagar plates (to isolate *Candida)* and the MacConkey Agar plates (to isolate aerobic gram negative bacteria) were incubated at 37°C for 48 hours aerobically. After the incubation period, the colonies were manually counted and expressed as colony forming

unit/ml (cfu/ml), similar to a study that was done by Ahmed *et al* (2013). One millilitre of oral rinse from all cancer patients and healthy individuals were labelled and stored in Eppendorff tubes at -20°C for the endotoxin assay.



Figure 2.1: Layout of agar plates for the microbiological analysis

2.3.2 Colony counts

Blue colonies were counted (cfu/ml) to obtain the total bacterial count of *Streptococcus mutans* on the Mitis Salivarius Agar plate (Figure 2.2). Counts were multiplied with the dilution factors and expressed as counts per ml of oral rinse.

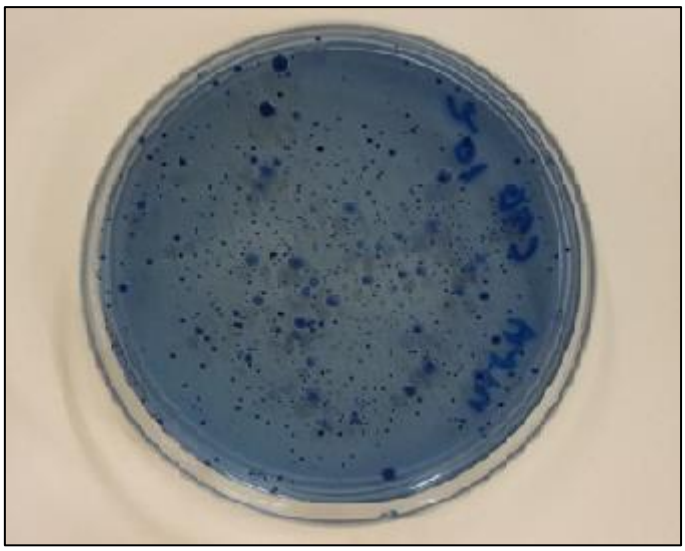


Figure 2.2: Colonies of *Streptococcus mutans* on a Mitis Salivarius Agar plate

Black colonies with a halo around the colony were counted to obtain the total bacterial count of *Staphylococcus aureus* (cfu/ml) from the Baird Parker Agar plate (Figure 2.3).

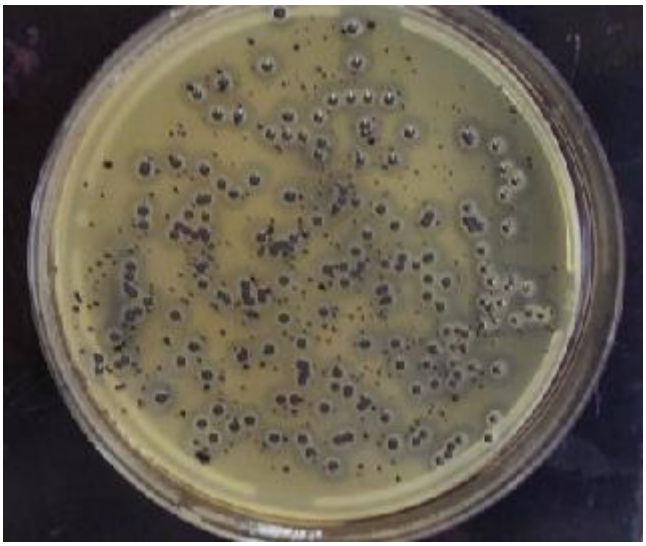


Figure 2.3: Colonies of *Staphylococcus aureus* on a Baird Parker Agar plate

Green, purple and blue colonies of *Candida* species were counted (cfu/ml) on the Candida Chromagar plates (Figure 2.4).

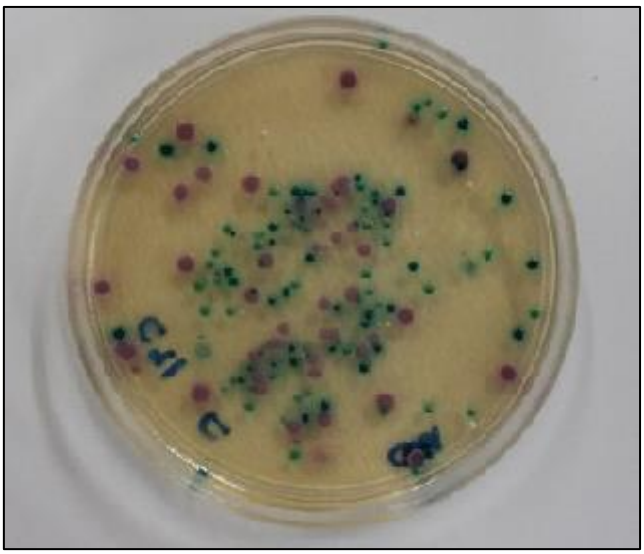


Figure 2.4: Mixed culture of *Candida* species on a Chromagar plate

The light and dark pink colonies of aerobic gram negative bacteria were counted (cfu/ml) from the MacConkey Agar plates. After incubation, the BHI broth showed turbidity from the growth of microorganisms. This was sub-cultured on MacConkey Agar plates and incubated at 37°C for a further 48 hours by the streaking technique using a cooled sterilized loop. The Gram staining technique was preliminary performed on certain isolated colonies, similar to a technique described in a study by Ahmed *et al*, (2003) to identify the presence of gram positive and gram negative cocci and bacilli.

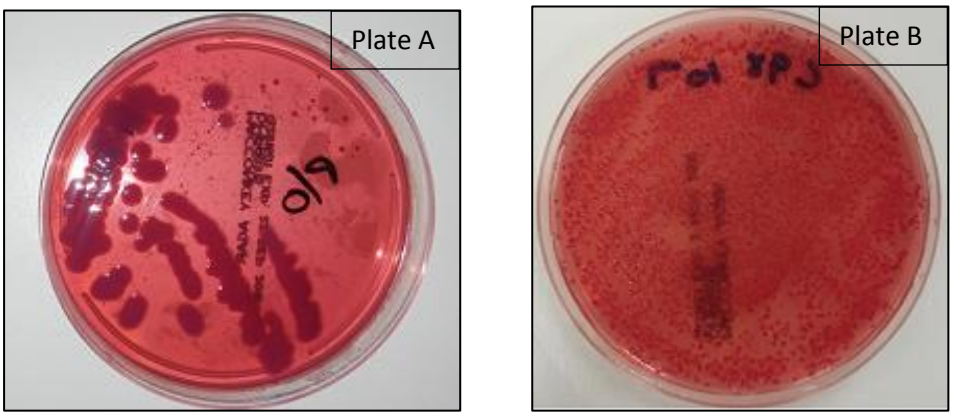


Figure 2.5 Plate A and B: Colonies of aerobic gram negative bacteria on a MacConkey Agar plate

2.3.3 The isolation and identification of *Candida spp.*

An isolated colony of *Candida spp*. from the Chromagar was randomly selected and plated using the streaking technique. A cooled sterilized loop was used to streak a Sabouraud Agar plate, which was used as an isolation plate and incubated for a further 48 hours at 37°C (Figure 2.6). These cultures were then identified using the API® 20 C AUX (bioMérieux) system. All the different colour colonies were isolated and identified separately.



Figure 2.6: Isolated colonies of *Candida spp.* on Sabouraud agar

The API® 20 C AUX system was used for the precise identification of *C. albicans* and other yeasts that were encountered which was similarly also used in a study by Ahmed *et al*, (2013). The API® 20 C AUX strip consists of 20 cupules (Table 2.1), containing dehydrated substrates which enable the performance of 19 assimilation tests. A suspension constituting of a young culture of a portion of a well isolated yeast colony from the isolation Sabouraud Agar plate and 2ml of the API C Medium, was mixed with turbidity equal to 2 McFarland standard. This is done to standardize the turbidity of the yeast solution tested by obtaining a solution with a specific optical density.

This was used to inoculate the cupules using a Pasteur pipette. The strips were tilted forward and the Pasteur pipette was placed against the side of the cupule to prevent the formation of air bubbles at the base of the tubes and care was taken not to overfill and underfill the cupules. The yeasts only grew if they were capable of utilizing each substrate as their sole carbon source. After 48 and 72 hours of incubation at 37°C, growth was read by comparing its turbidity to growth controls (Figure 2.7). Identification was obtained by referring to the Analytical Profile Index or using the identification software.

Table 2.1: Contents of the cupules of the API® 20 C tray

Tests	Active Ingredients	Quantity(mg/cup)
0	None	-
GLU	D-GLUcose	1.2
GLY	GLYcerol	1.2
2KG	Calcium 2-Keto-Gluconate	1.2
ARA	L-ARAbinose	1.2
XYL	D-XYLose	1.2
ADO	ADOnitol	1.2
XLT	XyLiTol	1.2
GAL	D-GALactose	1.9
INO	INOsitol	2.36
SOR	D-SORbitol	1.2
MDG	Methyl-αD-Glucopyranoside	1.2
NAG	N-Acetyl-Glucosamine	1.2
CEL	D-CELlobiose	1.2
LAC	D-LACtose (bovine origin)	1.2
MAL	D-MALtose	1.2
SAC	D-SACcharose (sucrose)	1.2
TRE	D-TREhalose	1.2
MLZ	D-MeLeZitose	1.2
RAF	D-RAFfinose	1.9



Figure 2.7: API® 20 C AUX system for the identification of *Candida species.*

2.3.4 The isolation and identification of aerobic gram negative bacteria

On each MacConkey Agar plate where growth of aerobic gram negative bacteria had been found from cancer patients and healthy subjects, isolated colonies of this bacteria were randomly selected and plated using a cooled sterilized loop onto another MacConkey Agar plate and incubated for 48 hours at 37°C (Figure 2.8).

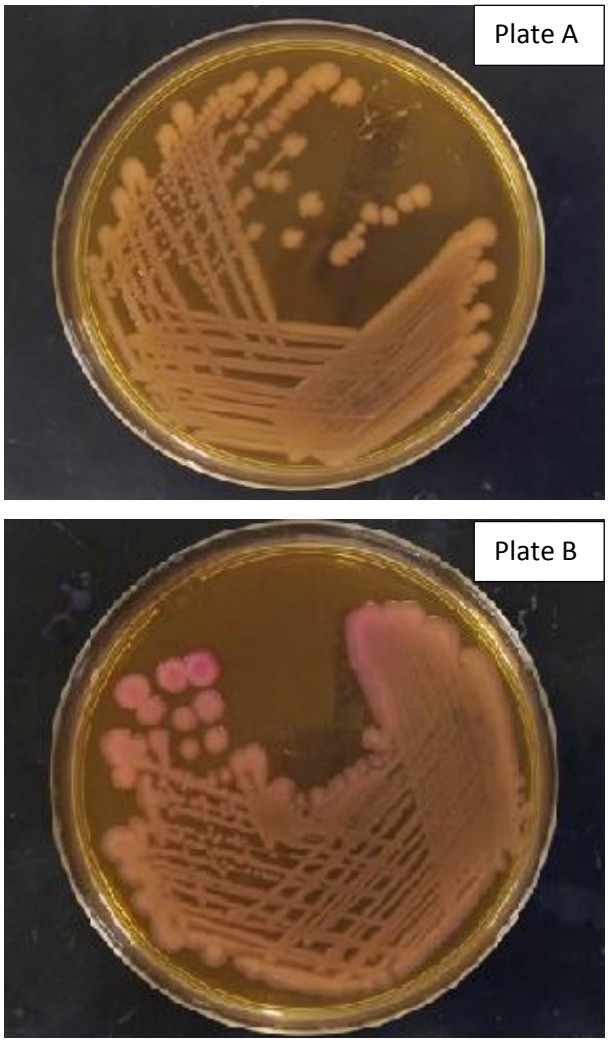


Figure 2.8 Plate A and B: Isolation of aerobic gram negative bacteria on MacConkey Agar plates

An oxidase test was performed to determine the presence of the bacterial cytochrome oxidase enzyme by using the wet filter paper method, similarly, as performed in a study by Ahmed *et al*, (2003). Using a sterile cool loop, a small portion of a pure colony was transferred onto a filter paper saturated with oxidase reagent and rubbed onto the reagent. A positive reaction was indicated by an intense deep-purple hue within 10- 30 seconds. A negative test resulted in a light-pink or absence of colouration (Figure 2.9).



Figure 2.9: Oxidase Test for the aerobic gram negative bacteria

These cultures were identified using the API® 20 E (bioMérieux) system. This system was similarly used in a study by Conti *et al*, (2009) to identify *Enterobacteriaceae* on the dorsum of the tongue and a study by Leenstra *et al*, (1996) in the identification of gram negative aerobic bacteria. It consists of 20 microtubes containing dehydrated chromogenic substrates. A bacterial suspension consisting of a well isolated colony from the isolation MacConkey Agar plate was added to 5ml of sterile distilled water without additives and emulsified. The tests were inoculated with the bacterial suspension using a Pasteur pipette, tilting the strips forward and placing the Pasteur pipette against the side of the cupule to prevent the formation of air bubbles at the base of the tubes. They were incubated at 37°C for 24 hours. Colour changes occurred either spontaneously during incubation from metabolism or after the addition of reagents after the incubation period (Figure 2.10). These reactions were read according to the Reading Table and the identification was obtained by referring to the Analytical Profile Index or using the identification software.



Figure 2.10: API® 20 E System for the identification of aerobic gram negative bacteria

Table2.2: Reading Table: API® 20E System (Adapted from API® 20E System reading manual)

READING TABLE

TESTS	ACTIVE INGREDIENTS	QTY (mg/cup.)	REACTIONS/ENZYMES	RESULTS	
				NEGATIVE	POSITIVE
ONPG	2-nitrophenyl-ßD-galactopyranoside	0.223	ß-galactosidase (Ortho NitroPhenyl-ßD-Galactopyranosidase)	colorless	yellow (1)
ADH	L-arginine	1.9	Arginine DiHydrolase	yellow	red / orange (2)
LDC	L-lysine	1.9	Lysine DeCarboxylase	yellow	red / orange (2)
ODC	L-ornithine	1.9	Ornithine DeCarboxylase	yellow	red / orange (2)
CIT	trisodium citrate	0.756	CiTrate utilization	pale green / yellow	blue-green / blue (3)
H_2S	sodium thiosulfate	0.075	H_2S production	colorless / greyish	black deposit / thin line
URE	urea	0.76	UREase	yellow	red / orange (2)
TDA	L-tryptophane	0.38	Tryptophane DeAminase	TDA / immediate	
				yellow	reddish brown
IND	L-tryptophane	0.19	INDole production	JAMES / immediate	
				colorless pale green / yellow	pink
VP	sodium pyruvate	1.9	acetoin production (Voges Proskauer)	VP 1 + VP 2 / 10 min	
				colorless	pink / red (5)
GEL	Gelatin (bovine origin)	0.6	GELatinase	no diffusion	diffusion of black pigment
GLU	D-glucose	1.9	fermentation / oxidation (GLUcose) (4)	blue / blue-green	yellow / greyish yellow
MAN	D-mannitol	1.9	fermentation / oxidation (MANnitol) (4)	blue / blue-green	yellow
INO	inositol	1.9	fermentation / oxidation (INOsitol) (4)	blue / blue-green	yellow
SOR	D-sorbitol	1.9	fermentation / oxidation (SORbitol) (4)	blue / blue-green	yellow
RHA	L-rhamnose	1.9	fermentation / oxidation (RHAmnose) (4)	blue / blue-green	yellow
SAC	D-sucrose	1.9	fermentation / oxidation (SACcharose) (4)	blue / blue-green	yellow
MEL	D-melibiose	1.9	fermentation / oxidation (MELibiose) (4)	blue / blue-green	yellow
AMY	amygdalin	0.57	fermentation / oxidation (AMYgdalin) (4)	blue / blue-green	yellow
ARA	L-arabinose	1.9	fermentation / oxidation (ARAbinose) (4)	blue / blue-green	yellow
OX	(see oxidase test package insert)		cytochrome-OXidase	(see oxidase test package insert)	

2.4 Antibiotic Sensitivity Test

The inoculum was prepared by making a saline suspension of young culture of isolated colonies from the MacConkey Agar plate of cancer patients and healthy individuals with a positive identification for aerobic gram negative bacteria. The suspension was adjusted to match the 0.5 McFarland turbidity standard, using a spectrophotometer at an absorbance of 625nm.

The surface of a Müeller-Hinton agar plate was inoculated by streaking a sterile buccal swab over the entire agar surface of three agar plates per sample. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed. Each of the three different labelled agar plates received six different commercially-prepared, fixed concentration paper antibiotic discs, which were placed evenly and distributed on the inoculated agar surface by a disc dispenser. The plates were incubated at 37°C for 24 hours. The plates were read after 24 hours using transmitted light by looking for any growth within the zone of inhibition. The susceptibility patterns were noted.

The zones of growth and inhibition around each of the antibiotic discs were measured to the nearest millimetre using sliding callipers (Figure 2.11). The diameter of the zone was related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The zone diameters of each drug were interpreted using the criteria published by the Clinical and Laboratory Standards Institute (CLSI, 2016). The qualitative results were based on the category of susceptibility, namely Susceptible (S), Intermediate (I), or Resistant (R) to the agents. The antibiotic discs on agar plate one (N1) had 10µg Ampicillin, 30µg Cefotaxime, 30µg Cefuroxime, 30µg Cefoxitin, 10µg Piperacillin-tazobactam and 10µg Gentamicin. Agar plate two (N2) contained 30µg Cefepime, 10µg Ertapenem, 10µg Imipenem, 10µg Meropenem, 30µg Ceftazidime and 10µg Amoxicillin- clavulanate. Agar plate three (N3) contained 30µg Nalidixic, 10µg Tobramycin, 30µg Amikacin, 30µg Chloramphenicol, 23.75µg Trimethoprim- sulfamethoxazole and 5µg Ciprofloxacin.

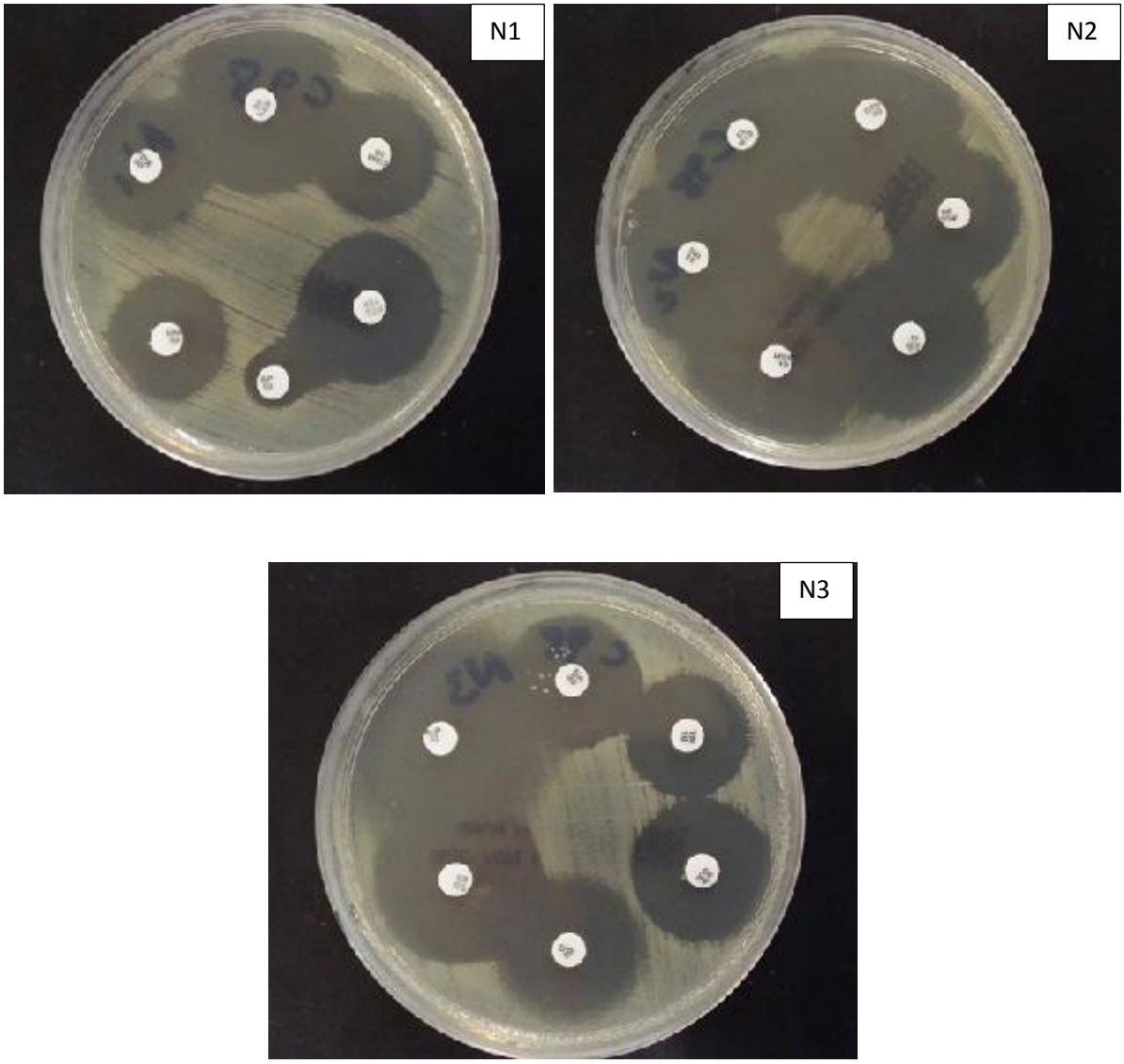


Figure 2.11: Antibiotic Susceptibility Test on Müeller-Hinton Agar plate

2.5 Endotoxin assay

The Hycult Biotech Limulus Amebocyte Lysate (LAL) assay was used to detect endotoxins in the oral rinse samples collected from 60 cancer patients and 16 healthy individuals. The oral rinse samples analysed from cancer patients were with or without aerobic gram negative bacteria and with or without oral mucositis. The oral rinse samples analysed from healthy individuals did not present with aerobic gram negative bacteria. This assay can measure the bacterial endotoxin in gram negative bacteria from various biological fluid (including sera), devices, (air) filters and tissue culture medium. It is a sensitive product that detects from as little as 0.04 EU/ml of endotoxin and ranges to 10 EU/ml. It is a specific and accurate product which uses the endotoxin standard from the kit to accurately quantify bacterial endotoxin.

The basis of this test was that these bacteria caused intravascular coagulation in the American horseshoe crab, Limulus Polyphemus by endotoxin, triggering the turbidity and gel-forming reaction enzymatically. The enzymatic reaction of this test will cause a yellow colour to develop upon cleavage of chromophore, p-nitroaniline (pNA), which is stopped by the addition of acetic acid as a stop solution. The absorbance at 405 nm is measured with a spectrophotometer. The endotoxin concentration of samples with unknown concentrations, which are run concurrently with the standards, can be determined from a standard curve. It uses an endotoxin standard of known concentration that is derived from the *E. coli* strain as stated on the Certificate of Analysis to create a standard curve. The developed colour intensity is proportional to the amount of endotoxin present in the sample and its concentration is determined by extrapolating the absorbance of an unknown sample against this standard curve.

The oral rinse that was stored at -20ºC in the Eppendorff tubes were warmed back to room temperature. Samples were centrifuged at 5000 rpm for 5 minutes, similar to a method described by Leenstra *et al*, (1996). The supernatants were collected (200µl) into another Eppendorff tube. These were placed on a heating block at 75ºC for 5 minutes to neutralize any endotoxin inhibiting compounds. From each sample of oral rinse, 25µl was diluted with 175µl of endotoxin free water (EFW) from the Hycult Biotech Limulus Amebocyte Lysate (LAL) assay kit to give a dilution of 1:8.

All materials in contact with the oral rinse such as pipette tips, the test material and its contents and reagents were endotoxin free. Depyrogenated test tubes were also utilized. All reagents were equilibrated to room temperature at 25°C.

The LAL reagent was prepared by adding 4ml of EFW to the LAL reagent vial. This was gently swirled until the LAL reagent dissolved into a colourless solution. The standard solution was reconstituted by pipetting 1.8ml of EFW (as stated on the Certificate of Analysis) to the concentrated endotoxin (*E. coli*) standard (Lot number 19819K0316-A), to give a concentration of 50 EU/ml after reconstitution. This was vortexed for 5 minutes. The Stop solution was prepared by adding 10ml of 2.5x concentrated stop solution to 15ml of distilled water.

The number of test wells required to assay the standards, controls and samples in duplicate was determined and labelled accordingly. For the standard series, 50µl of the reconstituted standard was added to 50µl of EFW using a calibrated micropipette, and vortexed for 30 seconds, to dilute it further to obtain a concentration of 25 EU/ml (Figure 2.12). For the duplicate standard curve, 16 wells of the plate were filled with 50µl of EFW. Thereafter, 33µl of the diluted standard was added to well A1 and diluted 1:1.5 further and mixed thoroughly. This was repeated by pipetting 33µl of this over to well B1 and again to the next well and so on until well number G1. From well G1, 33µl was discarded and well H1 was used as a control. This procedure was repeated for the standard dilution method for the A2-H2. This gave a concentration range of endotoxin as follows: 10, 4, 1.6, 0.64, 0.26, 0.10, and 0.04 EU/ml.

From each of the diluted samples, 50µl was transferred in duplicate to the assigned sample and control wells with a clean pipette for each transfer done. From the reconstituted LAL reagent, 50µl was added to each well, except in the control wells, where 50µl of EFW was added instead of LAL reagent. The plate was covered and incubated for 30 minutes at room temperature. The enzymatic reaction, triggered by endotoxin, caused a yellow colour to develop upon cleavage of the chromophore, p-nitroaniline. The samples were measured at 405nm using a calibrated ELISA plate reader (spectrophotometer). Similarly samples were processed (Figure 2.13).

If the standard concentrations between 10 and 4 EU/ml differed more than 10% in OD value, this was incubated for an additional 5 minutes. If it differed less than 10% in OD value, the reaction was halted by adding 50µl of stop solution. The absorbance was measured at 405nm with the spectrophotometer.

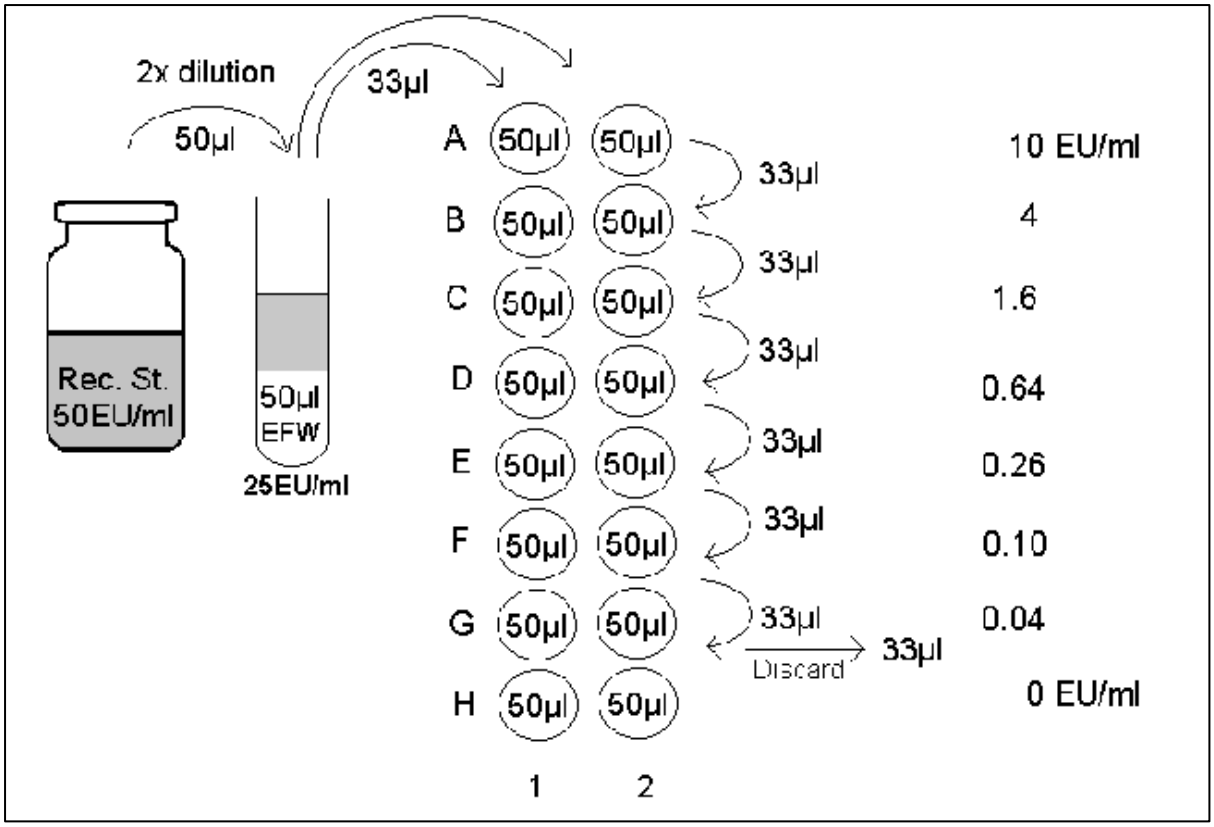


Figure 2.12: Preparation of the dilutions of standard series of *E. coli* endotoxin

Adapted from Limulus Amebocyte Chromogenic Endpoint Assay: Test for endotoxin detection- Product information and manual. Manual HIT302 Edition 04-13

If individual absorbance values differed by more than 15% from the corresponding mean value, the sample was retested. The mean absorbance of the zero standard should be less than 0.1 OD 405nm and the mean absorbance of the 10 EU/ml standard should be higher than 0.6 OD 405nm. A standard curve was obtained by plotting the absorbance (linear) versus the corresponding concentrations of the *E. coli* standards (log). The endotoxin concentration of the samples was read from the standard curve and this was multiplied by the dilution factor (Appendix E).

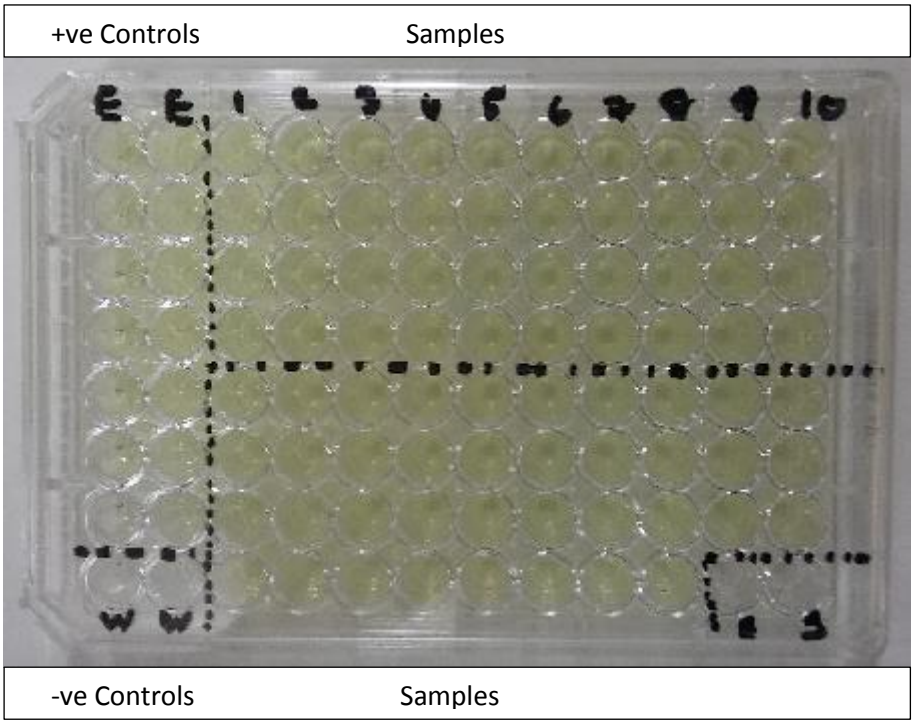


Figure 2.13 The assigned wells for the standard series, samples and controls

2.6 Statistical analysis of the data

The data was sorted and cleaned (identification, diagnosing and removal of missing variables). The data was imported into Statistica 12 for analysis. Results showed that by using the Shapiro Wilk test, there was no normality in the distribution in counts of *S. aureus, Streptococci, Candida* and endotoxin between the different study groups ($p>0.05$). Thus, the median and interquartile ranges were reported as measures of central tendency and the statistical significance was determined by using the Mann-Whitney test. The carrier rate between the different populations was computed on frequency tables of percentages and the chi-square test was used to check the statistical significance in the differences in percentage. P-values of $\leq 0.05\%$ were considered significant.

CHAPTER 3

RESULTS

3.1 Demographic data and clinical parameters

Demographic data and clinical parameters of cancer patients on treatment are shown in Table 3.1. The mean age of cancer patients was 52 years and 80% of cancer patients were females. These females mostly had either breast (58%) or cervical cancer (9%). Majority of these patients were on chemotherapy (61%). Patients were well into their chemotherapy treatment cycles and were on multiple chemotherapeutic drugs. The majority of them complained of nausea (61%). Most of them had no major other illness except for hypertension (21%). Some patients had oral mucositis (12%) which was classified as Grade I, Grade II, Grade III and Grade IV. Patients complained of dry mouth, altered taste and loss of appetite.

Table 3.1: Demographic data and clinical parameters of cancer patients on treatment

Parameters (*n*=100)	**Results**
Age Mean ±SD (Range)	51.87 ± 12.8 (25-92)
Gender	Male: 20%, Female: 80%
Type of Cancer	**Breast cancer: 58%, Cervical cancer: 9%,** Prostate cancer: 6%, Colon cancer: 5%, Rectal cancer: 4%, Laryngeal cancer: 3%, Pancreatic cancer: 2%, Skin cancer: 2%, Bone cancer, Brain cancer, Colon and Liver cancer, Gastric cancer, Liver cancer, Lung cancer, Nasal cancer, Ovarian cancer, Ovarian, Intestine, Liver, Vulva cancer, Palatal cancer, Spleen cancer each 1%
Treatment	Chemoradiation: 39%, Chemotherapy: 61%
Length of treatment completed	Chemoradiation: (*n*= 39) a. Chemotherapy cycles Mean ±SD (Range): 5.41 ±3.4 (2-14) b. Radiation sessions Mean ±SD (Range): 15.82 ±10.8 (1-35) Chemotherapy: (*n*= 61) a. Chemotherapy cycles Mean ±SD (Range): 7.97 ±18.4 (2-144)
Chemotherapy Medication	**Adriamycin + Cyclophosphamide: 24%** **Cisplatin: 10%** **Taxol: 7%**

	Adriamycin + Cyclophosphamide + Taxol: 6% 5 Fluorouracil + Leucovorin + Calcium: 5% 5 Fluorouracil + Adriamycin + Cyclophosphamide: 5% 5 Fluorouracil: 2% Adriamycin + Cyclophosphamide + Docetaxel: 2% Adriamycin, Adriamycin + Cyclophosphamide + Doxorubicin, Adriamycin + Cyclophosphamide + Tamoxifen, Cisplatin + Decadron, Docetaxel, Docetaxel + Decadron + Prednisone, Gemzar, Navelbine, Tamoxifen, Trial Chemo Drug, Xeloda + Oxaliplatin: each 1% Unknown: 28%
Nausea & Vomiting	Yes: 61%, No: 39%
Diabetes	No: 100%
HIV status	No: 83%, Unknown: 17%
Other medical conditions (n=100)	None: 70% High blood pressure: 21% Asthma, Cholesterol, Depression, arthritis, Blood Clotting, thyroid condition, Stomach Ulcers: 1% each
Smoker	No: 100%
Dentures	No: 100%
Antimicrobials	No: 100%
Oral Mucositis	History of oral mucositis: 27% Oral mucositis present: 12% None present or no history: 61%
Grade of oral mucositis	Grade 0: 69%, Grade I: 16%, Grade II: 11% Grade III: 3%, Grade IV: 1%
Loss of appetite	Yes: 57%, No: 43%
Difficulty to swallow	Yes: 33%, No: 67%
Altered taste	Yes: 74%, No: 26%
Halitosis	Yes: 22%, No: 78%
Swollen gums	Yes: 24%, No: 76%
Periodontitis	Yes: 42%, No: 58%
Dry mouth	Yes: 88%, No: 12%
Dental caries	Yes: 58%, No: 42%

3.2 Demographic data and clinical parameters of healthy individuals

Demographic data and clinical parameters of healthy individuals are shown in Table 3.2. The mean age of this study group was 31 years and they were mainly females (76%). They were relatively healthy with no illnesses (94%) and their oral health was also relatively good.

Table 3.2: Demographic data and clinical parameters of healthy individuals

Parameters (*n*=50)	**Results**
Age Mean ±SD (Range)	30.50 ± 11.6 (19-57)
Gender	Male: 24%, Female: 76%
Diabetes	No: 100%
Other systemic diseases	High blood pressure: 6%, Hypothyroidism: 2% None: 94%
Smoker	No= 100%
Dentures	No= 100%
Antimicrobials	No= 100%
Halitosis	No= 100%
Swollen gums	Yes: 16%, No: 80%
Periodontitis	Yes: 2%, No: 98%
Dry Mouth	Yes: 16%, No: 84%
Dental caries	Yes: 34%, No: 66%

3.3 Microbiological analysis of oral rinse samples collected from the cancer patients on treatment

The raw data of the microbiological analysis, endotoxin and the statistical analysis are presented in Appendix E, F and G.

The results of the microbiological analysis of oral rinse samples collected from the cancer patients on treatment are shown in Table 3.3. All the patients carried Streptococcus species in their oral cavity and the mean counts were 2.1 x 10^6 cfu/ml. *Staphylococcus aureus* and *Candida species* was carried by 52% and 54% of patients respectively. The mean *S. aureus* counts (1939 cfu/ml) were much higher than the *Candida* counts (693 cfu/ml). These cancer patients carried multiple species of *Candida.* The major species carried was *C. albicans,* followed by *C. glabrata* and *C. famata*. Six patients carried more than one species of *Candida.* Twenty four percent of patients carried aerobic gram negative bacteria in their oral cavity. Counts of these bacteria were not possible because some of them were identified after

the enrichment technique. Eleven different types of gram negative bacteria were identified and the most isolated bacteria were *Enterobacter cloacae.*

Table 3.3: Microbiological analysis of oral rinse samples collected from cancer patients on treatment

Organism	**Carriage (%)**	**Counts in carriers cfu/ml Mean ±SD (range)**	**Identification (no. of isolates)**
Streptococcus species	100	2142950 ± 3274759.1 (87000 - 24880000)	Not done
S. aureus	52	1938.90 ± 4705.9 (10-15000)	*S. aureus* (52)
Candida species (Isolates) (Patients)	54	692.59 ± 1732.5 (10- 10000)	 *Candida albicans* (44) *Candida glabrata* (9) *Candida famata* (5) *Saccharomyces cerevisiae* (3) Unidentifiable (3) [*C. albicans* + *C. glabrata* (6)] [*C. albicans* + *S. cerevisiae* (1)]
Aerobic gram negative bacteria	24	Not done	*Enterobacter cloacae* (6) *Escherichia coli* (4) *Klebsiella pneumonia* (3) *Pantoea Spp.(*2) *Pseudomonas aeruginosa* (3) *Aeromonas hydrophila* (1) *Klebsiella oxytoca* (1) *Citrobacter koseri* (1) *Serratia rubidaea* (1) *Kluyvera spp*. (1) *Pasteurella pneumotropica* (1)

3.4 Antimicrobial susceptibility of aerobic gram negative bacteria isolated from the oral cavities of cancer patients on treatment

The results of the antimicrobial susceptibility of aerobic gram negative bacteria isolated from the oral cavities of cancer patients on treatment are shown in Table 3.4. The data was very small to interpret, however resistance to antibiotics was high in *Pseudomonas species*.

Table 3.4 Antimicrobial susceptibility of aerobic gram negative bacteria isolated from the oral cavities of cancer patients on treatment

	Number of resistant strains (%)										
Ab *N*=18	*Enterobacter* spp. *n*= 6	*Klebsiella* spp. *n*= 4	*Escherichia* spp. *n*=4	*Pseudomonas* Spp. *n*= 3	*Pantoea* spp. *n*= 2	*Kluyvera* spp. *n*= 1	*Serratia* spp. *n*= 1	*Pasteurella* spp. *n*= 1	*Aeromonas* spp. *n*= 1	*Citrobacter* spp. *n*= 1	Total *n*= 24
AP	**1** (16.66)	**3** (75)	**3** (75)	**2** (66.66)	0	**1**(100)	0	0	0	**1** (100)	11 (45.83)
CTX	0	**1** (25)	0	**2** (66.66)	0	0	0	0	**1** (100)	0	4 (16.66)
CXM	0	**1** (25)	0	**2** (66.66)	0	0	0	0	**1** (100)	0	4 (16.66)
FOX	**4** (66.66)	0	0	**2** (66.66)	0	0	0	0	0	0	6 (25)
PTZ	**1** (16.66)	0	**1** (25)	0	0	0	0	0	0	0	2 (8.33)
GM	0	0	**1** (25)	0	**1** (50)	0	0	0	0	0	2 (8.33)
CPM	0	0	0	**1** (33.33)	0	0	0	0	**1** (100)	0	2 (8.33)
ETP	0	0	0	**1** (33.33)	0	0	**1** (100)	0	0	0	2 (8.33)
IMI	0	0	0	0	0	0	0	0	0	0	0
MEM	0	0	0	0	N/D	0	0	0	N/D	0	0
CAZ	**1** (16.66)	0	0	**1** (33.33)	**2** (100)	0	0	0	**1** (100)	0	5 (20.83)
AUG	**2** (33.33)	**1** (25)	**1** (25)	**2** (66.66)	0	0	0	0	0	0	6 (25)
NA	**1** (16.66)	0	**2** (50)	**2** (66.66)	**2** (100)	0	0	**1** (100)	**1** (100)	0	9 (37.5)
TN	0	**1** (25)	**1** (25)	0	**1** (50)	N/D	**1** (100)	0	0	0	4 (16.66)
AK	0	0	0	0	0	0	0	0	0	0	0
C	0	**1** (25)	0	**1** (33.33)	0	0	0	0	**1** (100)	0	3 (12.5)
TS	0	**2** (50)	**3** (75)	0	0	0	0	0	0	0	5(20.83)
CIP	0	**1** (25)	**1** (25)	0	0	0	0	0	0	0	2 (8.33)

Ab = Antibiotic, N/D = Not done, AP = Ampicillin, CTX = Cefotaxime, CXM = Cefuroxime, FOX = Cefoxitin, PTZ = Piperacillin-tazobactam, GM = Gentamycin, CPM = Cefepime, ETP = Ertapenem, IMI = Imipenem, MEM = Meropenem, CAZ = Ceftazidime, AUG = Amoxicillin- clavulanate potassium, NA = Nalidixic acid, TN= Tobramycin, AK = Amikacin, C= Chloramphenicol, TS = Trimethoprim- sulfamethoxazole, CIP = Ciprofloxacin

3.5 Microbiological analysis of oral rinse samples collected from healthy individuals

The results of the microbiological analysis of oral rinse samples collected from healthy individuals are shown in Table 3.5. Ninety eight percent of individuals carried Streptococcus species in their oral cavity and the mean counts were 1.1 x 10^6 cfu/ml. *Staphylococcus aureus* and *Candida species* was carried by 36% and 20% of individuals respectively. Mean *S. aureus* counts were 433 cfu/ml whereas the *Candida* counts were 1149 cfu/ml. These healthy individuals also carried more than one species of *Candida.* The major species carried was *C. albicans.* Fourteen percent of patients carried aerobic gram negative bacteria in their oral cavity. Counts of these bacteria were not possible because some of them were identified after the enrichment technique. Three different types of gram negative bacteria were identified and the most isolated bacteria were *Enterobacter cloacae.*

Table 3.5: Microbiological analysis of oral rinse samples collected from healthy individuals

Organism	**Carriage (%)**	**Counts in carriers cfu/ml Mean ±SD (range)**	**Identification (no. of isolates)**
Streptococcus species	98	1144616.33 ± 1110205.0 (1200 - 4600000)	Not done
S. aureus count	36	433.33 ± 1210.2 (10 - 4700)	*S. aureus* (52)
Candida species	20	1149 ± 2353.5 (10 - 6000)	*Candida albicans* (7) *Candida famata* (1) *Candida dubliniesis* (1) *Candida krusei* (1)
Aerobic gram negative bacteria	14	Not done	*Enterobacter cloacae* (3) *Klebsiella oxytoca* (1) *Klebsiella pneumoniae* (3)

3.6 Antimicrobial susceptibility of aerobic gram negative bacteria isolated from the oral cavities of healthy individuals

The results of the antimicrobial susceptibility of aerobic gram negative bacteria isolated from the oral cavities of healthy individuals are shown in Table 3.6. *Klebsiella species* was found to be resistant to many antibiotics.

Table 3.6: Antimicrobial susceptibility of aerobic gram negative bacteria isolated from the oral cavities of healthy individuals

	Number of resistant strains (%)		
Ab	*Enterobacter* spp. *n*= 2	*Klebsiella* spp. *n*= 4	Total *n*= 6
AP	0	4 (100)	4 (66.66)
CTX	0	2 (50)	2 (33.33)
CXM	0	2 (50)	2 (33.33)
FOX	2 (100)	2 (50)	4 (66.66)
PTZ	0	0	0
GM	0	2 (50)	2 (33.33)
CPM	0	0	0
ETP	0	0	0
IMI	1 (50)	0	1 (16.66)
MEM	0	0	0
CAZ	0	2 (50)	2 (33.33)
AUG	1 (50)	0	1 (16.66)
NA	0	2 (50)	2 (33.33)
TN	0	2 (50)	2 (33.33)
AK	0	0	0
C	0	2 (50)	2 (33.33)
TS	0	1 (25)	1 (16.66)
CIP	0	0	0

Ab = Antibiotic, AP = Ampicillin, CTX = Cefotaxime, CXM = Cefuroxime, FOX = Cefoxitin, PTZ = Piperacillin-tazobactam, GM = Gentamycin, CPM = Cefepime,
ETP = Ertapenem, IMI = Imipenem, MEM = Meropenem, CAZ = Ceftazidime, AUG = Amoxicillin-clavulanate potassium, NA = Nalidixic acid, TN= Tobramycin,
AK = Amikacin, C= Chloramphenicol, TS = Trimethoprim- sulfamethoxazole, CIP = Ciprofloxacin

3.7 Summary results of cancer patients and healthy individuals

Summary results of cancer patients and healthy individuals are shown in Table 3.7 and Figure 3.1. Cancer patients carried significantly high numbers of Streptococci compared to the healthy individuals. The carriage rate of *S. aureus* was not different between the two groups. The results also showed that the *Candida* carrier rate was significantly high in cancer patients compared to healthy individuals ($p<0.01$). Although the *Candida* counts were not different between the groups ($p>0.05$), cancer patients carried a variety of *Candida species* and some patients carried more than one *Candida species* in their oral cavity. In addition, *C. glabrata* was only found in cancer patients. Between the two groups, no significant difference in the carrier rate of aerobic gram negative bacteria was found ($p>0.05$). However, cancer patients carried a variety of aerobic gram negative bacteria. *Enterobacter cloacae* and *Klebsiella pneumonia* were isolated from the oral cavities of both the study groups.

Table 3.7: Summary results of cancer patients and healthy individuals

Organism	**Cancer patients**	**Healthy individuals**	**p- value**
Streptococcus species Carriage (%)	**100**	**98**	Not done
Counts (cfu/ml) Mean ±SD (range)	2142950 ± 3274759.1 (87000 - 24880000)	1144616.33 ± 1110205 (1200 - 4600000)	**P=0.020**
S. aureus Carriage (%)	**52**	**36**	P=0.064
Counts (cfu/ml) Mean ±SD (range)	1938.90 ± 4705.9 (10-15000)	433.33 ± 1210.2 (10 - 4700)	P=0.310
Candida species Carriage (%)	**54**	**20**	**P<0.01**
Counts (cfu/ml) Mean ±SD (range)	692.59 ± 1732.5 (10- 10000)	1149 ± 2353.5 (10 - 6000)	P=0.567
Species (Isolates)	*C. albicans* (44) *C. glabrata* (9) *C. famata* (5) *S. cerevisiae* (3) Unidentifiable (3)	*C. albicans* (7) *C. famata* (1) *C. dubliniesis* (1) *C. krusei* (1)	

(Patients)	*C. albicans+C. glabrata* (6) *C. albicans+S.cerevisiae* (1)		
Aerobic gram negative bacteria Carriage (%) Counts (cfu/ml) Mean ±SD (range) Species	**24** Not done *E. cloacae* (6) *E. coli* (4) *K. pneumonia* (3) *Pantoea Spp.(*2) *Ps. aeruginosa* (3) *A. hydrophila* (1) *K. oxytoca* (1) *C. koseri* (1) *S. rubidaea* (1) *Kluyvera spp*. (1) *P. pneumotropica* (1)	**14** Not done *E. cloacae* (3) *K. oxytoca* (1) *K. pneumoniae* (3)	P=0.154

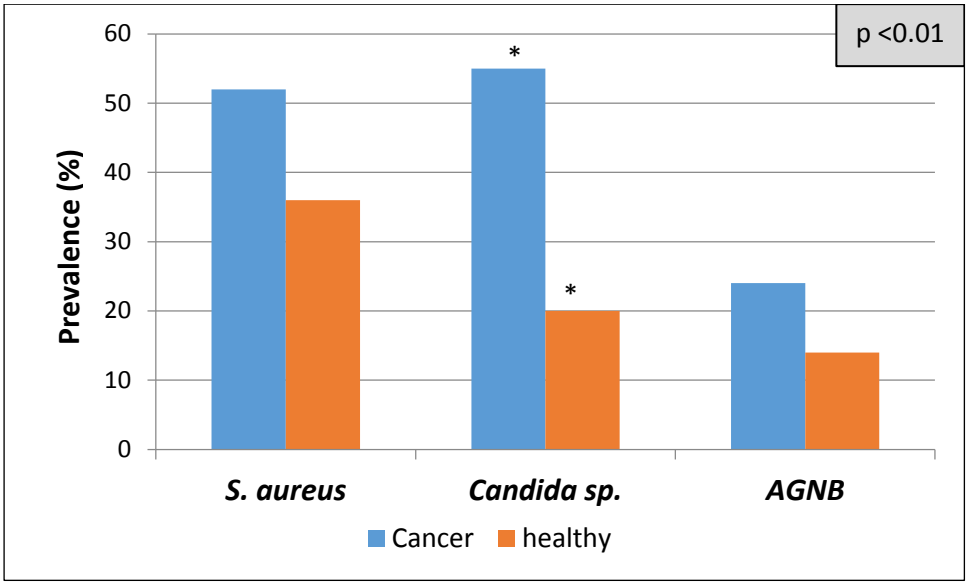


Figure 3.1 Prevalence of *S. aureus, Candida species* and aerobic gram negative bacteria (AGNB) in the oral cavities of cancer patients and healthy individuals

3.8 *S. aureus, Candida species* and aerobic gram negative bacteria (AGNB) in cancer patients with and without oral mucositis

The results of *S. aureus*, *Candida species* and aerobic gram negative bacteria (AGNB) in cancer patients with and without oral mucositis are shown in Table 3.8 and Figure 3.2. The results showed that the prevalence of *S. aureus* and the counts were not significantly different in patients with oral mucositis and patients without oral mucositis. Similarly percentage prevalence of aerobic gram negative bacteria was also similar in both study groups. However, percentage prevalence of *Candida species* was significantly high in patients with oral mucositis compared to the patients without oral mucositis.

Table 3.8: Presence of *S. aureus, Candida species* and aerobic gram negative bacteria (AGNB) in cancer patients with and without oral mucositis

Organism	Category	With oral mucositis (*n*=12)	Without oral mucositis (*n*=88)	P value
S. aureus	Prevalence (%)	7 (58.3)	45 (51.13)	p >0.05
	Mean counts (cfu/ml)	159.7	2215.7	p >0.05
Candida species	Prevalence (%)	9 (75)	47 (53.4)	**p <0.01**
	Mean counts (cfu/ml)	1021.1	616.35	p >0.05
AGNB	Prevalence (%)	4 (33.3)	20 (22.7)	p >0.05

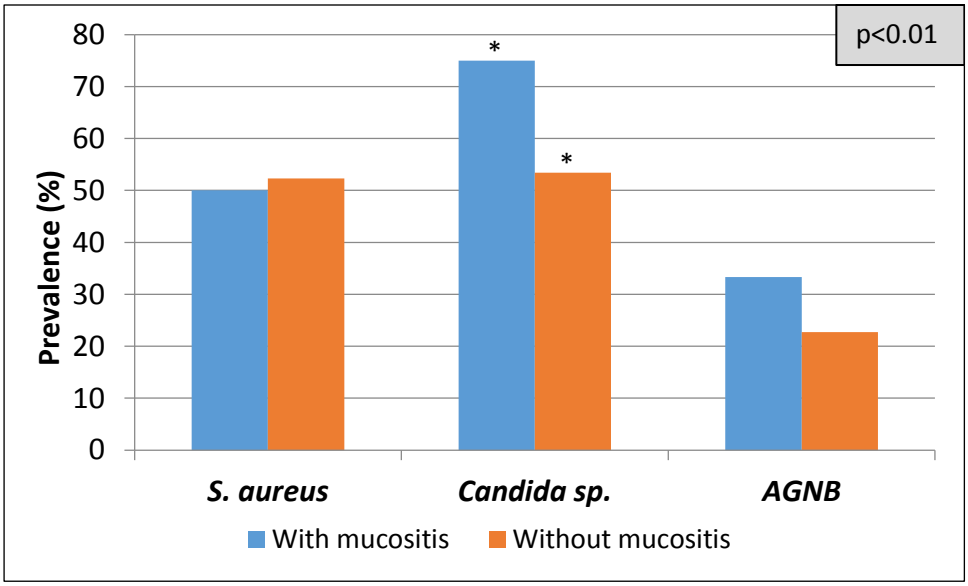


Figure 3.2 Prevalence of *S. aureus, Candida species* and aerobic gram negative bacteria (AGNB) in the oral cavities of cancer patients with and without oral mucositis

3.9 Endotoxin in the oral rinse samples of cancer patients and healthy individuals

The results of the endotoxin in the oral rinse samples of cancer patients and healthy individuals are shown in Table 3.9 and Figure 3.3. The range of endotoxins detected was 1.35ng/ml to 8.2ng/ml. In the oral rinse samples of cancer patients and healthy individuals, the mean endotoxin concentration of 3.65ng/ml and 3.37ng/ml was found respectively. The difference in the endotoxin between the two groups was not significant (p=0.5).

Table 3.9: Presence of endotoxin in the oral rinse samples of cancer patients on treatment and healthy individuals

Cancer patients (*n*=60)				**Healthy individuals (*n*=16)**	
Patient No.	**Endotoxin (ng/ml)**	**Patient No.**	**Endotoxin (ng/ml)**	**Patient No.**	**Endotoxin (ng/ml)**
C1	1.81	C33	3.9	H2	5.06
C2	3.63	C34	3.48	H3	4.9
C3	3.1	C36	1.58	H4	4.65
C4	3.19	C38	3.11	H6	4.51
C5	3.08	C41	1.6	H7	4.65
C6	5.87	C42	2.89	H9	3.32
C7	4.01	C45	5.17	H11	4.4
C8	3.65	C46	8.2	H12	4.51
C9	5.15	C47	3.34	H13	4.69
C10	4.15	C48	3.92	H14	4.28
C11	3.8	C50	3.45	H16	3.18
C12	4.87	C51	3.76	H18	3
C13	5.21	C53	3.6	H19	2.89
C14	4.77	C54	3.55	H20	1.76
C15	3.88	C56	3.49	H22	1.92
C16	3.53	C60	5.75	H23	2
C17	4.82	C61	5.24	**Mean**	***3.37**
C18	3.54	C62	4.64	**SD**	**1.11**
C19	1.89	C63	5.77		
C20	2.09	C65	8.16		
C21	2.24	C69	1.89		
C22	2.9	C70	1.35		
C23	2.7	C71	4.48		
C24	3.46	C75	4.01		
C25	3.3	C78	1.7		
C26	2.8	C80	1.77		
C28	4.36	C86	1.66		
C29	4.7	C87	1.71		
C30	2.87	C90	3.12		
C31	3.9	C98	3.18		
		Mean	***3.65**		
		SD	**1.43**		
***P=0.509**					

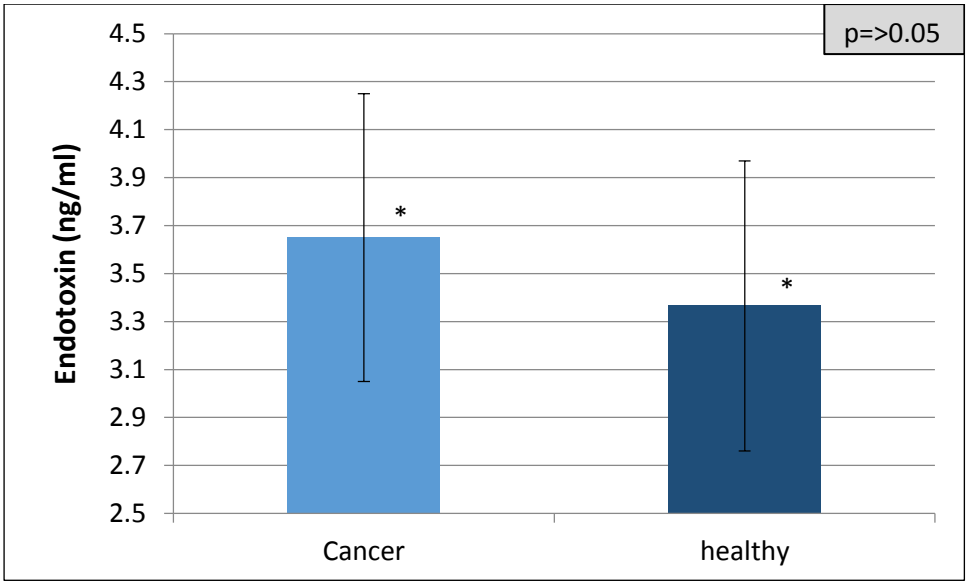


Figure 3.3: Presence of endotoxin in the oral rinse samples of cancer patients on treatment and healthy individuals

3.10 Endotoxin in the oral rinse samples of cancer patients with and without oral mucositis

The results of the endotoxin concentration in the oral rinse samples of cancer patients with and without oral mucositis are shown in Table 3.10 and Figure 3.4. In cancer patients with oral mucositis, the range of endotoxins was 1.35ng/ml to 8.2ng/ml, with a mean value of 4.1ng/ml. In cancer patients without oral mucositis, the range of endotoxins was 1.6ng/ml to 5.87ng/ml with a mean value of 3.53ng/ml. The difference in the quantities of endotoxins between the two groups was not significant (p=0.6).

Table 3.10: Presence of endotoxin in the oral rinse samples of cancer patients with and without oral mucositis

Cancer patients with oral mucositis (*n*=12)			**Cancer patients with no oral mucositis (*n*=48)**		
Category	**Patient No.**	**Endotoxin (ng/ml)**	**Category**	**Patient No.**	**Endotoxin (ng/ml)**
Grade I	C34	3.48	None	C1	1.81
	C42	2.89		C2	3.63
	C46	8.2		C3	3.1
	C62	4.64		C4	3.19
	C69	1.89		C5	3.08
	C70	1.35		C6	5.87
	C71	4.48		C7	4.01
	C75	4.01		C8	3.65
Grade II	C36	1.58		C9	5.15
	C65	8.16		C10	4.15
Grade III	C25	3.3		C11	3.8
Grade IV	C45	5.17		C12	4.87
	Mean	***4.1**		C13	5.21
	SD	**2.26**		C14	4.77
				C15	3.88
				C16	3.53
				C17	4.82
				C18	3.54
				C19	1.89
				C20	2.09
				C21	2.24
				C22	2.9
				C23	2.7
				C24	3.46
				C26	2.8
				C28	4.36
				C29	4.7
				C30	2.87
				C31	3.9
				C33	3.9
				C38	3.11
				C41	1.6
				C47	3.34
				C48	3.92
				C50	3.45
				C51	3.76
				C53	3.6
				C54	3.55
				C56	3.49
				C60	5.75
				C61	5.24
				C63	5.77
				C78	1.7
				C80	1.77
				C86	1.66
				C87	1.71
				C90	3.12
				C98	3.18
				Mean	***3.53**
				SD	**1.14**
***P=0.6501**					

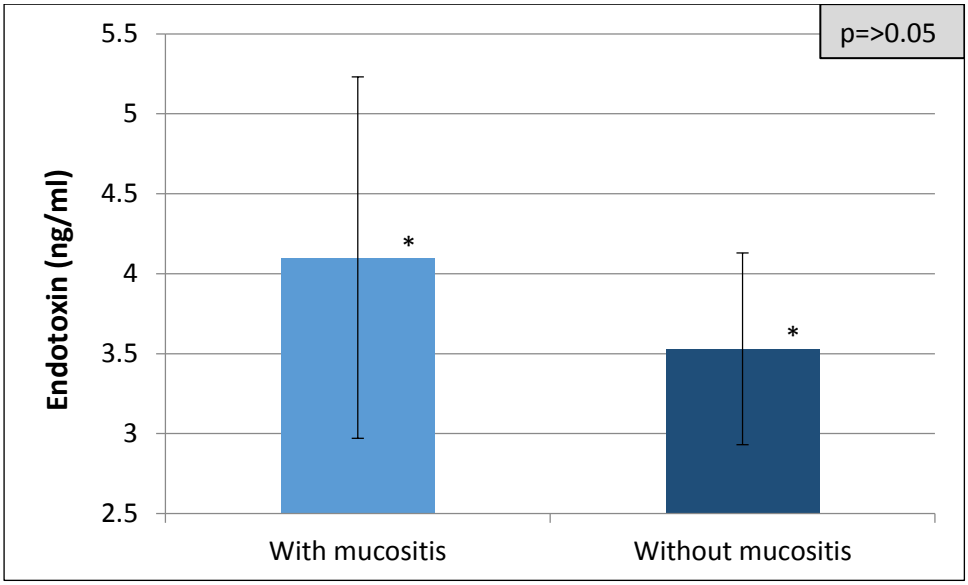


Figure 3.4: Presence of endotoxin in the oral rinse samples of cancer patients with and without oral mucositis

3.11 Endotoxin in the oral rinse samples of cancer patients with and without aerobic gram negative bacteria

The results of the endotoxin concentration in the oral rinse samples of cancer patients with and without aerobic gram negative bacteria are shown in Table 3.11 and Figure 3.5. In cancer patients with aerobic gram negative bacteria, the range of endotoxins was 1.7ng/ml to 5.77ng/ml with a mean value of 3.39ng/ml. In cancer patients without aerobic gram negative bacteria, the range of endotoxins was 1.58ng/ml to 8.16ng/ml with a mean value of 3.8ng/ml. The difference in the quantities of endotoxins between the two groups was not significant (p=0.45).

Table 3.11: Presence of endotoxin in the oral rinse samples of cancer patients with and without aerobic gram negative bacteria

Cancer patients (*n*=60)					
Category	**Patient No.**	**Endotoxin (ng/ml)**	**Category**	**Patient No.**	**Endotoxin (ng/ml)**
With AGNB	C1	1.81	**Without AGNB**	C2	3.63
(*n*=23)	C10	4.15	**(*n*=37)**	C3	3.1
	C16	3.53		C4	3.19
	C18	3.54		C5	3.08
	C30	2.87		C6	5.87
	C31	3.9		C7	4.01
	C34	3.48		C8	3.65
	C38	3.11		C9	5.15
	C48	3.92		C11	3.8
	C50	3.45		C12	4.87
	C51	3.76		C13	5.21
	C54	3.55		C14	4.77
	C56	3.49		C15	3.88
	C60	5.75		C17	4.82
	C62	4.64		C19	1.89
	C63	5.77		C20	2.09
	C70	1.35		C21	2.24
	C71	4.48		C22	2.9
	C78	1.7		C23	2.7
	C86	1.66		C24	3.46
	C87	1.71		C25	3.3
	C90	3.12		C26	2.8
	C98	3.18		C28	4.36
	Mean	***3.39**		C29	4.7
	SD	**1.19**		C33	3.9
				C36	1.58
				C41	1.6
				C42	2.89
				C45	5.17
				C46	8.2
				C47	3.34
				C53	3.6
				C61	5.24
				C65	8.16
				C69	1.89
				C75	4.01
				C80	1.77
				Mean	***3.8**
				SD	**1.56**
***P=0.456**					

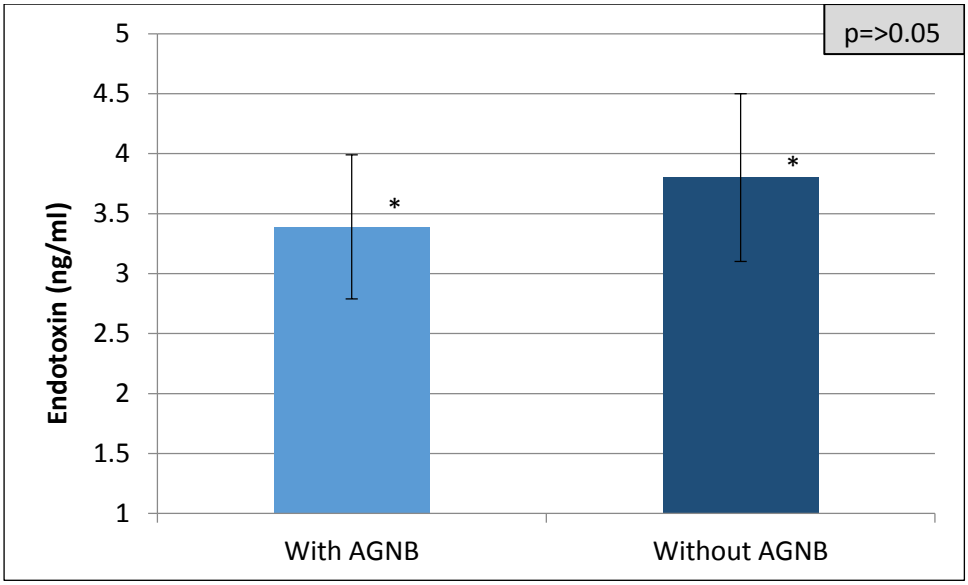


Figure 3.5: Presence of endotoxin in the oral rinse samples of cancer patients with and without aerobic gram negative bacteria

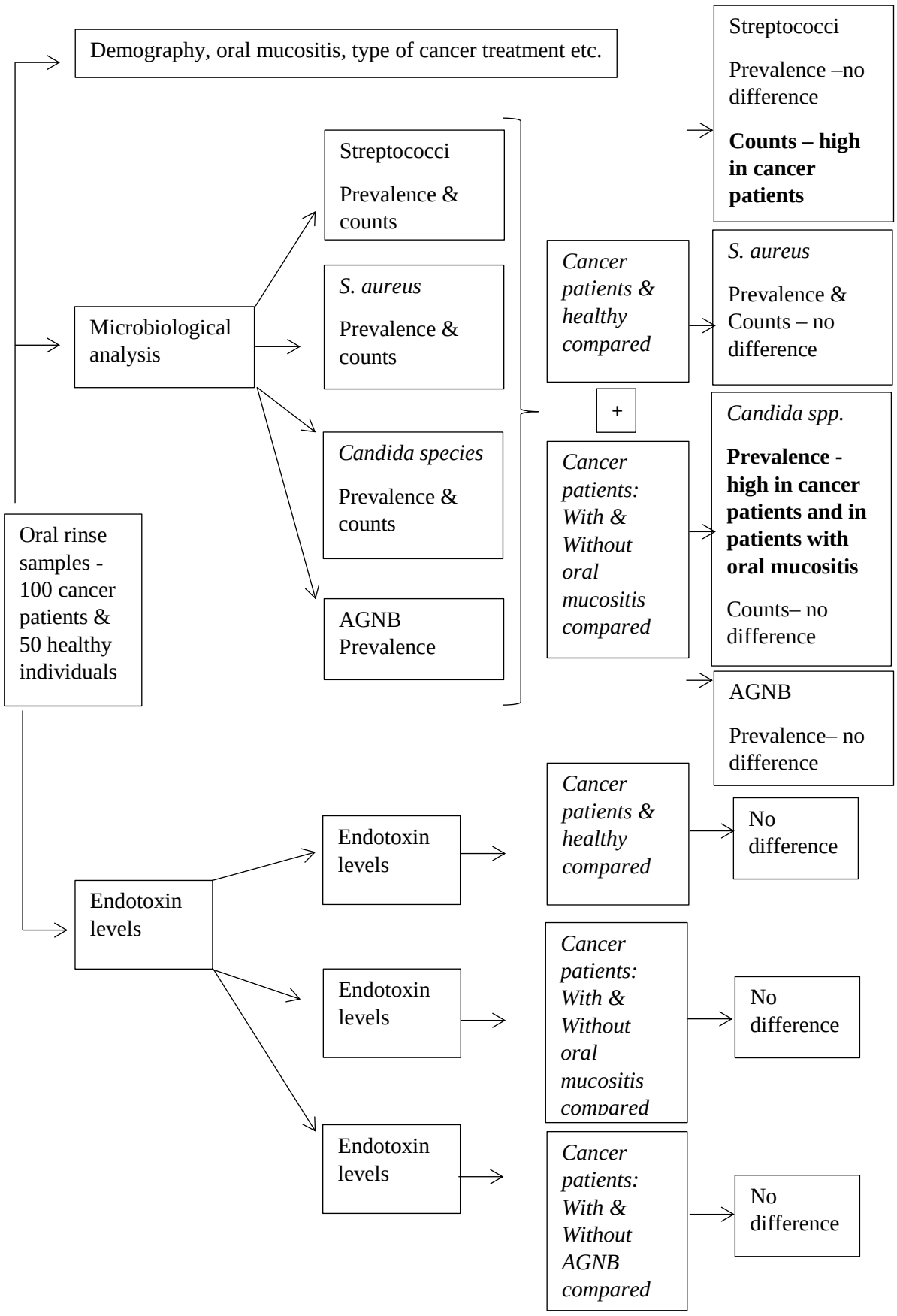
CHAPTER 4

DISCUSSION

The flow diagram of the study and the results are summarised in Figure 4.1. These results are discussed in this section.

Cancer patients develop oral mucositis due to the immunosuppression, cancer therapy and the therapy-related changes in the oral cavity. It causes pain, an altered taste sensation, painful swallowing, subsequent dehydration with malnutrition and hence affects the patient's quality of life. It also causes imbalance between commensal and opportunistic bacteria (Sixou et al, 1998). Some of these opportunistic bacteria such as aerobic gram negative bacteria, *Candida species,* Streptococci and Staphylococci become established in the lesions of oral mucositis and may contribute in the pathogenesis. Blood stream infections are common in patients with higher grades oral mucositis (Panghal et al, 2012). These aerobic gram negative bacteria are known to produce endotoxins as virulence factors. Although the involvement of endotoxins in the development of other systemic bacterial infections is well described, the role of endotoxins in oral mucositis is not known. Therefore, this study investigated the presence of aerobic gram negative bacteria and endotoxins in the oral cavities of patients receiving cancer treatment and their role in the development of oral mucositis. In addition, the presence of other microorganisms such as *Candida,* Streptococci and *S. aureus* was also investigated.

Figure 4.1 Flow diagram of the study and outcome



4.1 Streptococci and *S. aureus*

Streptococci and *S. aureus*

Oral Streptococci are the principle oral commensal bacteria of the oral cavity which usually occur in the form of biofilm. They are found in health and disease; however, increased numbers represent poor health and poor oral hygiene. In addition, high counts of Streptococci particularly *S. mutans* suggests caries activity in the oral cavity. The results in this study showed that healthy individuals (98%) as well as cancer patients (100%) carried Streptococci.

Complications of chemotherapy can illicit temporary impairment of the salivary gland function, changes in the quantity and quality of saliva and suppress the oral host's defence against microorganisms, thus allowing oral infections to develop. Consequently, this favours dental bacterial plaque accumulation, gingival inflammation and the abundance of cariogenic bacteria such as *S. mutans* and lactobacilli in these patients (Pendersen, 2016). This was similarly seen in the Streptococcal counts in cancer patients in this study, as they were significantly high compared to healthy individuals.

Literature on the number of Streptococci and Staphylococci during cancer treatment is controversial. Bergmann (1991) and Wahlin *&* Holm (1988) showed that during cancer treatment there was no increase in Streptococci and *S. aureus*, which may be explained by the concomitant use of antibiotics, antifungals and the use of chlorhexidine rinses during chemotherapy, as well as the cytotoxic effects of chemotherapy itself (O' Sullivan et al 1993). However, other studies have reported increases in Streptococci and *S. aureus* (Sixou et al, 1998, Main et al, 1984, Almståhl et al, 2008). The increase in these bacteria occurs as a result of hyposalivation and xerostomia and it can increase the risk of development of dental caries (Hu et al, 2013, Su et al, 2011, Keene and Fleming, 1987). Similarly, the cancer patients in this study were not on any topical or systemic antimicrobials and 88% of patients complained of a dry mouth with 58% of patients presenting with caries, thus contributing to the high counts of Streptococci in cancer patients. In addition, intergeneric co-aggregation and other symbiotic relationships also play a role in the occurrence of these bacteria in the oral cavity (Peterson et al, 1990).

Staphylococci are not considered to be part of the normal oral flora, although they have been found colonized in high numbers in healthy individuals. In this study, the oral carriage of *S. aureus* was 52% in cancer patients and 36% in healthy individuals. This colonization seems to coincide with the presence of other microorganism such as *Candida albicans* and *Streptococcus pyogenes* (Smith *et al*, 2001). This was similarly seen in this study, where the oral carriage of *S. aureus* (52%) and *Candida albicans* (54%) was not different.

S. aureus, in its pathogenic state has been found in oral mucositis associated with haematological malignancies and cancer treatment. Some strains of *S. aureus* produce and secrete enzymes and toxins, thereby enhancing its pathogenicity and exacerbating oral mucosal damage (Smith et al, 2001). As *S. aureus* may be part of a complex group of microorganisms, other microorganisms such as *Escherichia coli* strains can be found present in these ulcerated lesions, which can act symbiotically with *S. aureus* and further aggravate oral mucositis (Bagg et al, 1995). In this study, cancer patients with oral mucositis, although colonized with Staphylococci, presented with a higher prevalence for *Candida species* compared to aerobic gram negative bacteria, which may be related to the cancer treatment and xerostomia. As cancer patients on intensive cytotoxic anticancer treatment are associated with xerostomia and at any stage can present with neutropenia, the prevalence of Staphylococci and Streptococci is associated with great morbidity in oral mucositis. In the present study, the prevalence of *S. aureus* was not significantly different in patients with oral mucositis and patients without oral mucositis.

In patients undergoing myelosuppresive chemotherapy, oral Streptococci and *Staphylococcus spp.* are routinely found to cause bacteraemia originating from the oral cavity. In ulcerative oral mucositis, bacteraemia originating from oral *viridans Streptococci* in the oral cavity such as *Streptococcus mitis* and *Streptococcus oralis,* have been found (Raber-Durlacher et al, 2013). Therefore, although the role of oral Streptococci in oral mucositis is not clear, the risk of systemic infections, bacteraemia and septicaemia cannot be excluded (Elting et al, 1992, O'Sullivan et al, 1993, Panghal et al, 2012).

4.2 *Candida species*

Various species of *Candida* including *C. albicans* are known to cause oral candidiasis and they have been found in high numbers in the cancer treatment-related oral ulcers and angular chelitis (Wahlin & Holm, 1988).

In this study, *C. albicans* carriage was found to be 54% amongst patients undergoing radio- and chemotherapy. Patients treated with chemotherapy were noted to have an overall greater prevalence of *C. albicans* colonization, which may be due to the systemic nature of cytotoxic chemotherapy treatment and xerostomia related to the drugs and radiation (Redding et al, 1999, Al-Abeid et al, 2004, Ramirez-Amador et al, 1997). There is no definite colony count that has been established that allows for the difference between commensalism and disease. However, higher counts may predict the possibility of the development of infection. In this study it was shown that the prevalence of *Candida* carriage was high in patients undergoing cancer treatment compared to healthy individuals and cancer patients with oral mucositis compared to the cancer patients without oral mucositis. Therefore, the role of *Candida* in the development of infection and exacerbation of oral mucositis cannot be excluded. Similar results were reported by Gaetti-Jardim *et al* in 2011 where *Candida* was frequently noted in patients with grade III and IV oral mucositis. Other reasons that may have contributed to the *Candida* colonization would be from poor oral health care and the patient's nutritional status during cancer treatment, partly due to low socio-economic factors and poor education as seen in less developed countries (Panghal et al, 2012).

Malignancies and related chemotherapy and radiation are known to compromise the cell mediated host immunity which generally controls fungal infections. Both types of cancer therapies are able to cause oral mucositis resulting from hyposalivation and xerostomia which increases oral yeast proliferation, colonization and infection (Schelenz et al, 2011, Samaranayake et al, 1984). Therefore, to prevent infection it is important to maintain low counts of *Candida* in the oral cavity during cancer treatment.

The predominant species isolated was *C. albicans.* Nevertheless, the results showed that *Candida* species other than *C. albicans* colonized the oral cavities of cancer patients. The

presence of non-albicans species may be due to immunosuppression and their reduced ability to prevent colonization. Similar results have been reported by Gaetti-Jardim et al, 2011, Laheij et al, 2012, Panghal et al, 2012 and Ramla et al, 2016. The second most frequently isolated species was *C. glabrata* which is known to be found in patients undergoing cancer therapy and consistent with other reports (Schelenz et al, 2011, Al-Abeid et al, 2004, Safdar et al, 2001, Ramla et al, 2016). These results are important because many non-albicans *Candida species* are known to be resistant to many commonly used antifungal drugs (Pfaller and Diekema, 2012). In addition, these yeasts other than *C. albicans* are reported to cause candidemia in patients with malignancies as a result of probably the haploid nature and the ability to mutate rapidly (Hachem et al, 2008). Occurrence of more than one species of Candida was also noted only in the oral cavities of cancer patients which is similar to previous reports (Ramla et al, 2016, Davies et al, 2006). Management of infections in these patients can be challenging because many of these species other than *C. albicans* are often resistant to commonly used drugs (Schelenz et al, 2011). For example, 10-25% of *C. tropicalis*, 35% of *C. glabrata* and 75% of *C. krusei* are found to be resistant to fluconazole (Krcmery and Barnes, 2002).

Therefore, during cancer treatment, it is important to perform microbiological analysis of oral cavity samples.

4.3 Aerobic gram negative bacteria

The human oral cavity harbours approximately 700 different types of bacteria as commensals. However, aerobic gram negative bacteria (AGNB) particularly from the group of Enterobacteriacae do not form part of this flora. Nevertheless, it is generally known that during severe underlying disease, these bacteria are carried transiently into the oropharynx. Due to unknown reasons, these bacteria are also carried by generally healthy people, as also shown in this study. Denudement of the oral mucosa from the fibronectin exposes receptor sites required for the attachment of AGNB which is responsible for the colonization of these bacteria (Dal Nogare et al, 1987).

In literature, the prevalence rate of the carriage of AGNB is unclear. In this present study, the carriage rate of AGNB in cancer patients was 24% and in the healthy individuals it was 14%, which was not significantly different from one another. O'Sullivan *et al*, (1993) also found

that during chemotherapy, 29% of children with acute leukemia carried AGNB in their oral cavity. In contrast, Wahlin & Holm (1988) reported that 70% of cancer patients carried AGNB including *K. oxytoca, E. coli, P. aeruginosa, Serratia species, E. cloacae, P. vulgaris* and *E. sakazaki* in their oral cavity at some point during treatment. The same author reported a few years later that 86% of cancer patients carried AGNB in their oral cavity compared to 12% of healthy individuals (Wahlm et al, 1991). Similarly, 62.2% prevalence in cancer patients and 28% in healthy individuals was reported and it was attributed to the decreased white blood cell counts and the presence of oral mucositis (Galili et al, 1992). In acute leukemic patients during treatment, AGNB were present but after treatment the numbers decreased and the role in the infectious process was not established (Fainstein et al, 1981). These controversial results suggest that there are other physiological and habitual factors that might be contributing towards the colonization of the oral cavity with AGNB in diseased as well as healthy people. Although none of our patients were tobacco chewers, tobacco chewing among patients with oral squamous cell carcinoma is known to frequently carry AGNB compared to the non-chewers (Sonalika et al, 2012). Alcoholics and diabetics are also reported to carry AGNB frequently (Mackowiak et al, 1979).

Nevertheless, although in the present study, AGNB was not studied at time intervals during cancer treatment, studies have shown that often there is an imbalance of oral flora. The carriage and quantities of AGNB have been reported to increase during the treatment time period (Samaranayake et al, 1984 and Main et al, 1984). These results have been attributed to the decreased saliva flow and the reduction in salivary amylase and IgA (Main et al, 1984).

Minah *et al*, (1986) noted within 14 days of treatment, there was a sharp increase in AGNB and due to these AGNB, there was a reduction in indigenous flora. Similar results were reported by Renard *et al*, (1986) who studied these bacteria up to 28 days of treatment. Within twelve weeks of chemotherapy, patients also showed an increase in occurrence of *E. coli* and *Klebsiella spp.* (Main et al, 1984).

It is clear that cancer patients carry AGNB and the carriage can increase with the length of treatment time period. This does not necessarily mean that they contribute towards the

exacerbation of oral mucositis. In the present study, there was no difference in the prevalence of AGNB between the cancer patients with and without oral mucositis. In contrast Gaetti-Jardim et al, (2011) found that AGNB were frequently present in patients with of grade III and IV oral mucositis. Similarly, Soares *et al*, (2011) found *E. coli* and *K. pneumonia* less frequently in children with leukemia on treatment and in the children with oral mucositis. Up to 58% of cancer patients particularly those with oral mucositis carried AGNB in their oral cavity and the predominant species was *K. pneumonia* (Panghal et al, 2012), which suggest that AGNB may play some role in the pathogenesis of oral mucositis.

Oral mucositis develops from mucosal changes that occur from the thinning of oral tissues to ulceration (Redding, 2005). Even if AGNB may or may not cause any pathology, the mere presence of ulceration can allow these bacteria to enter into the deep tissues and into the blood stream. Therefore, the rate of blood stream infections due to AGNB in cancer patients has been found to be high (Gudiol et al, 2013 and Panghal et al, 2012). Blood stream infections are more common in patients with haematological malignancies compared to the patients with solid tumors. Patients with oral mucositis are known to carry their oral flora in their blood stream. For example 23% of leukemia patients carried AGNB in their oral cavity out of which 18% carried these bacteria were found in their bloodstream (Anirudhan et al, 2008). This transmission was much higher with AGNB compared to the fungi and gram positive bacteria (Anirudhan et al, 2008). Similarly 57% of blood cultures were positive for AGNB in cancer patients with fever (Panghal et al, 2012).

The results in this study suggests that in cancer patients compared to the healthy individuals, whether the carrier rate of AGNB is high or not, they are present and can increase with the treatment time period and there is the likelihood of systemic and bloodstream infections, due to the presence of these bacteria.

AGNB produce endotoxins which are known to interact with the host’s cells which produce proinflammatory cytokines and cause pathology. Therefore, the presence of endotoxins in the oral cavities of cancer patients and healthy individual was also studied.

4.4 Endotoxin

Endotoxins are lipopolysaccharide produced by aerobic gram negative and anaerobic bacilli. In the oral cavity mostly anaerobic gram negative bacilli exist and are implicated in periodontal diseases. Periodontal pathogens present are *Porphyromonas gingivalis, Prevotella intermedia, Fusobacterium nucleatum, Treponema denticola* and many more. These bacteria also produce endotoxins however, the endotoxicity is 1000 times lower than the endotoxins produced by *E. coli* which is an aerobic gastrointestinal bacillus and generally not found in the oral cavity. Endotoxin detection tests for the detection of anaerobic bacteria are not available but the *E. coli* endotoxin kit-based tests have been developed and these are commercially available. It is for this reason the chromogenic Limulus gelation assay test which uses the *E. coli* endotoxin, was used.

In our study groups (60 cancer patients and 16 healthy individuals), the range of endotoxins detected was 1.35ng/ml to 8.2ng/ml. In the oral rinse samples of cancer patients and healthy individuals, a mean endotoxin concentration of 3.65ng/ml and 3.37ng/ml was found respectively. The difference in the endotoxin between the two groups was not significant (p=0.5). The Hycult Biotech Limulus Amebocyte Lysate (LAL) assay kit that was used in this study is based on a similar kit that was used by Leenstra *et al*, (1996). Our endotoxin levels were found to be lower than the endotoxins reported by Leenstra *et al*, (1996). In their study, they measured endotoxin levels in oral rinse samples of 15 healthy individuals which were found ranging between 3ng/ml and 30ng/ml with a mean oral endotoxin level of 20ng/ml. However, in their study group these healthy individuals did not carry AGNB, therefore the results were multiplied by 1000 to adjust for the potency of anaerobic oral bacteria. This means that our endotoxin levels were much higher than those reported by Leenstra *et al*, (1996).

In Milln's study, the endotoxin concentration range was between 5.8ng/ml and 36ng/ml with a mean concentration of 19.1ng/ml for the paediatric cancer patients, which was similar to Leenstra *et al*, (1996) findings in healthy individuals of 20ng/ml. The healthy paediatric population had a mean endotoxin range of 3.1ng/ml and 20.7ng/ml with a mean salivary endotoxin concentration of 12.75ng/ml. Millns *et al*, (1999) describes that the false positive results in the leukemic patients were possible due to either bacterial clearance with the release of endotoxins at the same time or due to the absence of routine neutralizing effects from

platelets, proteins and leucocytes on endotoxins especially in immunocompromised, neutropenic patients or due to antibiotic-induced release of endotoxins (Goto and Nakamura 1980; Shenep et al, 1985 and Shenep and Mogan, 1984). Millns *et al*, (1999) findings showed that salivary endotoxins were a poor predictor of sepsis and they did not correlate with the ANGB carriage in the oropharynx, but rather that a relationship existed between neutropenia and the raised salivary endotoxins. Similarly in the present study no correlation existed between the carriage of AGNB and endotoxin levels.

In the present study, the range of endotoxins in cancer patients with oral mucositis was 1.35ng/ml to 8.2ng/ml with a mean value of 4.1ng/ml, where as in cancer patients without oral mucositis, the range of endotoxins was 1.6ng/ml to 5.87ng/ml with a mean value of 3.53ng/ml. Although the endotoxin levels were slightly higher in patients with oral mucositis, it was not significantly higher compared to the levels in patients without oral mucositis. Only 12 out of 100 cancer patients had oral mucositis. The sample size was too small for any inferences to be made. Nevertheless, given the limitations, endotoxins may not have contributed towards the pathogenesis of oral mucositis. This result could only be compared to the studies done by Leenstra *et al*, (1996) and Millns *et al*, (1999**),** as there are no other studies available on PUBMED.

These endotoxin levels, although not very high, raised the question of the origin of endotoxin. Gram negative anaerobic oral bacteria also produce endotoxins but they are slightly different in the composition and potency. The endotoxin from anaerobic bacterium lack two essential structures i.e. 2-keto-3 deoxyoctonate and heptose (Johne and Bryn, 1986) and therefore requires a concentration of a 1000 times higher to cause lethality in chick embryos and mice (Kasper, 1976, John and Bryn, 1986). Endotoxins from oral bacteria such as *Porphyromonas gingivalis* (previously called Bacteroides *gingivalis*), *Prevotella melaninogenicus* (previously called *Bacteroides melaninogenicus*), Fusobacterium and Veillonella have been characterised and tested against chick embryos, rabbits and mice (Sveen et al, 1977, Koga et al, 1985, Hofstad and Sveen, 1979 and Grehn, 1980). The results showed that much higher concentrations of endotoxins were required to cause lethality. This justifies the commensalism of these anaerobic bacteria. In the present study, the detected endotoxins may have been produced by aerobic or anaerobic oral bacteria and in those who were carriers of

AGNB, it may have been produced by these oral bacteria as well as AGNB. Therefore an attempt was made to compare the endotoxin levels in cancer patients with and without AGNB.

In cancer patients with aerobic gram negative bacteria, the range of endotoxins was 1.7ng/ml to 5.77ng/ml with a mean value of 3.39ng/ml. In the cancer patients without aerobic gram negative bacteria, the range of endotoxins was 1.58ng/ml to 8.16ng/ml with a mean value of 3.8ng/ml. The difference in the quantities of endotoxins between the two groups was not significant ($p=0.45$). These results suggest that these endotoxins were not produced by the AGNB and that the oral anaerobic bacteria may have been responsible for them. Considering this, the patient's general oral hygiene is important. During cancer treatment, the increase in periodontal pathogens has been reported and their role in the oral mucositis has been suggested (Sixou et al, 1998 and Laheij et al, 2012).

The normal amount of endotoxins levels circulating in a healthy individual is 0.3-10.4 pg/ml (O'Brien and Bruce, 2007). There are recommendations for the allowable endotoxin limit for biological products, drugs and devices. According to the Food and Drug Administration (FDA), water for Injection, sterile water for Injection and sterile water for irrigation have an allowable endotoxin limit of 0.025ng/ml. The maximum allowable endotoxin exposure for humans is calculated as 0.5 ng/Kg/Hour (or 350 EU or 35ng/ml per adult for 70Kg person per hour) to avoid fever and hypotension from endotoxin contamination (FDA, 2012). Our oral endotoxin readings were much lower than these allowable limits and the potency of this endotoxin would be much lower because the majority produced were by the anaerobic bacteria.

In addition, in the oropharyngeal and gastrointestinal tract area there are potent defense mechanisms which generally controls the absorption and clearance of toxins. The first line of defense, which is the intactness of tissues, may have failed in the case of oral mucositis. However, once absorbed, macrophages of the oropharynx and lymphatic tissue neutralises endotoxin. In addition, platelets, proteins and leucocytes present in the blood also further neutralise endotoxins. Not forgetting, there is the physical clearance and removal of

endotoxins and AGNB via physical mechanisms of reflex, tongue movements and the salivary flow. Salivary mucins are also known to neutralise endotoxin from the oral cavity (Daly et al, 1980). Nevertheless, high counts of AGNB in the oral cavity may increase the quantities of endotoxin and the sub-functioning oropharynx-associated lymphatic tissues in the immunosuppressed patient may not cope with the natural clearance. In some of our patients, AGNB was detected only after an enrichment technique meaning that the counts of AGNB in the actual oral rinse were very low.

4.5 Prevention, treatment and recommendations

While a number of the adverse effects associated with cancer treatment are well managed, oral mucositis remains an area for debate in the efficient management of this condition (De Sanctisa et al, 2016). Although several approaches have been recommended for the treatment of oral mucositis, no specific prophylactic agent or intervention has been devised for the effective prevention and management of this condition (Köstler et al, 2001), also considering that these patients already are taking multiple cancer drugs.

A systematic literature review was done in relation to the prophylaxis and management of oral mucositis over the past 20 years and this was assessed by clinical experts and a consensus by 40 different multidisciplinary experts such oncologists, infectious disease physicians, nurses, nutritionists and oral health professionals. Their aim was to reach consensus to the prophylaxis and management of the condition (De Sanctisa et al, 2016). Although oral mucositis can develop as a complication of the cytotoxic therapy, patient risk factors have been highlighted to influence the progression of oral mucositis, such as poor oral hygiene, periodontal disease, alcohol, tobacco use and immunosuppression related to the treatment or from other diseases such as diabetes mellitus (Meurman et al., 1997).

In this multidisciplinary approach, oral health professionals are recommended for the maintenance of good oral hygiene, periodontal disease and dental disease during pre-treatment, during treatment and post treatment of cancer (Lalla et al, 2008). The practice of strict oral hygiene regimens, have been associated with lowering the incidence of oral

mucositis with the reduction in the colonization of opportunistic bacteria (Yoneda et al, 2007).

Numerous topical agents like barrier-protective agents which have been suggested to cover the ulcerated region to reduce symptoms, have been recommended for the prevention and treatment of oral mucositis, although these have been proven ineffective (De Sanctisa et al, 2016). Topical antibacterial mouthwashes such chlorhexidine gluconate have been proposed. Many studies have been done to emphasize the positive effects of chlorhexidine to significantly reduce the incidence and treat this pathology (Ferretti et al, 1990, McGaw and Belch, 1985), but other randomised trials have proven them to be ineffective (Spijkervet et al, 1989c and Weisdorf et al, 1989). In the study by Ferretti *et al*, (1990), evidence of the reduction of streptococci and yeasts were noted, although in the study by Spijkervet *et al*, (1989c), colonization of *Candida spp*, *Streptococci spp*, *Staphylococci* and aerobic gram negative bacteria were not reduced after five weeks of using chlorhexidine rinses compared to a placebo rinse. Evidence of the presence of gram negative bacteria persisting despite the use this oral rinse (Raybould et al, 1994) was found due to the low susceptibility of gram negative bacilli to chlorhexidine, the discomfort and possible aggravation of the condition associated with the mouthwash (Wahlin, 1989). It has also been found that the inefficacy of certain antifungals such as nystatin in combination with chlorhexidine against *C. albicans* in vitro, have been reported (Barkvoll and Attramadal, 1989). These and other antibacterial agents have been recommended but none impacting on a better quality of life.

By selectively eliminating certain oral flora with antibiotic and antifungal lozenges (containing polymixin, tobramycin and Amphotericin B; or bacitracin, clotrimoxazole and gentamicin) in the hope that fewer patients would need a feeding tube and combat malnutrition, this did not reduce the severity of oral mucositis (Stokman et al, 2003 and Wijers et al. 2001). Polymyxin is used for the selective treatment for gram negative bacteria and Tobramycin has a role in treating bacterial infections, particularly gram negative infections. Amphotericin B is an antifungal used to treat systemic fungal infection (Saunders et al, 2013). Clotrimazole and fluconazole have been shown to reduce the candida colonization in patients with oropharyngeal candidiasis (Saunders et al, 2013), although fluconazole did not reduce the incidence of oral mucositis. This suggested that bacteria might

not be involved as the main aetiological factor in the pathogenesis of oral mucositis (Wijers et al, 2001). As candidiasis, such as the acute atrophic variant and oral mucositis can clinically present similar, fluconazole has not been recommended in the management of oral mucositis but rather for prophylaxis in patients with a high risk for mycotic infections and immunocompromised patients, such as diabetics (De Sanctisa et al, 2016). The long term use of systemic antibiotics for oral mucositis prophylaxis in patients without neutropenia was not recommended as these bacteria may become resistant to the antibiotics (Saunders et al, 2013).

In the present study, the antimicrobial sensitivity of AGNB shows that resistance to antibiotics was high in the *Pseudomonas species* found in cancer patients and *Klebsiella species* found in healthy individuals. *Pseudomonas spp.* are opportunistic human pathogens, where antibiotics such as fluoroquinolones, aminoglycosides, and imipenems are effective against these bacteria, however resistance towards these antibiotics have been developing rapidly (Yau et al, 2001).

As oral mucositis develops due to multiple factors, several management approaches have been proposed, but no definite solution has finalized. Miscellaneous treatments such as cryotherapy, growth factors, pure natural honey and many more agents have been suggested but none have been effective either. De Sanctisa *et al*, (2016) highlights the importance of a multidisciplinary approach from health professionals of different disciplines and the need for more evidence-based studies on the prevention and treatment of oral mucositis.

CHAPTER 5

CONCLUSIONS, LIMITATIONS AND FUTURE RESEARCH

5.1 Conclusions

The carriage of *Candida species* was found to be high in the oral cavities of cancer patients on radio and/or chemotherapy compared to the healthy individuals. However, carriage of Streptococci, *S. aureus* and aerobic gram negative bacteria was not different between the two groups. Cancer patients on radio and/or chemotherapy carried high number of Streptococci in their oral cavities compared to the healthy individuals. However, no difference in the counts of *S. aureus* and *C. albicans* was noted. The prevalence of *Candida* was high in the cancer patients with oral mucositis compared to the patients without oral mucositis. Cancer patients also carried a variety of *Candida species* including *C. albicans* and *C. glabrata*, and multiple species in their oral cavities compared to the healthy individuals. Cancer patients also carried a variety of aerobic gram negative bacteria compared to healthy individuals. *Enterobacter cloacae* and *Klebsiella pneumonia* were isolated from the oral cavities of both study groups. The antimicrobial sensitivity test results showed that aerobic gram negative bacteria were resistant to many antibiotics in cancer patients and healthy individuals.

Endotoxin test results showed no significant difference in the endotoxins present in the oral rinses samples collected from cancer patients and healthy individuals, cancer patients with and without oral mucositis and cancer patients with and without aerobic gram negative bacteria.

These results suggest that although cancer patients carry aerobic gram negative bacteria and endotoxins in their oral cavities, they may not contribute in the exacerbation of oral mucositis. However, *Candida species* may contribute in the exacerbation of oral mucositis and therefore, during cancer treatment, it is important to take preventative measures to prevent colonization of *Candida*.

5.2 Limitations

- It would have been ideal to have a larger sample size. Due to the time and financial constraints only 100 cancer patients and 50 healthy individuals were included.
- Thorough oral hygiene assessment was not possible as these cancer patients are often too ill to comply. It may have assisted in the interpretation of the microbiology results.
- Endotoxin test was performed only on 76 oral rinse samples (60 cancer patients and 16 healthy individuals) in duplicate. The cost of the endotoxin test was R 345.96 per sample and therefore more samples could not be tested. In addition, it is a kit based test.
- Unfortunately only 12/100 cancer patients had oral mucositis. Ideally, it would have been better to have more patients with oral mucositis in order to have a reasonable comparison.

5.3 Future research

- A similar study can be undertaken with a larger sample size
- Antifungal susceptibility tests can be performed on all the Candida isolates to determine a resistance pattern in our population
- Anaerobic gram negative bacteria which are generally dental plaque-associated periodontal pathogens can be isolated and identified from this study population
- Endotoxin testing can be performed more than twice per sample to eliminate any errors.
- Aerobic gram negative bacteria from faecal samples and oral rinse can be compared in cancer patients to establish the origin of these bacteria as they do not form part of true oral flora**.**

CHAPTER 6

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CHAPTER 7

APPENDICES

Appendix A: Data collection sheets

Data Collection sheet – Cancer patients

Laboratory no. Age: Male: Female:

Type of Cancer: _____

Type of Treatment: Chemotherapy and Radiation/ Chemotherapy

Length of treatment: _____

Present Stage of treatment (weeks): _____

Name of Chemotherapy Drugs: _____

Nausea and vomiting: Yes No

HIV status: positive Negative unknown

Diabetes: Yes No

Other medical conditions: _____

Smoker: Yes No

Dentures: Yes No

Antimicrobials: Yes No

Other medication: Yes No

Oral exam:	Oral Mucositis:	Yes		No		
	Bleeding ulcers:	Yes		No		
	Degree of Pain:	Mild	Moderate			Severe
	Loss of appetite:	Yes		No		
	Difficulty to swallow:	Yes		No		
	Altered taste:	Yes		No		
	Grade of Oral Mucositis:	0	1	2	3	4
Oral Hygiene:	Halitosis	Yes		No		
	Plaque:	Yes		No		
	Swollen gums:	Yes		No		
	Periodontitis:	Yes		No		
	Dry mouth:	Yes		No		

	Dental caries:	Yes	No
Consent form signed:		Yes	No
Oral rinse collected:		Yes	No

Data Collection sheet – Healthy individuals

Laboratory no. Age: Male: Female:

Diabetes: Yes No

Other systemic illnesses: _____

Smoker:	Yes	No
Dentures:	Yes	No
Antimicrobials:	Yes	No
Other medication:	Yes	No

Oral Hygiene:	Halitosis	Yes	No
	Plaque:	Yes	No
	Swollen gums:	Yes	No
	Periodontitis:	Yes	No
	Dry mouth:	Yes	No
	Dental caries:	Yes	No

Consent form signed:	Yes	No
Oral rinse collected:	Yes	No

Appendix B: Consent form

University of the Witwatersrand, Faculty of Health Sciences, Johannesburg

Consent form

Good Day,

How are you? I am Dr Juliana Mathews from the Oral Microbiology at the Wits Dental School. My colleagues and I are doing a study on germs that are found in our mouth. These germs cause painful sores in our mouth especially in cancer patients who are under treatment. These germs are bacteria and fungi and sometimes patients have to take tablets and mouthwashes to cure it. Sometimes these medicines do not help to cure the sores in our mouth. We would like to study these germs and the study will show us how powerful they are in causing sores and whether the treatment will work or not.

In order to do this study, I will check your mouth to see if you have these sores and how severe these sores are. We would like to collect a sample of oral rinse from your mouth. This will not cause any pain or discomfort. All you have to do is take this clean sterile water, put it in your mouth, rinse and collect it back into the jar given to you. It will not cause any harm. The sample will be processed at a laboratory. I may need to look at your medical records to see what cancer treatment you are on and how long you have been receiving it.

You may or may not participate and it is entirely up to you. What you decide will not affect your treatment. If you agree to participate, you may withdraw from the study at any time without affecting your treatment. The sample will be collected once only during your normal visit. If we find germs that cause sores in your mouth, your doctor will be notified. He will advise you on how to look after your mouth so that you don't get sores. We will also know if you have developed resistance to the medicines used to treat the sores in South Africa or not and everybody will benefit from the knowledge.

Your sample will be given a number and will be processed under a number. Your name will not appear anywhere on the results or on any publications. This study has been approved by the University Ethics Committee. Should you have any problems or queries with regards to ethics of this study, please contact Prof. P. Cleaton-Jones at 011 717 2301.

If you are 18 years old or older, not diabetic (sugar-disease), do not smoke, do not wear any false teeth, are not taking antibiotics and are already on cancer treatment for at least two weeks, you may take part in the study.

Patient's name: Investigator's name:

Signature: Signature:

Contact: Dr Juliana Mathews: cell no 083 799 2665, Prof M. Patel 011 717 2110

Appendix C: Ethics clearance certificate

R14/49 Dr Juliana Susan Mathews

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M160562

NAME: (Principal Investigator)	Dr Juliana Susan Mathews
DEPARTMENT:	Oral Biological Sciences Charlotte Maxeke Johannesburg Academic Hospital
PROJECT TITLE:	Detection of Aerobic Gram Negative Bacteria and Bacterial Endotoxins from the Oral Cavities of Cancer Patients on Cancer Therapy
DATE CONSIDERED:	27/05/2016
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Prof Mrudula Patel
APPROVED BY:	Professor P. Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	01/07/2016

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 10004,10th floor, Senate House/2nd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/We fully understand the the conditions under which I am/we are authorised to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated,from the research protocol as approved, I/we undertake to resubmit to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. in this case, the study was initially review in May and will therefore be due in the month of May each year.

Principal Investigator Signature Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix D: Composition and preparation of Media

Mitis Salivarius Agar

90g	Mitis Salivarius Agar
1000ml	Sterile distilled water
0.25ml	Potassium tellurite solution

Weigh 90g of Mitis Salivarius Agar. Suspend in 1000ml of sterilised distilled water. Boil to dissolve. Dispense medium into 250ml Boston bottles. Sterilize at 15 lb, 121 ºC for 15 minutes in an autoclave. Cool the media to 50-55 ºC. Add 0.25ml Potassium tellurite solution, mix and pour plates.

Chromagar Plates

47.7 g	Chromagar™Agar
1000 ml	Sterile distilled water

Weigh 47.7g of Chromagar™ *Agar*. Dissolve this into one litre of sterile distilled water. Boil the media, with the temperature not being greater than 100ºC. Cool the media until it reaches about 45ºC-50ºC and pour plates.

Sabouraud Agar

65g	Sabouraud agar
1000 ml	Distilled water

Suspend Sabouraud agar in half the distilled water. Heat half the water to boiling point. Mix the two and heat to boiling point. Pour into large bottles and sterilize at 15lb, 121°C for 15 minutes in an autoclave. Cool at 45°C and pour plates.

Phosphate Buffered Saline (PBS)

4.2 g	Sodium chloride
0.078 g	Sodium dihydrogen phosphate
0.64 g	Sodium hydrogen phosphate
500 ml	Distilled water

Suspend the solids and then autoclave at 15 lb, 121°C for 15 minutes and dispense.

MacConkey Agar

50.0 g medium

1000ml distilled water

Heat half the water to boiling point

Dissolve the powder in rest of the water and add to the boiling water. Pour into larger bottles to sterilize at 15 1bs for 15 min in autoclave, cool at 45°C and pour plates.

Mueller Hinton Agar

2.0 g	Beef extract
17.5 g	Acid hydrolysate of casein
1.5 g	Starch
17 g	Agar
1000 ml	Distilled water

Dissolve solids into distilled water, boil, adjust pH to 7.3, autoclave at 121°C, 15 lb for 15 minutes. Cool to 45°C and pour plates.

Baird Parker Agar

12.0g	Glycine
10.0g	Pancreatic Digest of Casein
10.0g	Sodium Pyruvate
5.0g	Beef Extract
5.0g	Lithium Chloride
1.0g	Yeast Extract
60.0ml	Egg Yolk Tellurite Enrichment
20.0g	Agar

Final pH 7.0 +/- 0.2 at 25ºC.

* Adjusted and/or supplemented as required to meet performance criteria.

Suspend desired quantity (as per manufacturer's instruction) of the medium in 940 ml deionized water. Boil to dissolve the medium and sterilise by autoclaving at 121°C for 15 minutes. Cool to 50°C and aseptically add 60ml of Egg Yolk Tellurite Emulsion. Mix well before pouring into sterile Petri Dishes.

Appendix E: Raw data of microbiological results

Laboratory Results- Cancer Patients

Laboratory no.	**S. *mutans* count: cfu/ml**	**S. *aureus* count: cfu/ml**	***Candida* count: cfu/ml**	***Candida* API 20C**	**AGNB API 20E**	**Endotoxin ng/ml**
C1	512000	0	85	*Candida famata*	*Enterobacter cloacae*	1.81
C2	1444000	12400	0	*N/A*	*N/A*	3.63
C3	1417000	0	20	*Candida famata*	*N/A*	3.1
C4	207000	10	0	*N/A*	*N/A*	3.19
C5	5080000	40	0	*N/A*	*N/A*	3.08
C6	452000	0	10	*Candida famata*	*N/A*	5.87
C7	960000	0	0	*N/A*	*N/A*	4.01
C8	2320000	10	0	*N/A*	*N/A*	3.65
C9	144000	0	0	*N/A*	*N/A*	5.15
C10	5520000	0	210	*Candida glabrata*	*Klebsiella pneumoniae*	4.15
C11	208000	0	0	*N/A*	*N/A*	3.8
C12	950000	10	50	*Candida famata*	*N/A*	4.87
C13	300000	0	20	*Candida famata*	*N/A*	5.21
C14	87000	10	0	*N/A*	*N/A*	4.77
C15	370000	30	0	*N/A*	*N/A*	3.88
C16	5500000	0	20	*Unidentifiable*	*Enterobacter cloacae*	3.53
C17	2680000	0	0	*N/A*	*N/A*	4.82
C18	3840000	50	0	*N/A*	*Klebsiella pneumoniae*	3.54
C19	470000	90	0	*N/A*	*N/A*	1.89
C20	2080000	1200	0	*N/A*	*N/A*	2.09
C21	1800000	165	0	*N/A*	*N/A*	2.24
C22	1040000	0	80	*Unidentifiable*	*N/A*	2.9
C23	3600000	0	0	*N/A*	*N/A*	2.7
C24	502000	10	280	*Candida albicans*	*N/A*	3.46
C25	633000	18	30	*Candida albicans*	*N/A*	3.3
C26	4350000	0	10	*Candida albicans*	*N/A*	2.8
C27	840000	15000	290	*Unidentifiable*	*N/A*	N/A
C28	1820000	0	30	*Candida albicans*	*N/A*	4.36
C29	1220000	0	20	*Candida albicans*	*N/A*	4.7

C30	810000	15000	1570	*Candida albicans*	*Klebsiella pneumoniae spp. ozaenae*	2.87
C31	812000	400	30	*Candida albicans*	*Escherichia coli*	3.9
C32	554000	0	30	*Candida albicans*	*N/A*	N/A
C33	490000	10	0	*N/A*	*N/A*	3.9
C34	650000	0	0	*N/A*	*Kluyvera spp.*	3.48
C35	500000	0	0	*N/A*	*N/A*	N/A
C36	7340000	20	260	*Candida albicans*	*N/A*	1.58
C37	2300000	10	0	*N/A*	*N/A*	N/A
C38	2180000	2140	180	*Candida albicans, Candida glabrata*	*Escherichia coli*	3.11
C39	432000	50	2300	*Candida albicans*	*N/A*	N/A
C40	2600000	0	1400	*Candida albicans*	*N/A*	N/A
C41	1060000	0	0	*N/A*	*N/A*	1.6
C42	2880000	20	210	*Candida albicans*	*N/A*	2.89
C43	120000	0	20	*Candida albicans*	*N/A*	N/A
C44	5480000	0	0	*N/A*	*N/A*	N/A
C45	2140000	40	1040	*Candida albicans*	*N/A*	5.17
C46	720000	0	10	*Candida albicans*	*N/A*	8.2
C47	6032000	80	560	*Candida albicans*	*N/A*	3.34
C48	1700000	30	260	*Candida glabrata*	*Enterobacter cloacae*	3.92
C49	2280000	0	0	*N/A*	*N/A*	N/A
C50	5060000	15000	10000	*Candida albicans, Candida glabrata*	*Enterobacter cloacae*	3.45
C51	1340000	30	230	*Candida glabrata*	*Escherichia coli*	3.76
C52	3960000	90	10	*Saccharomyces cerevisiae*	*N/A*	N/A
C53	770000	0	0	*N/A*	*N/A*	3.6
C54	460000	20	0	*N/A*	*Aeromonas hydrophila*	3.55
C55	1088888	15000	40	*Candida albicans*	*N/A*	N/A
C56	2320000	90	0	*N/A*	*Enterobacter cloacae*	3.49
C57	400000	0	40	*Candida albicans*	*N/A*	N/A
C58	3480000	15000	50	*Candida albicans*	*N/A*	N/A
C59	1440000	110	0	*N/A*	*N/A*	N/A
C60	1080000	130	90	*Candida albicans, Saccharomyces cerevisiae*	*Pantoea*	5.75
C61	150000	20	0	*N/A*	*N/A*	5.24
C62	150000	280	0	*N/A*	*Pseudomonas aeruginosa*	4.64
C63	630000	10	0	*N/A*	*Pseudomonas aeruginosa*	5.77
C64	930000	0	0	*N/A*	*N/A*	N/A
C65	1310000	0	40	*Candida albicans*	*N/A*	8.16
C66	140000	0	10	*Candida albicans*	*N/A*	N/A
C67	1840000	70	0	*N/A*	*N/A*	N/A

C68	440000	0	0	*N/A*	*N/A*	N/A
C69	9520000	730	0	*N/A*	*N/A*	1.89
C70	1420000	0	3840	*Candida albicans*	*Escherichia coli*	1.35
C71	1140000	10	3700	*Candida albicans, Candida glabrata*	*Pseudomonas aeruginosa*	4.48
C72	16720000	190	0	*N/A*	*N/A*	N/A
C73	24880000	130	1880	*Candida albicans*	*N/A*	N/A
C74	1820000	0	0	*N/A*	*N/A*	N/A
C75	960000	0	60	*Candida albicans*	*N/A*	4.01
C76	2640000	0	0	*N/A*	*N/A*	N/A
C77	172000	0	20	*Candida albicans*	*N/A*	N/A
C78	180000	430	400	*Candida albicans, Candida glabrata*	*Pantoea*	1.7
C79	360000	0	20	*Candida albicans*	*N/A*	N/A
C80	281000	0	0	*N/A*	*N/A*	1.77
C81	6360000	0	6770	*Candida albicans, Candida glabrata*	*N/A*	N/A
C82	2100000	10	0	*N/A*	*N/A*	N/A
C83	515000	10	0	*N/A*	*N/A*	N/A
C84	107000	0	30	*Candida albicans*	*N/A*	N/A
C85	1280000	0	0	*N/A*	*N/A*	N/A
C86	1030000	0	0	*N/A*	*Serratia rubidaea*	1.66
C87	2540000	30	30	*Candida albicans*	*Enterobacter cloacae*	1.71
C88	2440000	0	50	*Candida albicans*	*N/A*	N/A
C89	860000	0	10	*Candida albicans*	*N/A*	N/A
C90	720000	60	20	*Candida albicans*	*Pasteurella pneumotropica*	3.12
C91	1720000	0	1390	*Candida albicans, Candida glabrata*	*N/A*	N/A
C92	130000	0	130	*Candida albicans*	*N/A*	N/A
C93	3920000	240	0	*N/A*	*Klebsiella oxytoca*	
C94	1280000	30	70	*Candida albicans*	*N/A*	N/A
C95	1280000	0	0	*N/A*	*N/A*	N/A
C96	3540000	10	180	*Candida albicans*	*N/A*	N/A
C97	4180000	0	20	*Saccharomyces cerevisiae*	*N/A*	N/A
C98	740000	10	610	*Candida albicans*	*Citrobacter koseri*	3.18
C99	854000	6240	0	*N/A*	*N/A*	N/A
C100	200000	0	20	*Candida albicans*	*N/A*	N/A

Lab Results- Healthy Individuals

Laboratory no.	S. *mutans* count: cfu/ml	S. *aureus* count: cfu/ml	*Candida* count: cfu/ml	Candida API 20C	AGNB API 20E	Endotoxin
H1	1551000	0	0	N/A	N/A	N/A
H2	1040000	0	50	*Candida albicans*	N/A	5.06
H3	1760000	0	0	N/A	N/A	4.9
H4	830000	0	0	N/A	N/A	4.65
H5	1440000	0	6000	*Candida krusei*	*Klebsiella pneumoniae*	N/A
H6	11000	0	0	N/A	N/A	4.51
H7	700000	100	10	*Candida famata*	N/A	4.65
H8	253000	0	0	N/A	*Enterobacter cloacae*	N/A
H9	696000	0	0	N/A	N/A	3.32
H10	1136000	0	0	N/A	*Klebsiella pneumoniae*	N/A
H11	3560000	0	0	N/A	N/A	4.4
H12	150000	0	0	N/A	N/A	4.51
H13	2280000	20	0	N/A	N/A	4.69
H14	196000	0	0	N/A	N/A	4.28
H15	292000	30	0	N/A	*Klebsiella oxytoca*	N/A
H16	180000	10	10	*Candida albicans*	N/A	3.18
H17	24000	0	0	N/A	N/A	N/A
H18	700000	10	5200	*Candida albicans*	N/A	3
H19	3080000	110	0	N/A	N/A	2.89
H20	1500000	0	0	N/A	N/A	1.76
H21	1860000	10	0	N/A	N/A	N/A
H22	0	0	0	N/A	N/A	1.92
H23	1200	0	40	*Candida albicans*	N/A	2
H24	176000	0	0	N/A	N/A	N/A
H25	2000000	0	0	N/A	N/A	N/A
H26	270000	0	0	N/A	N/A	N/A
H27	1320000	0	0	N/A	N/A	N/A
H28	680000	70	0	N/A	N/A	N/A
H29	1580000	0	40	*Candida albicans*	N/A	N/A
H30	1060000	0	0	N/A	N/A	N/A
H31	570000	0	0	N/A	N/A	N/A
H32	2000000	90	50	*Candida dubliniesis*	N/A	N/A
H33	1440000	0	0	N/A	N/A	N/A
H34	490000	0	0	N/A	N/A	N/A
H35	740000	20	0	N/A	N/A	N/A
H36	1720000	30	0	N/A	N/A	N/A
H37	360000	0	10	*Candida albicans*	N/A	N/A
H38	124000	0	0	N/A	N/A	N/A

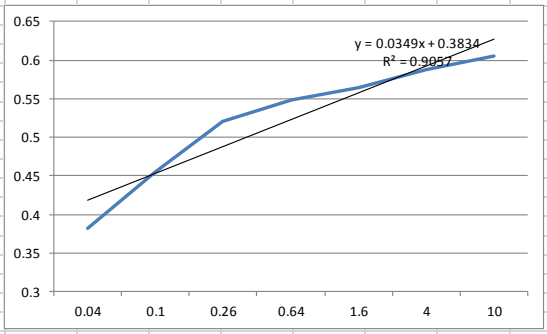
H39	84000	0	0	N/A	N/A	N/A
H40	2040000	0	0	N/A	N/A	N/A
H41	560000	0	0	N/A	*Klebsiella pneumoniae*	N/A
H42	900000	20	80	*Candida albicans*	N/A	N/A
H43	340000	0	0	N/A	*Enterobacter cloacae*	N/A
H44	140000	30	0	N/A	*Enterobacter cloacae*	N/A
H45	1480000	0	0	N/A	N/A	N/A
H46	3320000	20		N/A	N/A	N/A
H47	520000	40	0	N/A	N/A	N/A
H48	132000	2480	0	N/A	N/A	N/A
H49	4600000	4700	0	N/A	N/A	N/A
H50	4200000	10	0	N/A	N/A	N/A

Appendix F: Endotoxin Assay Results

Raw Data{Wavelength:415.0}	Reading 1											
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.601	0.555	0.58	0.572	0.586	0.554	0.535	0.57	0.628	0.637	0.568	0.566
B	0.583	0.549	0.561	0.552	0.573	0.528	0.543	0.523	0.595	0.608	0.552	0.557
C	0.56	0.54	0.555	0.557	0.585	0.544	0.543	0.55	0.532	0.498	0.586	0.541
D	0.526	0.506	0.524	0.551	0.546	0.51	0.533	0.537	0.593	0.619	0.591	0.46
E	0.435	0.43	0.558	0.539	0.566	0.513	0.542	0.54	0.583	0.594	0.598	0.546
F	0.298	0.307	0.566	0.54	0.56	0.546	0.505	0.531	0.588	0.592	0.589	0.565
G	0.232	0.193	0.532	0.538	0.55	0.518	0.513	0.533	0.607	0.595	0.572	0.598
H	0.038	0.04	0.484	0.511	0.521	0.518	0.511	0.515	0.589	0.597	0.564	0.57

	Reading 2											
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.605	0.557	0.582	0.576	0.591	0.556	0.538	0.571	0.635	0.644	0.583	0.589
B	0.587	0.552	0.562	0.555	0.576	0.531	0.544	0.524	0.601	0.615	0.578	0.582
C	0.564	0.544	0.557	0.56	0.585	0.544	0.544	0.551	0.594	0.597	0.613	0.559
D	0.548	0.528	0.533	0.552	0.551	0.524	0.535	0.541	0.597	0.624	0.596	0.46
E	0.52	0.511	0.558	0.54	0.565	0.511	0.543	0.54	0.586	0.597	0.603	0.548
F	0.455	0.451	0.564	0.541	0.561	0.546	0.506	0.531	0.592	0.595	0.592	0.568
G	0.382	0.317	0.531	0.538	0.551	0.519	0.513	0.532	0.61	0.598	0.576	0.6
H	0.037	0.038	0.493	0.51	0.521	0.517	0.522	0.513	0.593	0.601	0.567	0.573

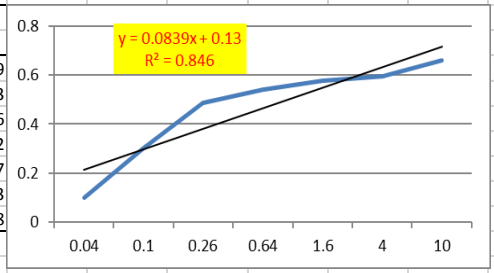
EU	Absorbance
0.04	0.382
0.1	0.455
0.26	0.52
0.64	0.548
1.6	0.564
4	0.587
10	0.605



Sample	Repeat	OD	EU	EU/sample	ng/ml	Mean	Sample	Repeat	OD	EU	EU/sample	ng/ml	
C71	1	0.582	5.690544	45.52	4.55	4.48	C2	1	0.543	4.573066	36.58	3.66	3.63
	2	0.576	5.518625	44.15	4.41			2	0.54	4.487106	35.90	3.59	
C75	1	0.562	5.117479	40.94	4.09	4.01	C3	1	0.506	3.512894	28.10	2.81	3.1
	2	0.555	4.916905	39.34	3.93			2	0.531	4.229226	33.83	3.38	
C7	1	0.557	4.974212	39.79	3.98	4.01	C4	1	0.513	3.713467	29.71	2.97	3.19
	2	0.56	5.060172	40.48	4.05			2	0.532	4.25788	34.06	3.41	
C8	1	0.533	4.286533	34.29	3.43	3.65	C5	1	0.522	3.971347	31.77	3.18	3.08
	2	0.552	4.830946	38.65	3.86			2	0.513	3.713467	29.71	2.97	
C11	1	0.558	5.002865	40.02	4.00	3.8	C6	1	0.635	7.209169	57.67	5.77	5.87
	2	0.54	4.487106	35.90	3.59			2	0.644	7.467049	59.74	5.97	
C15	1	0.564	5.174785	41.40	4.14	3.88	C9	1	0.601	6.234957	49.88	4.99	5.15
	2	0.541	4.515759	36.13	3.61			2	0.615	6.636103	53.09	5.31	
C24	1	0.531	4.229226	33.83	3.38	3.46	C12	1	0.594	6.034384	48.28	4.83	4.87
	2	0.538	4.429799	35.44	3.54			2	0.597	6.120344	48.96	4.90	
C26	1	0.493	3.140401	25.12	2.51	2.71	C13	1	0.597	6.120344	48.96	4.90	5.21
	2	0.51	3.627507	29.02	2.90			2	0.624	6.893983	55.15	5.52	
C28	1	0.591	5.948424	47.59	4.76	4.36	C14	1	0.586	5.805158	46.44	4.64	4.77
	2	0.556	4.945559	39.56	3.96			2	0.597	6.120344	48.96	4.90	
C33	1	0.576	5.518625	44.15	4.41	3.9	C17	1	0.592	5.977077	47.82	4.78	4.82
	2	0.531	4.229226	33.83	3.38			2	0.595	6.063037	48.50	4.85	
C10	1	0.585	5.776504	46.21	4.62	4.15	H2	1	0.61	6.492837	51.94	5.19	5.06
	2	0.544	4.601719	36.81	3.68			2	0.598	6.148997	49.19	4.92	
C16	1	0.551	4.802292	38.42	3.84	3.53	H3	1	0.593	6.005731	48.05	4.80	4.9
	2	0.524	4.028653	32.23	3.22			2	0.601	6.234957	49.88	4.99	
C18	1	0.565	5.203438	41.63	4.16	3.54	H4	1	0.583	5.719198	45.75	4.58	4.65
	2	0.511	3.65616	29.25	2.92			2	0.589	5.891117	47.13	4.71	
C31	1	0.561	5.088825	40.71	4.07	3.9	H6	1	0.578	5.575931	44.61	4.46	4.51
	2	0.546	4.659026	37.27	3.73			2	0.582	5.690544	45.52	4.55	
C34	1	0.551	4.802292	38.42	3.84	3.48	H7	1	0.613	6.578797	52.63	5.26	4.65
	2	0.519	3.885387	31.08	3.11			2	0.559	5.031519	40.25	4.03	
C38	1	0.521	3.942693	31.54	3.15	3.11	H9	1	0.596	6.091691	48.73	4.87	3.32
	2	0.517	3.82808	30.62	3.06			2	0.46	2.194842	17.56	1.76	
C48	1	0.538	4.429799	35.44	3.54	3.92	H11	1	0.603	6.292264	50.34	5.03	4.4
	2	0.571	5.375358	43.00	4.30			2	0.548	4.716332	37.73	3.77	
C50	1	0.544	4.601719	36.81	3.68	3.45	H12	1	0.592	5.977077	47.82	4.78	4.51
	2	0.524	4.028653	32.23	3.22			2	0.568	5.289398	42.32	4.23	
C51	1	0.544	4.601719	36.81	3.68	3.76	H13	1	0.576	5.518625	44.15	4.41	4.69
	2	0.551	4.802292	38.42	3.84			2	0.6	6.206304	49.65	4.97	
C54	1	0.535	4.34384	34.75	3.48	3.55	H14	1	0.567	5.260745	42.09	4.21	4.28
	2	0.541	4.515759	36.13	3.61			2	0.573	5.432665	43.46	4.35	

Raw Data{Wavelength:415.0}	Second reading											
	1	2	3	4	5	6	7	8	9	10	11	12
A	0.658	0.386	0.316	0.323	0.31	0.936	0.571	0.389	0.329	0.328	0.335	0.58
B	0.593	0.334	0.352	0.305	0.295	0.567	0.621	0.395	0.264	0.28	0.34	0.587
C	0.542	0.342	0.314	0.385	0.318	0.324	0.606	0.387	0.297	0.317	0.344	0.583
D	0.577	0.37	0.336	0.394	0.348	0.243	1.062	0.404	0.313	0.304	0.321	0.569
E	0.486	0.406	0.366	0.502	0.329	0.267	0.881	0.478	0.317	0.315	0.293	0.574
F	0.303	0.321	0.421	0.405	0.479	0.388	0.701	0.532	0.308	0.3	0.316	0.313
G	0.099	0.079	0.574	0.379	0.446	0.898	0.844	0.626	0.314	0.305	0.301	0.361
H	0.039	0.043	0.54	0.309	1.044	0.937	1.131	0.84	0.364	0.336	0.33	0.349

Control OD
0.099
0.303
0.486
0.542
0.577
0.593
0.658



Sample	Repeat	OD	EU	EU/sampl	ng/ml	Mean		Sample	Repeat	OD	EU	EU/sampl	ng/ml	
C1	1	0.316	2.216925	17.7354	1.77354	1.81		C61	1	0.881	8.951132	71.60906	7.160906	5.24
	2	0.323	2.300358	18.40286	1.840286				2	0.478	4.147795	33.18236	3.318236	
C19	1	0.352	2.646007	21.16806	2.116806	1.89		C62	1	0.701	6.805721	54.44577	5.444577	4.64
	2	0.305	2.085816	16.68653	1.668653				2	0.532	4.791418	38.33135	3.833135	
C20	1	0.314	2.193087	17.5447	1.75447	2.09		C63	1	0.844	8.510131	68.08105	6.808105	5.77
	2	0.385	3.039333	24.31466	2.431466				2	0.626	5.9118	47.2944	4.72944	
C21	1	0.336	2.455304	19.64243	1.964243	2.24		C65	1	1.131	11.93087	95.44696	9.544696	8.16
	2	0.394	3.146603	25.17282	2.517282				2	0.84	8.462455	67.69964	6.769964	
C22	1	0.366	2.812872	22.50298	2.250298	2.9		C69	1	0.329	2.371871	18.97497	1.897497	1.89
	2	0.502	4.43385	35.4708	3.54708				2	0.328	2.359952	18.87962	1.887962	
C23	1	0.421	3.468415	27.74732	2.774732	2.7		C70	1	0.264	1.597139	12.77712	1.277712	1.35
	2	0.405	3.277712	26.22169	2.622169				2	0.28	1.787843	14.30274	1.430274	
C25	1	0.574	5.292014	42.33611	4.233611	3.3		C71	1	0.297	1.990465	15.92372	1.592372	1.69
	2	0.379	2.967819	23.74255	2.374255				2	0.317	2.228844	17.83075	1.783075	
C27	1	0.54	4.88677	39.09416	3.909416	2.8		C78	1	0.313	2.181168	17.44934	1.744934	1.7
	2	0.309	2.133492	17.06794	1.706794				2	0.304	2.073897	16.59118	1.659118	
C29	1	0.31	2.145411	17.16329	1.716329	4.7		C80	1	0.317	2.228844	17.83075	1.783075	1.77
	2	0.936	9.606675	76.8534	7.68534				2	0.315	2.205006	17.64005	1.764005	
C30	1	0.295	1.966627	15.73302	1.573302	2.87		C86	1	0.308	2.121573	16.97259	1.697259	1.66
	2	0.567	5.208582	41.66865	4.166865				2	0.3	2.026222	16.20977	1.620977	
C34	1	0.318	2.240763	17.9261	1.79261	1.82		C87	1	0.314	2.193087	17.5447	1.75447	1.71
	2	0.324	2.312277	18.49821	1.849821				2	0.305	2.085816	16.68653	1.668653	
C36	1	0.348	2.598331	20.78665	2.078665	1.58		C89	1	0.364	2.789035	22.31228	2.231228	2.1
	2	0.243	1.346841	10.77473	1.077473				2	0.336	2.455304	19.64243	1.964243	
C41	1	0.329	2.371871	18.97497	1.897497	1.6		C90	1	0.335	2.443385	19.54708	1.954708	3.12
	2	0.267	1.632896	13.06317	1.306317				2	0.58	5.363528	42.90822	4.290822	
C42	1	0.479	4.159714	33.27771	3.327771	2.89		C98	1	0.34	2.50298	20.02384	2.002384	3.18
	2	0.388	3.075089	24.60072	2.460072				2	0.587	5.446961	43.57569	4.357569	
C45	1	0.446	3.766389	30.13111	3.013111	5.17		H16	1	0.344	2.550656	20.40524	2.040524	3.18
	2	0.898	9.153754	73.23004	7.323004				2	0.583	5.399285	43.19428	4.319428	
C46	1	1.044	10.89392	87.15137	8.715137	8.2		H18	1	0.321	2.27652	18.21216	1.821216	3
	2	0.937	9.618594	76.94875	7.694875				2	0.569	5.23242	41.85936	4.185936	
C47	1	0.571	5.256257	42.05006	4.205006	3.34		H19	1	0.293	1.942789	15.54231	1.554231	2.89
	2	0.389	3.087008	24.69607	2.469607				2	0.574	5.292014	42.33611	4.233611	
C53	1	0.621	5.852205	46.81764	4.681764	3.6		H20	1	0.316	2.216925	17.7354	1.77354	1.76
	2	0.395	3.158522	25.26818	2.526818				2	0.313	2.181168	17.44934	1.744934	
C56	1	0.606	5.673421	45.38737	4.538737	3.49		H22	1	0.301	2.038141	16.30513	1.630513	1.92
	2	0.387	3.06317	24.50536	2.450536				2	0.361	2.753278	22.02622	2.202622	
C60	1	1.062	11.10846	88.8677	8.88677	5.75		H23	1	0.33	2.38379	19.07032	1.907032	2
	2	0.404	3.265793	26.12634	2.612634				2	0.349	2.61025	20.882	2.0882	

Appendix G: Statistical analysis

Question 1: Do cancer patients carry a higher count of *S. aureus* compared to healthy individuals (cfu/ml)?

Question 2: Do cancer patients carry a higher count of *Streptococci* compared to healthy individuals (cfu/ml)?

Question 3: Do cancer patients carry a higher count of *Candida* compared to healthy individuals (cfu/ml)?

Question 4: Do cancer patients carry higher count of endotoxin compared to healthy individuals (ng/ml)?

Summary of results

Count	**Cancer: Median (IQR)**	**Healthy: Median (IQR)**	**p-value (Mann- Whitney test)**
S. mutans (cfu/ml)	1110000 (1879000)	720000 (1327000)	0.020
S. aureus (cfu/ml)	50 (246)	30 (70)	0.310
Candida (cfu/ml)	55 (265)	45 (70)	0.567
Endotoxins (ng/ml)	4 (1)	4.3 (2)	0.509

Descriptive statistics of cancer patients: organism and endotoxin count

Variable	Patients=1 Descriptive Statistics (Stats table)									
	Valid N	Mean	Median	Minimum	Maximum	Lower Quartile	Upper Quartile	Range	Quartile Range	Std.Dev.
S. aureus count cfu/ml	52	1939	50	10.00	1500(	14.0	260	1499(	246	4706
S. mutans counts cfu/ml	100	214295(	111000(	87000.0(	2488000(	501000.(	238000(	2479300(	187900(	327475(
Candida counts cfu/ml	56	693	55	10.00	1000(	20.0	285	999(	265	1733
Endotoxin count ng/ml	61	4	4	1.35	8	2.9	4	7	1	1

Descriptive statistics of healthy individuals: organism and endotoxin count

Variable	Patients=0 Descriptive Statistics (Stats table)									
	Valid N	Mean	Median	Minimum	Maximum	Lower Quartile	Upper Quartile	Range	Quartile Range	Std.Dev.
S. aureus count cfu/ml	18	433	30.0	10.0000(	4700	20.0	90	469(	70	1210
S. mutans counts cfu/ml	50	112172(	720000.(	0.0000(	460000(	253000.(	158000(	460000(	132700(	111067(
Candida counts cfu/ml	10	1149	45.0	10.0000(	6000	10.0	80	599(	70	2354
Endotoxin count ng/ml	16	4	4.3	1.7600(	5	2.9	5	3	2	1

Mann-Whitney test for cancer patients and healthy individuals: organism and endotoxin count

variable	Mann-Whitney U Test (w/ continuity correction) (Stats table) By variable Patients Marked tests are significant at p <.05000 Rank Sum Group 1	Rank Sum Group 2	U	Z	p-value	Z adjusted	p-value	Valid N Group 1	Valid N Group 2	2*1sided exact p
S. aureus count cfu/ml	1922.00(	563.00(	392.00(	1.01454:	0.31032-	1.02373:	0.30596(	52	18	0.31327:
S. mutans counts cfu/ml	8131.50(	3193.50(	1918.50(	2.31629:	0.02054:	2.31635(	0.02054(	100	50	
Candida counts cfu/ml	1908.50(	302.50(	247.50(	0.572281	0.567132	0.57446:	0.565652	56	10	0.566541
Endotoxin count ng/ml	2326.00(	677.00(	435.00(	-0.65914(	0.50980(	-0.65917(	0.509787	61	16	0.513435

Question 5: Do cancer patients with gram negative bacteria (GNB) have a higher count of endotoxin compared to cancer patients without GNB (ng/ml)?

Summary of results

Count	**GNB: Yes- Median (IQR)**	**GNB: No- Median (IQR)**	**p-value (Mann-Witney test)**
Cancer Patients: Endotoxin (ng/ml)	3.490000 (1.050000)	3.615000 (1.970000)	0.456

Descriptive statistics of endotoxin count (ng/ml): cancer patients with GNB

Variable	GNB/no GNB=1 Descriptive Statistics (Stats table) Include condition: v2=1 Valid N	Mean	Median	Minimum	Maximum	Lower Quartile	Upper Quartile	Range	Quartile Range	Std.Dev.
Endotoxin count ng/ml	23	3.38782	3.49000	1.35000	5.77000	2.87000	3.92000	4.42000	1.05000	1.18943

Descriptive statistics of endotoxin count (ng/ml): cancer patients without GNB

Variable	GNB/no GNB=0 Descriptive Statistics (Stats table) Include condition: v2=1 Valid N	Mean	Median	Minimum	Maximum	Lower Quartile	Upper Quartile	Range	Quartile Range	Std.Dev.
Endotoxin count ng/ml	38	3.76105	3.61500	1.58000	8.20000	2.80000	4.77000	6.62000	1.97000	1.55999

The Mann-Witney test for endotoxin count (ng/ml): cancer patients with and without GNB

variable	Mann-Whitney U Test (w/ continuity correction) (Stats table) By variable GNB/no GNB Marked tests are significant at p <.05000 Include condition: v2=1 Rank Sum Group 1	Rank Sum Group 2	U	Z	p-value	Z adjusted	p-value	Valid N Group 1	Valid N Group 2	2*1sided exact p
Endotoxin count ng/ml	662.500	1228.50	386.500	-0.74406	0.45684	-0.74409	0.45682	23	38	0.45533

Question 6: Do cancer patients with oral mucositis have a higher count of endotoxin compared to healthy individuals (ng/ml)?

Summary of results

Count	**Cancer: Mucositis Median (IQR)**	**Healthy**: **Median (IQR)**	**p-value (Mann- Whitney test)**
Endotoxin (ng/ml)	4 (3)	4.3 (2)	0.889

Descriptive statistics of endotoxin count (ng/ml): cancer patients with oral mucositis

Variable	Patients=1 Descriptive Statistics (Stats table) Exclude condition: v9=0 and v2=1 Valid N	Mean	Median	Minimum	Maximum	Lower Quartile	Upper Quartile	Range	Quartile Range	Std.Dev.
S. aureus count cfu/ml	7	160	20	10.0	730	18.0	280	720	262	269
S. mutans counts cfu/ml	12	240525(	122500(	150000.(	952000(	685000.(	251000(	937000(	182500(	294302(
Candida counts cfu/ml	9	1021	210	10.0	3840	40.0	1040	3830	1000	1591
Endotoxin count ng/ml	12	4	4	1.4	8	2.4	5	7	3	2

Descriptive statistics of endotoxin count (ng/ml): healthy individuals

Variable	Patients=0 Descriptive Statistics (Stats table) Exclude condition: v9=0 and v2=1 Valid N	Mean	Median	Minimum	Maximum	Lower Quartile	Upper Quartile	Range	Quartile Range	Std.Dev.
S. aureus count cfu/ml	18	433	30.0	10.0000(	4700	20.0	90	469(	70	1210
S. mutans counts cfu/ml	50	112172⁴	720000.(	0.0000(	460000(	253000.(	158000(	460000(	132700(	111067⁷
Candida counts cfu/ml	10	1149	45.0	10.0000(	6000	10.0	80	599(	70	2354
Endotoxin count ng/ml	16	4	4.3	1.7600(	5	2.9	5	3	2	1

Mann-Whitney test for endotoxin count (ng/ml): cancer patients with oral mucositis and healthy individuals

variable	Mann-Whitney U Test (w/ continuity correction) (Stats table) By variable Patients Marked tests are significant at p <.05000 Exclude condition: v9=0 and v2=1 Rank Sum Group 1	Rank Sum Group 2	U	Z	p-value	Z adjusted	p-value	Valid N Group 1	Valid N Group 2	2*1sided exact p
Endotoxin count ng/ml	170.500(	235.500(	92.5000(	-0.13927	0.88923(	-0.13932(	0.88919(	12	16	0.87305(

Question 7: Do cancer patients with oral mucositis have a higher count of endotoxin compared to cancer patients without oral mucositis (ng/ml)?

Summary of results

Count	**Cancer: Oral mucositis: Median (IQR)**	**Cancer: No oral mucositis: Median (IQR)**	**p-value (Mann- Whitney test)**
Endotoxin (ng/ml)	3.745 (2.515)	3.53 (1.14)	0.6501

Question 8: Do cancer patients have a higher carrier rate of *S. aureus* compared to healthy individuals (%)?

Summary of results

Population	***S. aureus* (%)- Present**	***S. aureus* (%)- Not Present**	**p- value (chi-squared test)**
Cancer Patients	52	48	P=0.064
Healthy Individuals	36	64	

Frequency tables: *S. aureus* in cancer patients and healthy individuals

	Summary Frequency Table (Stats table) Marked cells have counts > 10 (Marginal summaries are not marked)			
	Patients	S. aureus 0	S. aureus 1	Row Totals
Count	0	32	18	50
Row Percent		64.00%	36.00%	
Count	1	48	52	100
Row Percent		48.00%	52.00%	
Count	All Grps	80	70	150

Question 9: Do cancer patients have a higher carrier rate of *Candida* compared to healthy individuals (%)?

Summary of results

Population	**Candida (%)- Present**	***Candida* (%)- Not Present**	**p- value (chi-squared test)**
Cancer Patients	56	44	P=0.000
Healthy Individuals	20	80	

Frequency tables for *Candida* in cancer patients and healthy individuals

	Summary Frequency Table (Stats table) Marked cells have counts > 10 (Marginal summaries are not marked)			
	Patients	Candida 0	Candida 1	Row Totals
Count	0	40	10	50
Row Percent		80.00%	20.00%	
Count	1	44	56	100
Row Percent		44.00%	56.00%	
Count	All Grps	84	66	150

Question 10: Do cancer patients have a higher carrier rate of GNB compared to healthy individuals (%)?

Summary of results

Population	**GNB (%)- Present**	***GNB* (%)- Not Present**	**p- value (chi-squared test)**
Cancer Patients	24	76	P=0.154
Healthy Individuals	14	86	